UNIVERSITAT DE BARCELONA

Design and synthesis of peptides that neutralize bacterial endotoxins as therapeutic agents for the treatment of sepsis

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Tesi Doctoral

DESIGN AND SYNTHESIS OF PEPTIDES THAT NEUTRALIZE BACTERIAL ENDOTOXINS AS THERAPEUTIC AGENTS FOR THE TREATMENT OF SEPSIS

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Als meus pares,
exemple de lluita i coratge

Ventana sobre la utopía:

Ella está en el horizonte - dice Fernando Birri-. Me acerco dos pasos, ella se aleja dos pasos. Camino diez pasos y el horizonte se corre diez pasos más allá. Por mucho que yo camine, nunca la alcanzaré. ¿Para qué sirve la utopía? Para eso sirve: para caminar.

Eduardo Galeano

Sempre havia pensat que la part dels agraïments seria la part més fàcil i més divertida del procés d'escriptura de la Tesi. Però fa dies que ho intento, i aquestes últimes pàgines se'm resisteixen. Els últims mesos han estat una mica agònics, de nits molt llargues i moments molt crítics, i tot i aconseguir fer el dipòsit de la Tesi *in extremis*, el procés de correcció del manuscrit sembla que no hagi d'acabar de mai (he desenvolupat un instint gairebé sobrenatural per detectar els més petits errors a qualsevol pàgina oberta a l'atzar). Diria que estic patint una espècie de síndrome d'Estocolm amb la meva pròpia Tesi, que em dificulta desprendre'm definitivament. També podria ser que m'hagi estat negant aquests últims mesos a reconèixer que una etapa meravellosa de la meva vida acaba, i que ben aviat en comença una altra. Així, aquests agraïments són en bona part també un acomiadament, la qual cosa no facilita les coses. Però a aquestes alçades crec que uns agraïments no seran res que no es pugui superar, així que sense més preàmbuls, m'agradaria començar aquest viatge, i compartir-lo amb tots vosaltres, els veritables protagonistes d'aquests últims 5 anys de la meva vida.

Tot va començar un dia (segurament) plujós a la màgica ciutat de Manchester, quan després d'una columna interminable al laboratori del Dr. Joule, una persona, segurament sense saber-ho, em va animar a fer un doctorat. Els primers agraïments doncs van per la persona que menys ho deu imaginar. Gràcies ***Carme*** (avui en dia ja mare i doctora) per donar l'empenta que necessitava aquell esbojarrat Erasmus que tenia massa dubtes al cap.

A la tornada vaig descobrir que existia el Parc Científic, i no vaig dubtar en parlar amb el ***Fernando***, professor de qui guardava un record especial, tant per la seva capacitat didàctica com per la seva personalitat. T'he d'agrair Fernando que em donessis l'oportunitat de treballar al teu costat, i per donar-me aquell projecte que parlava de la septicèmia i d'un munt de coses que no entenia. Com han canviat les coses, eh? Gràcies Fernando per ser molt més que un director de Tesi, per ensenyar-me tantes coses, per la teva confiança i la teva manera de preocupar-te pels teus estudiants, per fer-nos sentir importants i valorats. Per tenir aquest punt d'humanitat que molts pocs tenen i per tenir sempre un segon (i dos) per parlar de feina o del que sigui, un vespre al tard (sovint molt tard), dissabtes o diumenges i festes de guardar. Per donar-me tantes oportunitats, per aquell *tens un 60 % de probabilitats*.

Pensava instal·lar-me a (en aquella època) l'espaiós 300 amb les seves taules individuals amb calaix i prestatge, quan el Fernando em va portar a un altre laboratori. Així vaig arribar a PharmaMar. Laboratori que durant aquests anys s'ha convertit en una segona casa. Un lloc aparentment caòtic i desorganitzat, però amb una harmonia difícil d'aconseguir. On tot té el seu lloc, i on tots els seus integrants treballen a una, ajudant-se i recolzant-se mútuament. On els problemes i els èxits es comparteixen. La qualitat humana de la gent que hi treballa, o de la que hi ha passat, és difícil de descriure, i segurament unes poques línies aquí, no en faran justícia.

A PharmaMar doncs vaig trobar-me amb una minúscula taula, però envoltat de noies amb bata, i vaig pensar que el canvi de laboratori després de tot no seria tant dolent. D'aquesta primera etapa vull donar les gràcies a la ***Natàlia***, l'***Ari*** i la ***Carol***, per la seva càlida rebuda i per ajudar-me en els sempre desconcertants inicis a un laboratori. Al ***Luis Javier*** he d'agrair-li que em guiés en els meus primers passos pel món dels pèptids i de la fase sòlida, així com per algunes de les idees que van ajudar a que el projecte tirés endavant. Després hi havia dues noies increïbles. La ***Núria*** que es va convertir en un exemple de lluita i tenacitat. Gran científica i millor persona. Un referent a seguir. Amb qui hem compartit moments molt agradables lluny del lab, a València i aquella nit tant surrealista, l'inoblidable viatge a Montreal i la vostra visita a Boston (records al Mateu i ànims que quan presenti la tesi estaràs a punt de ser mare!!!). I la incansable ***Fayna***, l'única persona amb un amic de Sri Lanka (de verdad Fayna, aún no me lo creo) i capaç d'estudiar japonès, fer volley, dirigir un curt, discutir de política, fer pèptids de més de 40 aminoàcids i tenir sempre un moment per fer un cafè quan un amic ho necessita. Per cert, també una aposta segura per a qualsevol nit de festa (creo que aún tenemos un duelo entre manos...) Però això no havia fet més que començar, aviat va arribar la ***Leti***, amb qui he compartit molt bons moments, i que sense èxit va

intentar posar ordre al caos que regnava a PharmaMar, sense imaginar-se el que estava per arribar. Treballadora nocturna d'horaris poc ortodoxos que primer havia de fer un xip i que no volia treballar amb res bio ni amb res "petit" i va acabar fent "nanos". Et desitjo molta sort a Austràlia (tornaràs? o ja t'has lligat un australià buenorro??) Per la mateixa època, si la memòria no em falla (cosa bastant probable), va arribar el ***Juan***, que tot ho sabia, des de la IP del masses a on es trobaven els reactius. Company de cafès passades les 7, i un altre fix a la travessa de les nits més llargues. Lo único que me falta es llegar entender aquello del 4+4! I poc a poc van anar arribant les meves nenes. El millor motiu per anar a treballar amb un somriure, fins i tot en els pitjors moments, per tirar sempre endavant. Us he d'agrair molt especialment la vostra ajuda, el vostre recolzament i haver-me ensenyat tantes coses. Primer va arribar la ***Yesi***, que al començament semblava un mica tímida fins que va començar a parlar (*una cosa te voy a decir*...). Una persona a qui estimo moltíssim i que mai he sabut si considerar com la meva germana gran o petita. Una lluitadora nata i una persona amb molt més coratge del que creu. Amb la seva vitalitat, alegria i espontaneïtat, va convertir PharmaMar en el que és. Després va arribar la ***Marta***, de qui podria dir tantes coses, que em quedaré amb que només per conèixer una persona com ella (pura bondat) valdria la pena començar una altra Tesi. M'emporto una companya i amiga per sempre, de bromes inimaginables que la Yesi mai va arribar a comprendre (crec que amb quedo amb el *si hi ha algun doctor a la sala*). I després va arribar la ***Lor***, que ja havia passat l'estiu al parc, però que a efectes pràctics es va incorporar més tard. Una persona que encisa pel seu atractiu, la seva intel·ligència i la seva simpatia. Què més es pot demanar? Un altre exemple a seguir de constància i força. Companya de vitrina, i un altre valor segur a les nits de festa. Capaç de deixar anar qualsevol barbaritat quan menys t'ho esperes. Et trobaré a faltar! I després tenim a l'**Eli**, l'investigadora que tot laboratori necessitaria. I no ho dic per ser una mestra del Bricomania, per la seva creativitat sense límits (increïbles gimcanes), per la seva professionalitat en tot el que fa o per la seva excel·lent capacitat científica, sinó per ser la persona que he conegut amb un sentiment de justícia mes gran. Una persona a qui sempre admiraré. I finalment la ***Myri***. Quan pensava que les coses no podien anar millor del que anaven, va arribar ella. I les va canviar. No recordo un sol dia al laboratori que no m'hagi arrancat un somriure. El seu riure tant contagiós amaga una gran persona, una amiga que m'ha ajudat sense saber-ho en moments complicats i a qui em costarà molt de dir adéu. També vull agrair al ***Dani***, la seva grandària només la supera el tamany del seu cor, te acuerdas de la habitación del vicio en Santiago? Quina risa aquell cap de setmana amb *la droja en el colacao* o el *no está prohibido*, joooder tio...esto va por Montoya! I el ***Padi*** i l'***Òscar***, que demostrem que darrera d'una gran dona, sempre hi ha un gran home (o era al revés?)

Però per PharmaMar ha passat molta gent, uns hi han estat temporades curtes, d'altres en canvi, sempre en formaran part. Deixeu-me que destaqui a la ***Judit***, la nostre mami científica, a qui cal acudir per rebre un bon consell, o el ***Gerardo***, el nostre estimat cubà, un nen atrapat en un cos gran, sempre a punt per narrar la crònica política internacional (*niña, has hecho ya los MALDIs?*). Una altra cubana a qui recordar és l'***Hortensia***, quina dona! El café que hicimos aquella tarde no tiene precio. Ha visto tu marido ya las últimas fotos?? Un beso! I la ***Montse***, a qui vaig acabar apreciant, amb unes grans dosis de paciència! A les Nacions Unides de PharmaMar també hem acollit durant aquests anys a un munt de gent molt especial. Mi primer recuerdo para mi querida ***Mariela***, creo que el secreto aún no ha sido revelado. Després van arribar mi *pana* ***Manu***, *chamo*, sabes que tarde o temprano nos tomaremos unos Sta. Teresa en Venezuela!, la meva altra germana petita, la dolça ***Ivonne***, te extraño muchísimo, no te preocupes que aquella foto nunca saldrá a la luz, i l'adorable ***Ele***, l'italiana més guapa d'Ascoli que sempre m'alegrava amb el seu somriure. Més recentment van arribar la ***Kate***, una altra mami, amb una capacitat incomprensible per entendre el cubà, que sempre intentava tenir cura de nosaltres (thank you Kate for all your wise advices, I miss you) i el ***Thanos***, un grec que va reinventar la petanca, un gran amic de qui sempre recordaré els dies a Finlàndia i les nostres converses (you are the real Big guy, my friend). Un record també per la ***Cami***, mi argentino sigue sin mejorar, i la ***Irene***.

A la porta del costat, al Lab 100, habiten els sintètics, mestres del work-up i de les columnes, sempre disposats a donar un cop de mà. En primer lloc em tocaria parlar del ***Pau***, que en poc temps s'ha convertit en un gran amic. Una persona que val la pena descobrir poc a poc, per deixar que et vagi sorprenent. Ànims Pau, és qüestió de temps que ho treguis, tots els que et coneixem sabem que vals molt. També hi van passar l'***Abdú***, el ***Pablo*** (gracias por tu ayuda en Boston), la ***Núria***, l'agradable ***Estela***, l'inesgotable ***Antonio***, l'irònic ***Roger***, la ***Delia***, qué bueno el ceviche y los licores mejicanos de aquella tarde en tu casa, el ***Carles*** i la **Teresa**, que van animar el laboratori amb el seu humor, i la última promesa incorporada, el ***Xavi***.

Del Lab 300 també m'emporto molts bons amics. De la banda del Fernando tenim un dels últims fitxatges, el ***Ramón***, imitador dels personatges més variats, amb qui he rigut fins a l'esgotament. Treballador incansable i un bon amic de tertúlies futbolístiques i cafès dels dissabtes. La ***Laia***, amb qui *cigarro* rere *cigarro* he forjat una maca amistat (ànims que el pitjor ja ha passat), la ***Marta*** que amb la seva simpatia i el seu riure va revolucionar tot el 300, el ***Tomasso***, el *free-lance* de la recerca, sempre políticament incorrecte i irreverent i el ***Jan***, amb el seu humor tant peculiar, amb qui vaig passar uns moments molt divertits a Finlàndia. I als que ja van marxar: l'***Albert***, el titu més crack del grup, que té aquesta virtut que molt poca gent tant brillant com ell té, la humilitat (aquest any fem triplet fijo!! ens veiem a Cambrils!), el ***Javi*** (alias el navarro), l'entranyable ***Martina*** que irradiava serenitat, el ***Frank***, el ***Jesús***, el ***Pierre***, ...

Abans de passar al Giralt's world, parada obligatòria i reconeixement més que merescut per a l'***Eva***, la persona que fa que tot funcioni com cal, sense la qual, el vaixell s'enfonsaria.

De l'entorn Giralt, tenim al ***Nessim***, amb qui sempre és un plaer tenir una conversa, narrador de les històries més surrealistes, que ha deixat algunes perles per la posteritat, *no me vengas con que a Chuchita la bolsearon*, *te cargó el payaso*! i descobridor d'un dels *hits* mes corejats (el Disco...). ¿Nos vemos en Chicago? També vaig tenir la sort de conèixer als cursos a l'explosiva ***Laia***, la *chacha miringuela* (inoblidable la festeta a Montpellier) sempre a punt per animar el cotarro. Ànims Laia que ja quasi ho tens, un cop triada la lletra, la resta s'escriu sola. A l'***Steph*** i el ***Renaud*** (una parella feta l'un per l'altre), els francesos més sans, esportius i *cool*. Gràcies Steph per ser el nostre Time Out particular i per les primeres masies. Després hi ha la ***Silvia***, una gran científica (arribaràs on vulguis) i cinèfila, sempre disposada a ajudar, amb qui hem patit (i de quina manera) tot el procés del dipòsit i les reiterades visites a la Manoli. No sé jo si sol me n'hagués sortit... La ***Susana***, *who fell in love with Boston*, gràcies per tots els consells Bostonians i el teu PNT, com ja et vaig dir (take it easy!). La ***Giovana***, la meva mentora del masses (la de tardes que nos hemos peleado con nuestro hijo). I molta altra gent: les simpàtiques i agradables ***Laura*** i ***Irene***, l'***Eduard*** (bon chance en Suïsse) l'***Oscar*** (sempre recordaré aquella corba a Finlàndia, vaig veure passar la vida davant meu!!), l'altra mami, la ***Tere***, una gran professional, gràcies pels consells de l'Índia, a la **Txell** gràcies també per la Lonely de l'Índia, l'***Esther***, guardiana de l'invent, que es preocupa per a que el lab rutlli, el ***Roger*** (titu, a la *maison de la chemie* ja saps que toca...), el ***Morteza***, la ***Muriel***, la ***Dolors***, la ***Pili***, el ***Sergio***, el ***Ricard*** (gràcies per l'ajuda a Boston), l'adorable ***Birgit*** i un llarguíssim etc.

Als soterranis del Parc tenim finalment UQC. Capitanejat per la ***Miriam***, que ha tingut sempre l'habilitat de detectar pels passadissos quan estava baix de moral i donar-me els ànims que necessitava. Allà hi tenim la ex-PharmaMar Natàlia, l'encantadora ***Sònia***, el ***Dani Carbajo*** i el ***Dani Pulido***, l'***Anabel*** i el ***Farrera*** (que com no sabia ben bé on posar-te, crec que el millor serà aquí, sort a St Quintin!!). A l'***Ángela***, con quien he tenido el placer de trabajar en el apasionante mundo de los dendrimeros, ya verás como triumfamos con el OBC (Miriam pone el champagne). I els que ja van marxar, com la incombustible ***Glòria*** i el divertit ***Marc*** i a les noves incorporacions, com l'***Alba*** (companya de festivals Apoleros), sort amb la Tesi! Un record també per l'***Aina*** d'Almirall, bona companya de cursos i congressos.

Dels Serveis Cientificotècnics vull agrair a la ***Nieves*** la seva ajuda amb els cultius cel·lulars, a la ***Yolanda*** per fer tant agradables les interminables hores al confocal, a la ***Carmen*** i la ***Gemma*** per cedir-me un espai on preparar les mostres del TEM, i a l'***Elisenda***, professionalitat i amabilitat en una sola persona, per la seva dedicació i paciència amb el TEM. També he de donar les gràcies a l'***Alberto***, per deixar-me utilitzar el seu preuat liofilitzador, en les situacions d'emergència, i a la ***Lia***, per deixar-me la seva targeta, per entrar al laboratori de l'Alberto a liofilitzar a hores més intempestives. A l'equip Lia, també hi ha el ***David*** i l'***Antònia***, sempre disposats a donar un cop de mà i a fer petar la xerrada, fent més amenes les avorrides tardes al MALDI.

Que aquest projecte hagi tirat endavant, ha estat en gran part gràcies a la col·laboració amb el ***Quique*** del Príncipe Felipe de València. Gràcies ***Quique***, perquè de cada reunió amb tu, en sorgia una bona idea (Carles, podries provar a fer...) i a la ***Puig***, que em va introduir en el món del LPS i la septicèmia, la de vegades que m'he rellegit la teva Tesi! També vull agrair a tot el seu laboratori per fer-me sentir com a casa en les meves mini-estades a València. En especial, a la ***Laura***, un esperit lliure que viu la ciència i la vida amb idèntica passió. Laura gracias por acogerme en tu casa, por todas las jornadas maratonianas en el lab, seguidas siempre de cervecitas en los más diversos bares de la noche valenciana (cuanto ambientillo hay en Valencia), costó mucho, pero al final sacamos los papers adelante! Te deseo lo mejor en Australia. I a la ***Lucile***, amb qui, ves tu per on, vam acabar compartint projecte de peptoids, gràcies a la ***María Jesús***, també per accedir a aquesta col·laboració i per la seva predisposició i professionalitat. Però la meva estada de veritat, la vaig fer una mica més lluny, a Boston, la ciutat més "europea" diuen, dels States. I would like to thank ***Professor Dennis Kasper*** for giving me such a great opportunity to work in the amazing field of immunology. I am also in debt with ***Sanna***, from whom I learned everything about LPS-stimulation assays. Things would have been much harder without ***Hachung***, my bench mate, thanks for your every-day support and for helping me that much those months. There were also a bunch of other people to acknowledge: ***Mark***, who showed me how to work with mice (thanks for that Halloween's party), ***Rockan*** (the Iranian-American Eva), ***Julia*** and ***Kate*** (for the European lunches at Children's), ***Chris***, ***Dazza***, ***Fikri***, ***Sun***, ***Ronit***, ***Rachel*** and ***Trent*** and his useful hints with ELISA assays. Però Boston no hagués estat el mateix sense els meus amics *cracks* telecos, el ***JM*** i la ***Patri***. Gràcies per tots els viatges, l'escapada a Canadà, el finde a NY (*don't block the box*, *r u talkin' to me buddy?*), les lobster a Cape Cod, les festes Clandestino, i les post-parties a Fullkerson, les house-warming parties, els dimecres d'alitas al MIT, i els diumenges de fideuàs, sauvingnon blanc i cine al MIT (with butter honey? yes please), per les tornades de festa amb bici, les partides de Wii i les converses caminant per la neu, per Broadway i Mass. Ave.

A la carrera també vaig conèixer gent molt especial, i tot i que hem acabat desperdigats pel món, els any passats a la uni van ser memorables. A l'***Anita***, per tot el que vam arribar a compartir i a l'***Anne***, perquè tot i estar lluny sempre l'he sentit molt a prop. Amb la meva amiga ***Raquel***, podria passar-me nits senceres parlant i rient (*yes we can*) fins arribar a descobrir quins són els temazos que els metges porten a l'Ipod, ànims Reich que tu vals molt! I el ***Carlos***, el químic més brillant i *parres* que hagi conegut mai. Amb qui vaig compartir habitació durant 6 mesos a Manchester. Aquella etapa em va marcar, i sempre he pensat que és la culpable d'haver-me convertit en un cul inquiet. All the best for my dear lost, ***Chiara*** (my little sister, TVB), ***Xtina*** and ***Ditte***. I un petó per la meva ***Meri***, una persona molt especial de qui vaig aprendre moltes coses. Espero que siguis molt feliç.

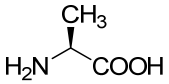
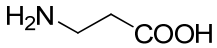
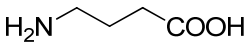
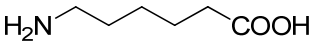
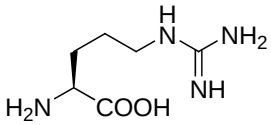
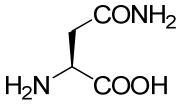
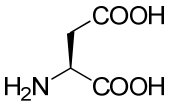
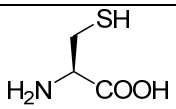
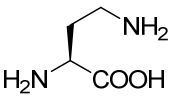
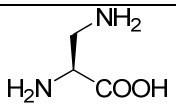
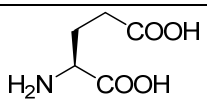
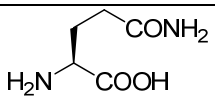
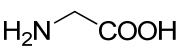
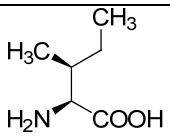
I fora del món de la Química, hi ha els meus amics, que poc a poc han anat entenent de que anava això del doctorat (però Carles quan començaràs a treballar?) i que no saben com mirar-se el tema del post-doc (però Carles ara ja treballaràs?) Crec que hem tingut molta sort de tenir-nos els uns altres, després de tant de temps. Hem crescut junts, i només és qüestió de temps que comencin a sonar les campanes (hagan sus apuestas). Per ordre alfabètic tenim a l'***Albert*** (l'especulador d'esquerres anti-sistema, un gran amic), la ***Carol*** (una bellíssima persona), l'***Edu*** (el

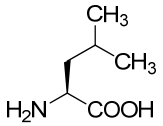
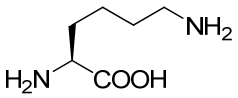
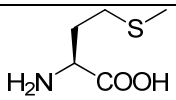
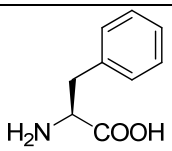
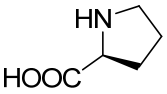
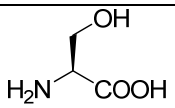
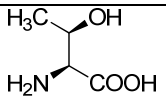
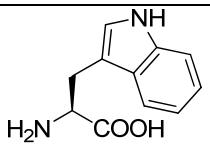
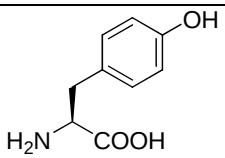
més creatiu i enginyós del grup, relax man!), l'***Infi*** (esta noche se lia), l'***Isaac*** (una noble persona, amic de la infància que sé que mai perdré) el ***Marc*** (qui ens hauria de dir que acabaria enamorat), la ***Mireia*** (con novias así, da gusto tener amigos!) l'***Uri*** (gran company de pis i millor persona), el ***Roy*** (una altra persona molt important amb qui sempre podré comptar), el ***Sergi*** (que per mi sempre serà el *melenas*, una gran persona, ja heu fet un pensament tu i la ***Cindy***??) i la ***Vicky*** (gran amiga des de fa molt anys, i companya de pis i moltes altres coses). També tenim el ***Lluís***, primer company d'habitació a Londres, i més tard company de pis a la Ronda i de mojitos a Cuba (fascinant viatge) i la sempre sorprenent ***Ari***.

Les últimes línies d'aquests agraïments van dedicades a les persones més importants de la meva vida. Als meus ***pares***, la meva petita gran família, a qui he d'agrair tot el seu esforç per donar-me les oportunitats que a ells els hi van ser privades, sense el seu amor i el seu suport incondicional mai hagués arribat a on sóc ara. Us estimo i admiro moltíssim. Mama, lo peor ya ha pasado y ha merecido la pena, tienes que seguir luchando, aun tengo que hacerte abuela (y Marta quiere tener unos cuantos).

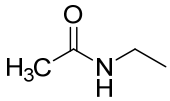
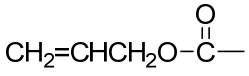
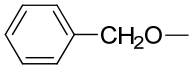
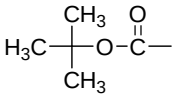
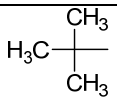
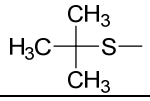
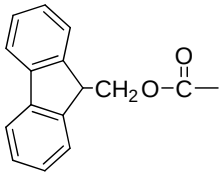
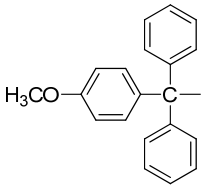
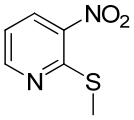
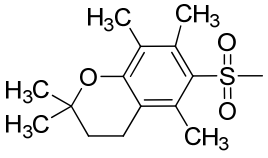
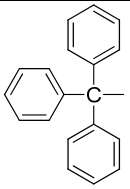
I després hi ha una noia que em va mirar d'aquella manera tant especial, una tarda a les classes d'anglès, i que ha fet que res a la meva vida no torni a ser el mateix. Gràcies ***Marta*** per donar-li més sentit a tot, per omplir-ho de color i música. Per agafar-me ben fort i no deixar-me caure MAI. Per fer-me sentir l'home més afortunat del món cada matí quan em llevo i et tinc al costat, i per començar aquest viatge plegats ja fa molt de temps, que ha viscut moltes etapes, i que encara n'ha de viure moltes, perquè tu fas que tot valgui la pena. T'estimo.

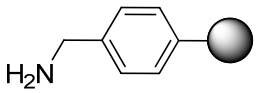
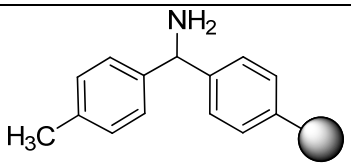
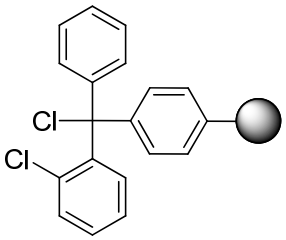
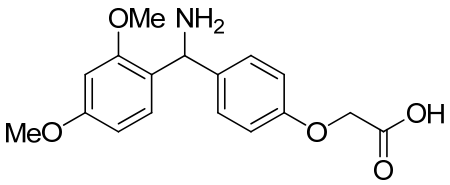
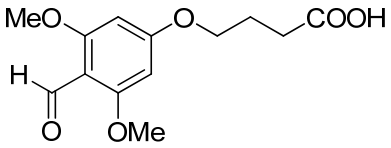
3. PEPTIDE-BASED LPS-INHIBITORS

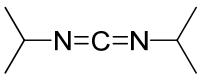
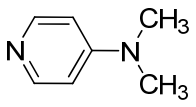
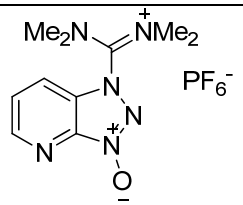
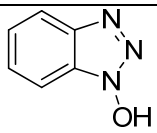
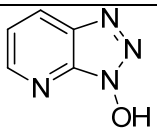
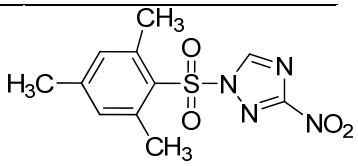
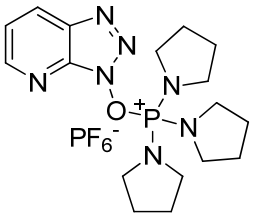
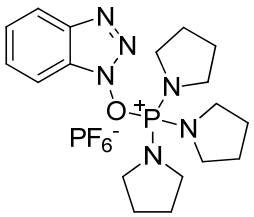
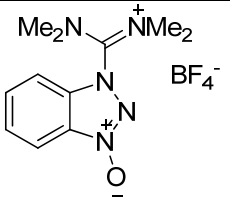
Amino acid	Code*	Chemical structure**
Alanine	Ala, A	
β-alanine	β-Ala	
4-aminobutyric acid	γ-Abu	
6-aminohexanoic acid	ε-Ahx	
Arginine	Arg, R	
Asparagine	Asn, N	
Aspartic acid	Asp, D	
Cysteine	Cys, C	
Diaminobutanoic acid	Dab	
Diaminopropionic acid	Dap	
Glutamic acid	Glu, E	
Glutamine	Gln, Q	
Glycine	Gly, G	
Isoleucine	Ile, I	

Amino acid	Code*	Chemical structure**
Leucine	Leu, L	
Lysine	Lys, K	
Methionine	Met, M	
Phenylalanine	Phe, F	
Proline	Pro, P	
Serine	Ser, S	
Threonine	Thr, T	
Tryptophan	Trp, W	
Tyrosine	Tyr, Y	

***** When possible, the three-letter and the one-letter code for each amino acid are expressed
****** If applicable, amino acids are represented on the L-configuration

Protecting group	Symbol	Chemical structure
Acetamidomethyl	Acm	
Allyl	All	
Allyloxycarbonyl	Alloc	
Benzyloxycarbonyl	Z	
t-Butoxycarbonyl	Boc	
t-Butyl	tBu	
t-Butylthio	tButhio	
9-Fluorenylmethoxycarbonyl	Fmoc	
4-methoxytrityl	Mmt	
3-Nitro-2-pyridinesulfenyl	Npys	
2,2,4,6,7-Pentamethyl-dihydrobenzofurane-5-sulfonyl	Pbf	
Trityl	Trt	

Resin/handle	Chemical structure
Aminomethylated polystyrene	
MBHA resin: Amino-(4-methylphenyl)methyl polystyrene	
2-Chlorotrityl chloride resin (CTC)	
Rink amide linker: 4-(2',4'-Dimethoxyphenyl-aminomethyl)-phenoxyacetic acid	
BAL linker: 4-(4-Formyl-3,5-dimethoxy-phenoxy)butyric acid	

Reagent	Abbreviation	Chemical structure
N,*N*'-Diisopropylcarbodiimide	DIPCDI (DIC)	
4-Dimethylaminopyridine	DMAP	
1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b]pyridinium hexafluorophosphate 3-oxide	HATU	
1-Hydroxybenzotriazole	HOBt	
7-Aza-1-hydroxybenzotriazole	HOAt	
1-(Mesitylenesulfonyl)-3-nitro-1,2,4-triazole	MSNT	
(7-Azabenzotriazol-1-yloxi)-tris(pirrolidino)phosphonium hexafluorophosphate	PyAOP	
Benzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate	PyBOP	
1-[Bis(dimethylamino) methylene]-1H-benzotriazolium tetrafluoroborate 3-oxide	TBTU	

Abbreviations:

Ac	Acetyl
ACH	α-Cyano-4-hydroxycinnamic acid
BSA	Bovine serum albumin
CD	Circular dichroism
CLSM	Confocal laser scanning microscopy
cmc	Critical micellar concentration
COSY	Correlated spectroscopy
DCM	Dichloromethane
DIPEA	*N*,*N*-Diisopropylethylamine (also DIEA)
DKP	Diketopiperazine
DMEM	Dulbecco's modified essential medium
DMF	*N*,*N*'-Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPH	1,3-Diphenyl-1,3,5-hexatriene
EDT	1,2-Ethanedithiol
ELISA	Enzyme-linked immunosorbent assay
equiv	Equivalent
ESI	Electrospray ionization
FBS	Fetal bovine serum
HPLC	High performance liquid chromatography
HR	High resolution
HSQC	Heteronuclear single quantum coherence
MALDI	Matrix-assisted laser desorption ionization
MS	Mass spectrometry
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Microwave
NMM	*N*-Methylmorpholine
NMR	Nuclear magnetic resonance
PAGE	Polyacrylamide gel electrophoresis
Palm	Palmitoyl
PBS	Phosphate buffered saline
PDA	Photo diode array
PEG	Polyethylene glycol
pNA	*p*-Nitroalanine
QSAR	Quantitative structure-activity relationship
RP	Reverse phase
SAR	Structure activity relationship
SPPS	Solid phase peptide synthesis
SDS	Sodium dodecylsulfate
TBME	*tert*-Butyl methyl ether
TEA	Triethylamine
TEM	Transmission electron microscopy
TIPS	Triisopropylsilane (also TIS)
TOF	Time of flight
TFA	Trifluoroacetic acid
UV	Ultraviolet

Note: Abbreviations used for amino acids, protecting groups, resins, linkers and coupling reagents can be found in the preceding annexes. Abbreviations for more specific terms will be properly described thorough the text.

1. INTRODUCTION AND OBJECTIVES

1. 1 INTRODUCTION

Life on the edge

At the 30th European Peptide Symposium (30EPS) held in Finland (Aug-Sept 2008) I attended a very interesting talk given by Christopher Dobson about the origins of protein misfolding and the diseases associated with this biological process. What really captured my attention in that seminar was the idea that the boundary between normal folding and aberrant behavior is extremely narrow. In other words, complex biological systems generate a vast range of functions with an astonishing degree of specificity in their chemical processes. But these systems work on the edge, close to the limit that separates health from disease.

Is not cell survival ruled by this same concept? Cells live, proliferate and die under a myriad of external stimuli and control mechanisms that often work in harmony. However, when the equilibrium between life and death is biased, a number of pathological conditions appear.

$$\text{Life} \Longleftrightarrow \text{Death}$$

If cells proliferate in an uncontrolled manner and the regulatory mechanisms fail, normal cells become immortal cancer cells. In contrast, if cells die prematurely, a series of neurodegenerative diseases, among other conditions, might arise.

This idea can also explain immune disorders. The immune system comprises a complex network of cells and proteins that protect organisms from potentially harmful invaders. One of the main functions of the immune system is to fight infections. This response aims to preserve health in living organisms.

$$\text{Infection} \Longleftrightarrow \text{Response}$$

If the immune system does not respond effectively to infection, several pathological conditions arise. The effects of immunodeficiency in humans are widely known. But what happens when the immune system is over-stimulated? What are the effects of an uncontrolled immune response?

Autoimmune diseases are just one example of an unspecific immune response. But there is another clinical syndrome that results from an exaggerated and unspecific immune response: **sepsis**.

In the following pages sepsis and related disorders will be introduced. The immunopathogenesis of sepsis will be described and the treatments and therapies assayed will be discussed. Finally, the aim of the present doctoral thesis will be presented.

1.1.1 SEPSIS: AN OVERVIEW

Sepsis is a syndrome very complex to define, diagnose and treat. The condition of sepsis results from a harmful or damaging host response to infection. Exposure to bacterial endotoxins induces a systemic inflammatory response that involves a series of clinical symptoms such as fever and increased heart and respiratory rates among others. This process may be self-limiting or proceed to severe sepsis or septic shock, life-threatening conditions that usually end with the death of the patient.

Defining a syndrome

Sepsis is as a clinical syndrome defined by a systemic response to infection. However, conflicting terminology and confusing semantics have long impaired communication in this field. Before 1992, the terminology used to define a systemic response to infection varied widely. This can be explained because despite the high incidence, mortality and costs of sepsis, there was (and still today exists) a lack of explicit patient phenotypes. Or in other words, the clinical diagnosis of sepsis depends on signs and symptoms. The inexistence of specific clinical criteria presented a serious problem as clinical trials multiply and researchers and physicians demanded a common language to unify the terminology and facilitate communication.

For this reason, in 1992 a consensus conference of the American College of Chest Physicians (ACCP) established a set of definitions for sepsis and related disorders.[1] The ACCP introduced the term "*systemic inflammatory response syndrome*" (**SIRS**) to describe any systemic inflammatory process independent of cause. SIRS may present a series of clinical symptoms such as hyper- or hypothermia, tachycardia, hypoventilation and leukocytosis. **Sepsis** was defined as a "*systemic inflammatory response to infection*", that is SIRS caused by a presumed or confirmed infection. **Septic shock**, a severe case of sepsis, was defined as "*sepsis-induced hypotension, persisting despite adequate fluid resuscitation, along with the presence of hypoperfusion abnormalities or organ dysfunction*". As sepsis progresses, at late stages of septic shock various organ dysfunctions may appear such as acute renal failure (ARF), acute respiratory distress syndrome (ARDS) or disseminated intravascular coagulation (DIC). The presence of

more than one of these organ dysfunctions is named multiple organ dysfunction syndrome (MODS). The interrelationship between SIRS, sepsis and infection is shown in Figure 1.1. Also, ACCP definitions are summarized in Table 1.1.

In 2001, in a second consensus conference, clinicians and researchers agreed in the usefulness of these definitions.[2] A better definition of sepsis, based on biological markers is still required. However, to date, there has not been sufficient data to provide new alternative definitions. In the present work ACCP definitions will be used.

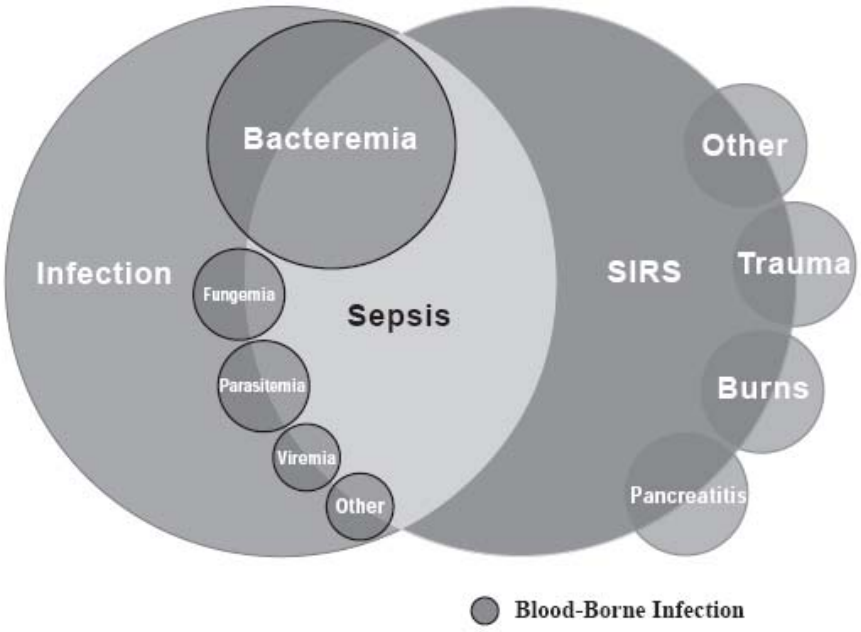


Figure 1.1: The systemic inflammatory response syndrome, SIRS, might have distinct causes, such as burns or trauma. When there is an infection, mainly bacterial, SIRS is termed sepsis. From ref [3].

Table 1.1: Consensus conference definitions of sepsis and related disorders[3,4]

Syndrome	Definition
SIRS	2 or more of the following: - Temperature > 38ºC or < 36ºC - Pulse > 90 beats/minute - Respiratory rate > 20 breaths/minute or $PaCO_2$ < 32 mm Hg - White blood cells > 12,000/mm^3 or < 4000/mm3 or > 10% immature forms
Sepsis	SIRS due to suspected or confirmed infection

Severe sepsis	Sepsis associated with organ dysfunction, hypoperfusion or hypotension
Septic shock	Sepsis-induced hypotension, persisting despite adequate fluid resuscitation along with the presence of perfusion abnormalities

Sepsis in numbers: the burden of sepsis

If even for experts it has been so difficult to meet a consensus in the terminology of sepsis, it is not strange that the concept "sepsis" usually remains unknown for the general public. However, the numbers of sepsis are serious. Sepsis represents the 1st cause of mortality in Intensive Care Units (ICU).[3,5] There are approximately 750,000 cases per year in the U.S. In this country sepsis accounts the 2% of all hospitalizations and it is estimated that in 2020 there will more than 1 million cases of sepsis annually.[6,7] According to the Centers for Disease Control and Prevention (CDC), septicemias were the 12th leading cause of death in the U.S. in 1997.[8] Only two years later, in 1999, sepsis was ranked as the 10th foremost cause of death.[9] Recently published preliminary data for 2006 still mentions sepsis as the 10th cause of death.[10] Other reports estimate that up to 215,000 deaths in the U.S and 135,000 in Europe are caused by sepsis every year.[7,11] Worldwide, over 18 million cases of severe sepsis per year are reported.[7]

The risk of death increases as sepsis progresses to severe sepsis and septic shock, phases in which hospital mortality reaches values of 45-50%.[12] Although the mortality rate has decreased over the last 20 years, the increase in the number of patients with sepsis has resulted in a tripling of the number of sepsis-related deaths. This is caused by the high lethality of sepsis that ranges from 20 to 40 % (on a 28-day mortality basis). The increase in the occurrence of sepsis over time can be explained, in part, as a result of i) the medical and technological advances associated with (more invasive) treatments ii) the increasing number of elderly or debilitated people (with compromised immune systems) and patients with underlying diseases (e.g. cancer), who require therapy, and iii) the widespread use of antibiotics, which is leading to the growth of drug-resistant microorganisms.[6,7]

The costs of sepsis and related-disorders are also notable. In the U.S. care for septic patients costs per year more than $16 billion, and near €7.6 billion in Europe.[3,7] Only in Spain, the economical burden is estimate to be of €345 million annually.[11] The fact that septic patients require an average of 20 hospital days, and that they need admission in ICUs in more than half of the cases, support this data.

1.1.2 THE IMMUNOPATHOGENESIS OF SEPSIS

The immune system represents a complex network of mechanisms that protects organisms against microorganisms and pathogens. The host's protection from infection is based on layered defenses of increasing specificity. The first line of protection consists of physical barriers (skin and mucosa) that prevent pathogens such as bacteria, viruses and parasites from entering the organism. If a pathogen breaches these barriers, the innate immune system provides an immediate but non-specific response. However if pathogens successfully evade the innate system, a third layer of protection is activated, the adaptive immune system. This system responds to the infection and adapts to it. The adaptive immune system improves its recognition of the pathogen and "memorizes" it (the so-called immunological memory), allowing the immune system to respond faster in future encounters. Both immune systems are correlated. The innate stimulates the response of the adaptive, which, in turn, uses the machinery of the innate system to attack pathogens.[13,14]

Recognition of bacterial endotoxins by the innate immune system

As introduced before, microorganisms or toxins that are able to enter into the organism will encounter the cells and mechanisms of the innate immune system. Recognition of these antigens may trigger the innate response. But how can the innate immune system discriminate between self (molecules within the organism) and non-self (pathogenic molecules)? Microorganisms are recognized via pattern recognition receptors (PRR) and serum proteins that identify often at high affinity what have been called *pathogen-associated molecular patterns* (PAMPs). [15] This recognition activates the signaling cascades that initiate the induced response. Without this response, the host will surely succumb to overwhelming infection. However, paradoxically, it is this

enhancement of immunity what eventually leads to the pathological condition of sepsis. In Gram-negative bacteria, lipopolysaccharide (LPS; also known as endotoxin) plays a major role.[16] However, other microbial components may elicit an immune response. Gram-positive bacteria do not present lipopolysaccharides in their structure but contain peptidoglycan and lipoteichoic acids (bacterial cell-wall components will be described later). Although their role in clinical sepsis is uncertain, evidences exist about their pro-inflammatory activity.[17,18] Other bacterial components that can induce septic shock are cell-wall structures such as flagellin,[19] and unmethylated CpG sequences in bacterial DNA.[20] Nevertheless, since the inhibition of LPS represents the principal aim of this Doctoral Thesis, this endotoxin will be described with particular interest.

Basic biology of lipopolysaccharide (LPS)

LPS is the molecule that origins sepsis and it is responsible of many infection diseases.[21] LPS is the major component of cell wall in Gram-negative bacteria. The bacterial cell wall is found around the outside of the plasma membrane and is mainly made of peptidoglycan (also called murein). Peptidoglycan is composed of amino-sugars (*N*-acetylglucosamine and *N*-acetylmuramic acid) that are crosslinked by short peptide chains attached to *N*-acetylmuramic acid. Among other functions, peptidoglycan gives structural strength and rigidity and counteracts osmotic pressure of the cytoplasm. This peptidoglycan layer presents several differences between Gram-positive and -negative bacteria. Gram-positive bacteria possess a thick peptidoglycan layer with several lipoteichoic and teichoic acids. In contrast, the cell wall in Gram-negative bacteria comprises a thinner peptidoglycan layer and an outer membrane containing lipopolysaccharides and lipoproteins. The LPS molecule is embedded in this outer membrane and it is anchored to the cell wall by its lipidic portion, the lipid A (Figure 1.2).

As shown in Figure 1.3, the molecular structure of LPS consists of two distinct regions:[22] a hydrophilic carbohydrate portion and a hydrophobic moiety, a glycolipid called lipid A.[23,24]

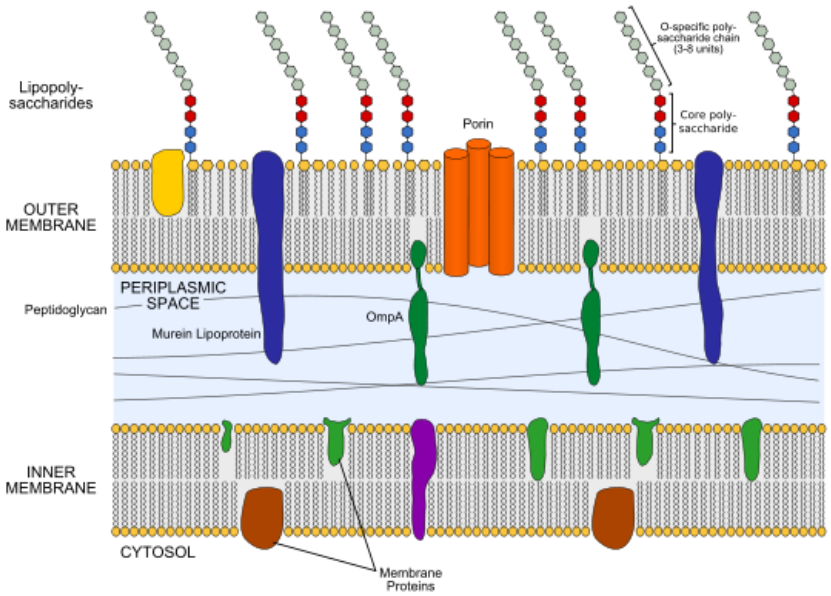


Figure 1.2: Enlarged section of bacterial cell wall in Gram-negative. LPS is anchored to lipidic environment of the outer membrane by its lipidic portion.

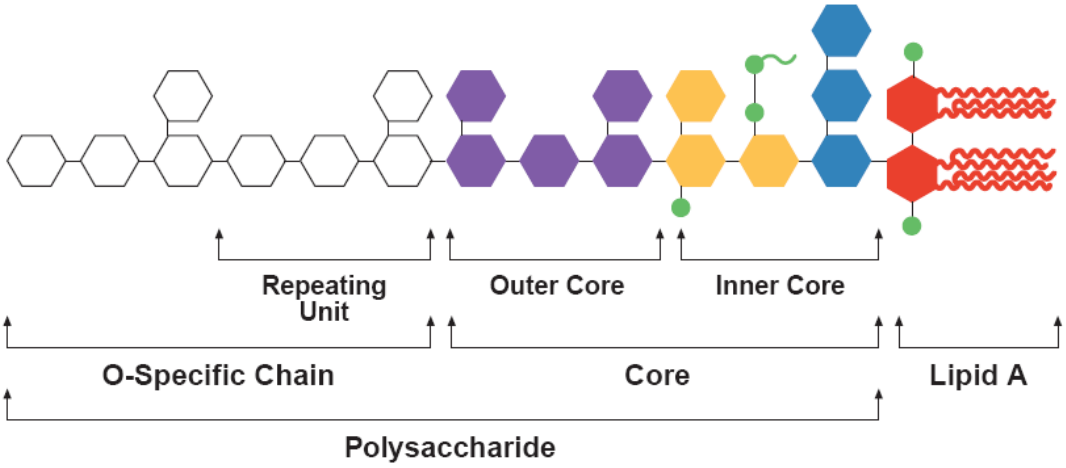


Figure 1.3: Schematic view of bacterial lipopolysaccharide (LPS) structure. From ref [22, 23].

The polysaccharide portion comprises an O-specific chain, an immunogenic, highly variable, repeating polysaccharide that extends into the external medium; and an inner and outer oligosaccharide core region. The O-specific chain confers resistance to phagocytosis and bactericidal agents and holds a considerable structural variability between bacterial species.[21,22] Variations in this chain also produce two different morphological types of Gram-negative bacterial growth in culture: the “rough” LPS, that contains a short O-specific chain; and the “smooth” LPS, that bears a long chain. This variation is responsible of the differences within several bacterial strains in terms of virulence and resistance.[22]

The lipid A is formed by a 1,4'-bis-phosphorylated glucosamine disaccharide that carries several amide- and ester-linked fatty acids (Figure 1.4). This moiety confers the endotoxin molecule its toxicity.[25,26] An unequivocal evidence of that was reported in a series of studies using synthetic molecules. In these, the lipid A, independent from all carbohydrate constituents, showed the same toxicity as the whole endotoxin.[27] The lipid A structure is highly conserved in all species of Gram-negative bacteria. Nonetheless, lipid A molecules of different organisms present some structural differences. The main differences are: i) the number of phosphates attached to the glucosamine backbone (0, 1 or 2), ii) the number of acyl chains (3 to 7), iii) the length of these acyl chains (C10 to C28), and iv) the presence or absence of branched, unsaturated or substituted acyl chains.[28,29]

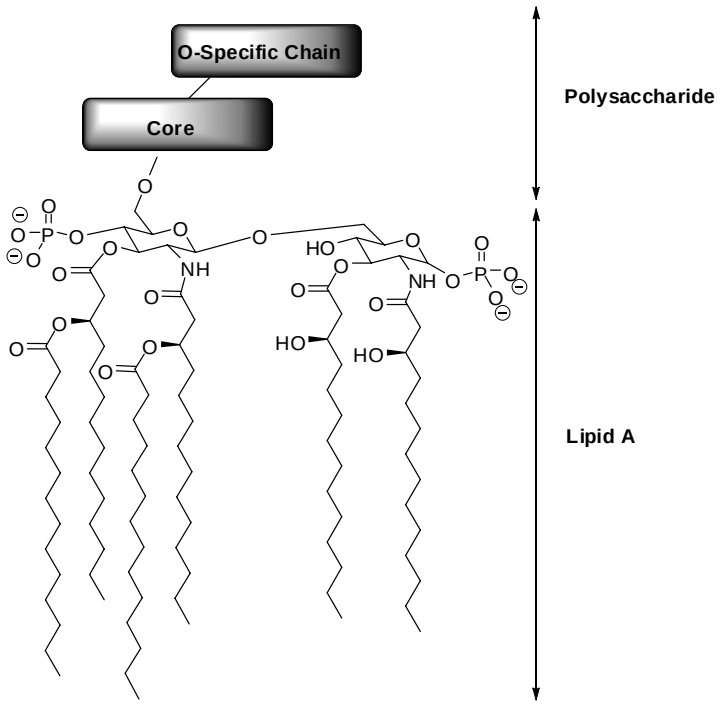


Figure 1.4: General structure of LPS showing the chemical detail of the lipid A moiety. This general representation corresponds to the lipid A of *Escherichia coli*.

Members involved in host recognition of microbial components

For many years the identification of an "LPS receptor" represented a challenge for researchers in order to understand how Gram-negative bacteria initiated the septic shock. It was not until 1990 that it was shown that activation of immune cells depended on the presence of LPS-binding protein (LBP) and the receptor CD14.[30] Hence, after

endotoxin release from Gram-negative bacteria into blood or serum, LPS is bound to LBP among other serum lipoproteins. LBP transfers LPS to the CD14 receptor. CD14 can be found in two forms: a membrane bound CD14 (mCD14), a glycosylphosphatidylinositol-linked molecule anchored in the cell surface; or in the circulation as a soluble CD14 receptor (sCD14).[16] This explains how cells that are constitutively CD14 negative (i.e. they do not present the CD14 receptor in their membranes) are still able to interact with LPS by sCD14. Moreover, levels of sCD14 rise in septic patients,[31] and antibodies to CD14 have shown protection in primates from septic shock.[32]

Toll-like receptors

Despite the great advance in the understanding of host response to LPS provided by the description of CD14, there was a question that remained unclear. The mCD14 presented no intracellular tail, thus another receptor was required for the LPS-LBP complex to elicit cell activation. The mystery was resolved by the discovery of the family of Toll-like receptors (TLR).[33,34] To date, a family of ten TLR has been identified with a wide range of ligand specificity for diverse sources of infection.[35] In this regard, TLR4 is the LPS receptor whereas TLR2 is mainly responsible for the recognition of Gram-positive cell-wall structures.[36] TLR5 recognizes flagellin,[37] and TLR9 is the receptor for bacterial DNA.[38] A summary of bacterial components and the TLR that recognize them is shown in Table 1.2.

Table 1.2: Ligand recognition in bacterial sepsis[39]

TLR/host receptors	Bacterial ligands
TLR4	Lipopolysaccharide
TLR1 + TLR2	Triacylated lipopeptides
TLR2 + TLR6	Diacylated lipopeptides, lipoteichoic acids (from Gram-positive bacteria)
TLR5	Flagellin
MHC class II	Bacterial superantigens
NOD2	Muramyl dipeptide (from peptidoglycan)
TLR9	Bacterial DNA

However, the association of one bacterial ligand with only one unique receptor is, in fact, an oversimplification. For instance, it has been described that a number of lipopolysaccharides, such as those from *Bacteroides fragilis*, *Chlamydia trachomatis* and *Pseudomonas aeruginosa*, signal via TLR2 and not via TLR4.[40] The structural differences in their lipid A molecules may explain this behavior.

The whole picture is finally completed with the cell-surface molecule MD-2. This molecule is required for activation of TLR4. Experimental data support that, since MD-2 knock-out mice do not respond to LPS challenge and therefore survive endotoxic shock.[41] It seems that MD-2 may have the function of positioning TLR4 correctly on the cell surface. The signaling pathway of LPS via TLR is depicted in Figure 1.5. The intracellular domain of TLR, TIR (Toll/IL-1 receptor homology domain) binds to IRAK (IL-1 receptor-associated kinase). This process is mediated by two adapter proteins, MyD88 (myeloid differentiation protein 88) and TIRAP (TIR domain containing adapter protein). This binding induces TRAF6 activation (TNF receptor-associated factor-6), what eventually leads to nuclear translocation of NF-κB (nuclear factor κB) an event that ultimately activates cytokine gene promoters.[16] Additional signaling pathways have also been proposed (Figure 1.5). However, some of these mechanisms are still unclear. For this reason, and for the sake of clarity, only the TLR signaling pathway has been described.

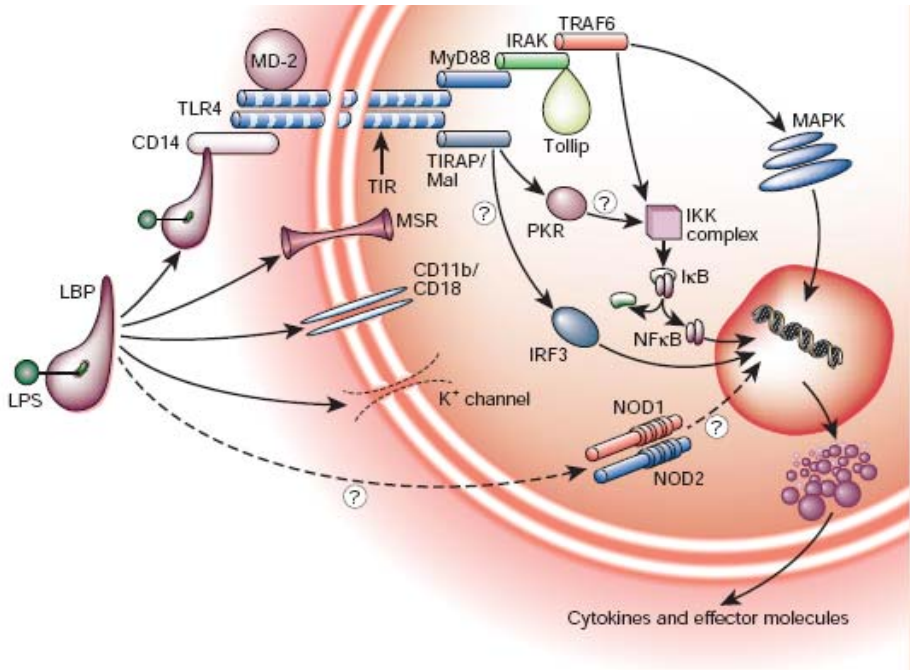


Figure 5: The complex LBP-LPS binds to the TLR4-MD2 receptor. This event activates a signaling pathway that eventually leads to the activation of cytokine expression. From ref [16]

Signal amplification

The initial host-microbial interaction is followed by the activation of the innate immune response. This process defines the pathological condition of sepsis and is extremely complex. A detailed description of the entire inflammatory cascade would be very extensive and is far beyond the scope of this introduction. For this reason, only the most important events will be reviewed:

i) the production of pro-inflammatory cytokines at early stages of the infection,
ii) the disorders in coagulation associated with sepsis,
iii) the immunosuppresion in acute sepsis
iv) and the fatal organ failure that represents the main cause of mortality in patients with septic shock

Moreover, together with the initial recognition of endotoxin by the innate immune system, all these processes, and the molecules involved therein, have been classically targeted for the treatment of sepsis. Therefore, they will be discussed from a therapeutical point of view, later in this introduction.

Pro-inflammatory cytokines and other mediators of inflammation

After recognition of microbial components in the circulation, innate immune cells such as macrophages release a number of pro-inflammatory cytokines and other mediators. Cytokines are a class of signaling proteins and glycoproteins that are used extensively in cellular communication. Cytokines are used to activate and recruit further immune cells to increase the system's response to the pathogen.

The pro-inflammatory cytokine tumor necrosis factor-alpha (TNF-α) is one of the earliest and most important mediators of the LPS-induced immune response.[42] TNF-α is mainly produced by macrophages and is released during the first 30-90 minutes after exposure to LPS. Another cytokine involved in immune defense against infection is interleukin-1 (IL-1), which has a pro-inflammatory activity similar to that of TNF-α. However, this cytokine is not able (alone) to induce septic shock. The third classic pro-

inflammatory cytokine is interleukin-6 (IL-6), an important mediator of fever and the acute-phase response.[43] These cytokines, in addition, activate a second level of inflammatory cascade that includes cytokines (IL-12, IL-15, IL-18), chemokines, lipid mediators and reactive oxygen species. The effects produced by these mediators contribute to the pathogenesis of sepsis. These effects are diverse and are summarized in Table 1.3.

Table 1.3: Mediators involved in the pathogenesis of sepsis[16]

Mediators	Main effects
Cytokines	Activate other cells of the immune system (e.g neutrophils and lymphocytes)
	Activate vascular endothelium
	Upregulate cellular adhesion molecules
	Synthesis of postraglandins, nitric oxide synthase and acute-phase proteins
	Induce fever
	Activate coagulation cascade
Chemokines	Mobilize and activate neutrophils and other inflammatory cells
	Activate macrophages
Lipid mediators	Activate vascular endothelium
	Regulate vascular tone
	Activate extrinsic coagulation cascade
Oxygen radicals	Antimicrobial properties
	Regulation of vascular tone

The primary role of sepsis-associated inflammatory mediators release is to enhance leukocyte infiltration. This process, also known as leukocyte *extravasation*, involves the penetration of immune system cells from their usual location in the blood to the site of injury or infection. The process of leukocyte movement from the blood to the tissues through the blood vessels is known as extravasation, and comprises three main steps: i) leukocyte localization and recruitment to the endothelium local site of inflammation (this step requires the activation of cellular adhesion molecules of the endothelium); ii) migration across the endothelium into the tissues, known as *transmigration* (this migration is stimulated by chemokines gradients that stimulate leukocyte movement; and iii) movement of leukocytes within the tissues via chemotaxis.

TLR4 activation via LPS stimulation also results in the production of inducible nitric oxide synthase (iNOS), in both immune cells and vascular tissue, leading to release of nitric oxide (NO).[44] NO-induced vasodilatation allows slowing of blood flow, what permits tethering of leukocytes such as neutrophils to the vessel walls. At the same time, TNF-α, and IL-1 activate the vascular endothelium, enhancing adhesion molecule expression. Neutrophil transmigration will help to bacterial clearance in affected tissues. However, this process is not exempt of complications. On the one hand, adherence of leukocytes to blood vessels may cause leukopenia (decrease in the number of circulating leukocytes in the blood) and blood vessel and capillary injury.[22] In the other hand, the pass of neutrophils through the vessel walls is accompanied by a significant amount of intravascular fluid that may provoke edema as observed in cases of severe sepsis. Also, the release of pro-inflammatory granules and enzymes by neutrophils directed to kill bacteria, can result in catastrophic damage in organs such as the lung.[39] Another harmful effect associated to NO release is hypotension in patients with shock or severe sepsis.[45,46] NO is a potent vasodilator, a vascular relaxing agent, and it is responsible of the precipitous drop in blood pressure associated with septic shock.[47] Figure 1.6 represents some of these inflammatory processes that occur in sepsis.

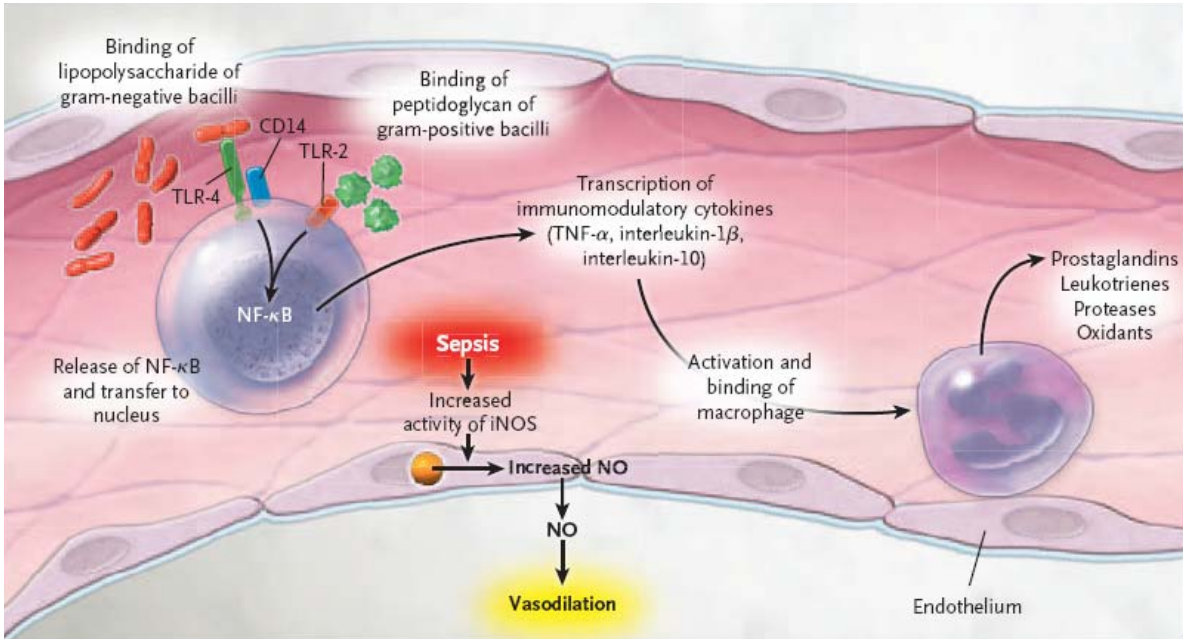


Figure 1.6: The inflammatory response of sepsis causes directly and indirectly widespread tissue injury. Bacterial challenge activates a signaling pathway that leads to the release of immunomodulatory cytokines such as TNF-α, IL-1 and IL-10 (IL-10 function will be described later). Sepsis increases the activity of iNOS, which increases the synthesis of NO, a potent vasodilator. Cytokines also activate endothelial cells by upregulation of cell adhesion molecules.

This induces the binding of cells like neutrophils and macrophages to endothelial cells. The products released by immune cells (proteases, oxidants …) injure endothelial cells impairing their normal functions: selective permeability and vasoregulation among others. This leads to increased permeability and further vasodilatation.[48] Figure from ref [48].

Furthermore, TNF-α, IL-1 and IL-6 coordinate initiation of the acute phase response (APR). This response aims to prevent ongoing tissue damage, isolate and destroy malignant infective organisms and activate the repair processes necessary to restore the host's normal function.[49] APR is characterized by leukocytosis (high number of neutrophils in blood), fever, alterations in the metabolism of many organs (increased gluconeogenesis,[50] muscle catabolism, altered lipid metabolism[51,52] and others) and activation of both complement and coagulation cascades.[53] APR involves the upregulation of a series of proteins called acute-phase proteins (APPs).[54] There are two types of APPs in function of the cytokine that induces their expression. A first group is comprised by proteins such as C-reactive protein and serum amyloid A. These proteins play a role in antibacterial immunity and are induced by TNF-α and IL-1. In the course of an infection their expression is enhanced up to 1000-fold. The second group includes fibrinogen and α2 macroglobulin, which are induced by IL-6. Some of these proteins have a clear role in immune stimulation while others have obscure and yet to be discovered functions.[39]

Finally, pro-inflammatory cytokines display also an important role in the activation of the coagulation cascade.

The coagulation cascade

Cytokines have an important role in inducing a procoagulant effect in sepsis. As previously introduced, septic shock patients often present disseminated intravascular coagulation (DIC). In fact, this severe clinical form is found in 30-50% of septic patients.[55] DIC is a pathological activation of coagulation (blood clotting) mechanisms that leads to the formation of small blood (fibrin) clots inside the blood vessels throughout the body. As a result of that, normal coagulation is disrupted and abnormal bleeding may occur (such as vascular leak) along with consumption of platelets and prolongation of clotting times. Also, these fibrin deposits disrupt adequate tissue perfusion (normal blood flow to organs) provoking organ failure. The formation of

fibrin clots is the net result of two different processes: the enhanced production of fibrin and the malfunction of normal regulatory fibrinolytic mechanisms (Figure 1.7).

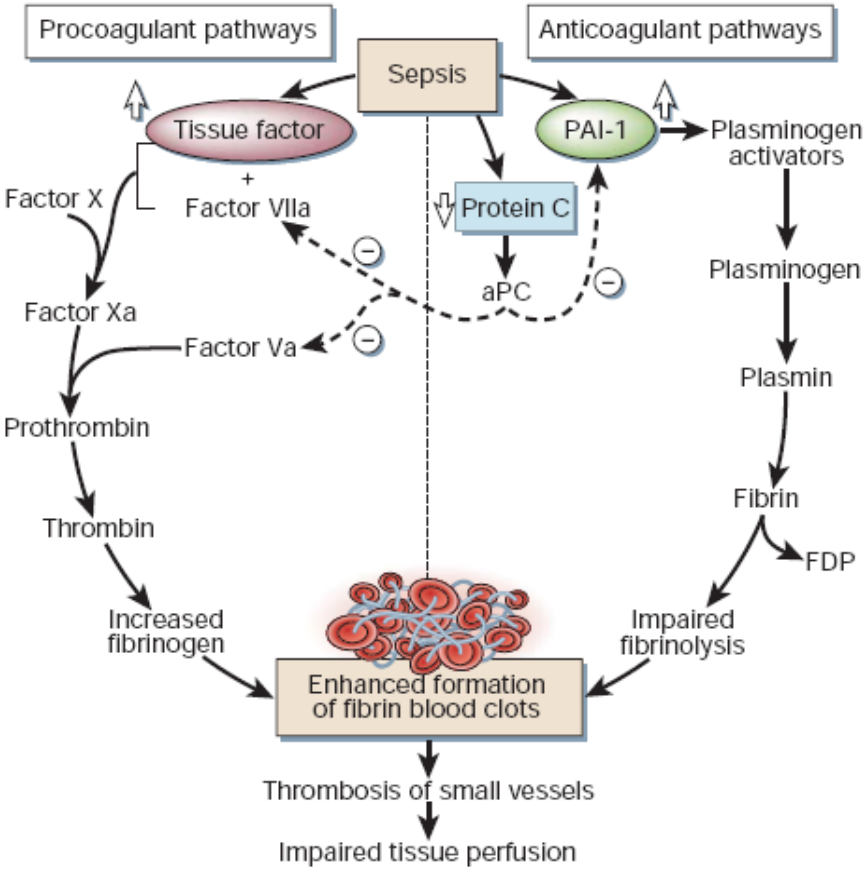


Figure 1.7: Distortion of the normal homeostatic balance between coagulant and anti-coagulant pathways in sepsis. From ref [16].

TNF-α, IL-1 and IL-6 are powerful inducers of coagulation. LPS-induced coagulation pathways start with the enhanced expression of tissue factor (TF), which leads to an increase in the production of prothrombin that is converted to thrombin. In turn, thrombin generates fibrin from fibrinogen. Simultaneously, the normal homeostatic anticoagulant pathway is impaired by high levels of plasminogen-activator inhibitor type-1 (PAI-1) that prevents the normal generation of plasmin from plasminogen, and thus failure of fibrinolysis. Another important cause of disorders in coagulation is the downregulation of three anticoagulant proteins: antithrombin, protein C and tissue factor pathway inhibitor (TFPI).[56] These proteins are not only important for being anticoagulant but also for their anti-inflammatory properties. These include for example the inhibition of the activation of the transcription factor NF-κB.[57] Of these three

proteins, protein C has gained a special interest. Protein C can be converted to its activated form (aPC) when thrombin complexes with thrombomodulin, an endothelial transmembrane protein. Once aPC is activated it dissociates from an endothelial protein C receptor (EPCR) before binding to protein S (Figure 1.8).[58] aPC is able then to inactivate factors Va[59] and VIIa[60] (coagulation factors involved in the coagulation cascade, see Figure 1.8) and also to inhibit PAI-1 activity.[61] So far, replacement of aPC in septic patients has been the only therapy approved by the FDA in the U.S. The use of aPC as a drug to treat sepsis will be discussed later on.

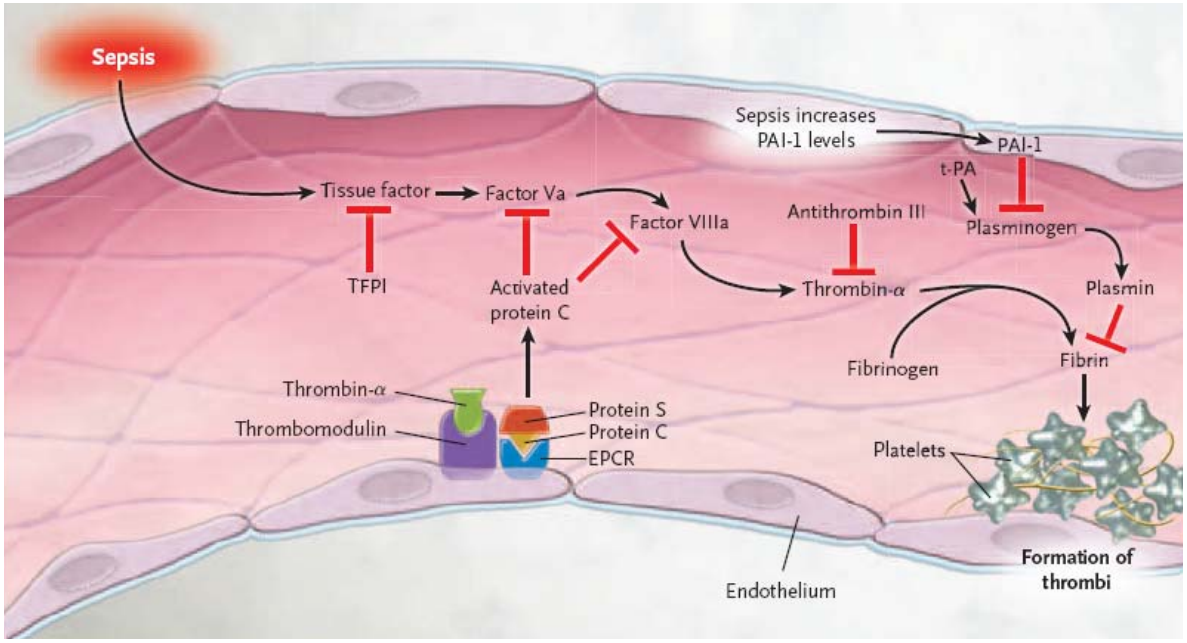


Figure 1.8: Schematic view of the procoagulant response in sepsis where the activation of aPC and the proteins involved are shown. From ref [48].

Immunosuppresion and apoptosis in sepsis

The pro-inflammatory response that occurs in sepsis is balanced by regulatory anti-inflammatory mechanisms that attempt to restore the immunological homeostasis. However, this counter-regulatory response is not necessarily always beneficial for the host. Within the molecules of the counter-inflammatory response are antagonists such as the soluble TNF receptors (sTNFR) and IL-1 receptor antagonist, inactivators of the complement cascade and anti-inflammatory cytokines, of which the most important is IL-10. [62] As a result of that, circulating monocytes from septic patients are

hyporesponsive to pro-inflammatory stimuli when compared with normal cells.[63] This state has been defined as an immunological exhaustion due to excessive systemic inflammation[64] or, as it has been named, the compensatory anti-inflammatory response syndrome.[65]

Another process that occurs in the downregulation of sepsis is the development of lymphocyte apoptosis and therefore septic patients are often lymphopenic (condition of having a low count of lymphocytes in blood).[66] Apoptosis plays an ambiguous role in sepsis. In addition to early removal of lymphocytes (cells that should not be removed), it also delays the removal of neutrophils (cells that should be removed). If neutrophils remain activated for prolonged times, they have the potential to cause organ injury.[67,68] This immunosuppressive state (diminished monocyte response and enhanced lymphocyte apoptosis) may be the cause of an inadequate host response to infection, and hence can be viewed as a potential "mediator" of sepsis in stead of a regulatory state. What is more, it may increase the risk of nosocomial infections, a cause of mortality in critically ill patients who survive the initial septic episode. While apoptosis may be intended to repair damaged tissues, increased cellular apoptosis also might contribute to organ dysfunction and immunosuppresion in sepsis.[66] Reversal of this state has been regarded to be of therapeutic value, as show studies where mice transfected with the human gene bcl-2 (a gene that overexpresses the protein Bcl-2, which has anti-apoptotic properties) are protected from death after LPS administration.[69,70] All in all, this contributes to add a further degree of complexity to the pathological condition of sepsis. The idea that immunosuppresion may origin death in septic patients, opposes the classical view of sepsis as a syndrome mainly caused by an enhanced immunostimulation. Today, several strategies aiming to immunostimulate septic patients have encountered different results and constitute the subject of extensive research.[71,72]

Mechanisms of organ failure

Organ failure represents the ultimate cause of death in severe cases of sepsis. Patients may first develop a single organ failure, such as ARF or ARDS (see pages 7-8) and progressively develop failure of other organs if the pathological condition persists. The severity of organ dysfunction is closely related to survival in ICUs. In general, if 4-5 organs fail, the mortality is greater of 90%, independent of the treatment.[16]

There are many factors involved in the mechanisms of organ failure, and these are generally complex and not totally understood. However there are two main responsible factors: tissue hypoperfusion and hypoxia. The term hypoperfusion refers to the shortage of the blood supply to an organ, whereas hypoxia denotes a shortage of the oxygen supply. Both restrictions can be originated by the coagulation disorders discussed above. In this sense, the accumulation of fibrin deposits cause microvascular occlusion and compromises a proper tissue perfusion. But there are also other causes. For example, the development of tissue exudates (fluids that filter from the circulatory system into lesions or areas of inflammation) prevents cells from an adequate oxygenation. Also, cellular infiltrates, such as neutrophils damage tissues directly by releasing lysosomal enzymes and superoxide-derived free radicals. Cytokines can also stimulate the expression of the nitric oxide synthase, which releases nitric oxide. Nitric oxide has several harmful effects: it can cause vascular instability and hypotension, myocardial depression and damage mitochondria.[73,74] The latter provokes dysoxia in cells. That is the inability to utilize available oxygen by cells.

Overall, the immunopathogenesis of sepsis is extremely complex and heterogeneous, and today still are mechanisms not totally understood. Remick made this point in a recently published review: “At this point in time, the literature richly illustrates that no single mediator/system/pathway/pathogen drives the pathophysiology of sepsis.”[75] The next section will try to discuss the principal strategies assayed for the treatment of sepsis and explain why there has been such a great deal of failure in this area so far.

1.1.3 STRATEGIES FOR THE TREATMENT OF SEPSIS

Given the extraordinary complexity and heterogeneity of the sepsis syndrome, the choice of an appropriate, clinically-relevant therapeutic target has long represented a major challenge for clinicians and researchers. Moreover, the lack of consistent and rapid diagnostic tools that difficult sepsis diagnosis, or the diversity of patients with distinct underlying diseases, have added additional difficulties to the finding of effective treatments. This has been translated to a considerable number of clinical trial fails and therefore notable frustration in the field.

Nevertheless, the continuous developments in understanding the immunopathology of sepsis are providing new insights into the disease mechanisms of this condition. In this sense, over the last ten years several new promising targets have been described, and one drug, activated protein C (aPC) is now on the market for the treatment of critically-ill severe sepsis patients.

Despite these advances, there is still a long way to walk. Numerous promising drugs with great activities in septic animal models are inactive in humans. And unfortunately, aPC presents serious side-effects that leave this drug too far from being the “ideal” drug.

The next section will focus on the principal strategies pursued for the treatment of sepsis. These strategies are classified in function of the biological mediator targeted for inhibition, and are listed below:

1. The inhibition of bacterial components
2. The blockage of pro-inflammatory cytokines
3. The inhibition of other inflammatory mediators
4. The inhibition of members of the coagulation cascade
5. The reversion of the immunosuppresion state and the inhibition of apoptosis
6. Non-pharmacological approaches

Special emphasis will be put in the biological rationale beyond these approaches and the reasons of failure.

1. The inhibition of bacterial components

Targeting bacteria

The administration of antibiotics directed to kill infecting organisms represents the first therapeutic approach in the current treatment of sepsis. However, in patients with sepsis, the site of infection and the type of microorganisms responsible are often unknown. The rising prevalence of fungi, Gram-positive bacteria and highly resistant to antibiotics Gram-negative bacteria make a good choice of antibiotics difficult. What is more, several studies indicate the outcome of sepsis is worse if the causative microorganisms are not sensitive to the original antibiotic treatment.[76,77] In addition, even if bacteria are successfully killed, their residual LPS may be able to continue triggering the immune response. In this regard it has been demonstrated that antibiotic treatment of Gram-negative bacteria might increase the amount of circulating LPS and therefore enhance the septic outcome.[78]

Targeting LPS

The inhibition of LPS has represented an interesting alternative and so far one of the most famous targets. LPS is the earliest and most important mediator of sepsis and its inhibition is, still today, considered to be a very attractive and promising strategy. Early in 1969, treatments with antiserum to endotoxin were shown to be beneficial in LPS-challenged mice,[79] but its application in numerous clinical trials did not provide convincing results.[80] Also, in addition to LPS antiserum, the lipid A has been targeted with mouse (E5)[81] or human (HA-1A)[82] monoclonal antibodies. These antibodies were evaluated in multicentre clinical trials. Data pooled from six published studies recently reviewed,[83] reveals a poor 1.1% reduction of the 28-day mortality. Only one study achieved a statically significant improvement in survival;[82] however, in the largest of these studies, there was a trend towards increased mortality for non-septic patients treated with anti-lipid A antibodies.[84]

Other strategies

Other strategies to neutralize endotoxin have been evaluated using LPS-binding proteins. This is the case of bactericidal permeability-increasing protein (BPI) or cationic antimicrobial protein 18 kDa (CAP 18). These and other proteins with endotoxin-neutralizing activity will be described in detail later in the introduction of the first chapter of this Thesis.

Finally, other approaches include the administration of high-density lipoprotein or lipid compounds to sequester circulating LPS[85] and the use of synthetic TLR4 antagonists.[86] There are currently two TLR4 antagonists in clinical phase: E5564 (eritoran) and TAK-242.[87] However, blocking TLR may lead to inappropriate immune responses such as allergies or immune tolerance, and thus the risks and benefits of manipulating TLR require further investigation.[88]

2. The blockage of pro-inflammatory cytokines

Targeting TNF-α and IL-1

For many years investigators and clinicians believed that the problem of sepsis was simply an excessive and uncontrolled production of pro-inflammatory molecules. The solution for sepsis seemed rather simple: block inflammation, save lives. For this reason, cytokines have classically constituted an attractive target for researchers. This was led by a series of evidences:

i) Increased levels of TNF-α in septic patients were correlated with an increased risk of death.[89]

ii) Injection of TNF-α into experimental animal models resulted in widespread inflammatory alterations[90] and tissue injury,[91] similar to that observed in septic patients.

iii) Animal models injected with lethal LPS doses displayed elevated levels of cytokines such as TNF-α.[92,93]

iv) And, inhibition of pro-inflammatory cytokines improved survival in animal shock models.[94]

These findings initiated a series of clinical trials aimed at blocking TNF-α or IL-1. The results are summarized in Tables 1.4 and 1.5.

Table 1.4: Clinical trials with TNF-α inhibitors[75,95] (and references cited therein)

Year	No. of patients	Inhibitor	Outcome
1993	80	Murine antibody	Safety study. Increased IL-6 predicted mortality
1995	42	Humanized antibody	Safety study. Reduction in circulating cytokines
1995	994	Monoclonal antibody	Significant reduction in mortality at day 3 but not day 28
1996	122	Antibody fragment	No improvement in survival, but patients with high IL-6 levels appeared to benefit
1996	141	p75-soluble receptor	Higher mortality with highest dose of receptor
1996	564	Monoclonal antibody	More rapid reversal of shock, but no significant reduction in mortality at day 28
1997	498	p55-soluble receptor	Trend towards reduced mortality, but not significant
1998	92	Chimeric antibody	No reduction in mortality or cytokine levels
1998	1879	Monoclonal antibody	No improvement in survival
2001	944	Antibody fragment	Patients classified by IL-6 levels, no improvement in survival
2001	1342	p55-soluble receptor	No improvement in survival
2004	2634	F(ab') monoclonal antibody	Patients classified by IL-6 levels, TNF-α inhibition resulted in improved survival
2006	81	Sheep antibody	No reduction in 28-day mortality, decreased circulating TNF-α and IL-6

Two main strategies targeting circulating TNF-α have been evaluated: soluble TNF receptors and specific neutralizing anti-TNF antibodies. TNF-α signals via one of two receptors named TNFR1 (p55) and TNFR2 (p75).[96] These receptors are cleaved from the cell membrane and released into the circulation where they compete for the binding of circulating TNF-α. Therefore, they could serve also to inhibit TNF-α bioactivity. In example, the p55 construct has been evaluated in two studies. Although there was an encouraging trend towards reduced mortality in a Phase II trial with 498 patients,[97] the

therapeutic signal in a larger Phase III trial (1342 patients) was weak and statistically insignificant (see Table 1.4).[98] In another study, the administration of p75 resulted in an increase in mortality.[99] The therapy using monoclonal anti-TNF antibodies has had a somewhat slightly greater success. As summarized in Table 1.4, several trials have been completed. And despite the differences in the outcome for each study, on average they show a statistically significant reduction of 3.5% in mortality.[83] The largest of these trials, with 2634 patients, reported a 3.5% reduction of 28-day mortality and a 5.6% reduction of mortality in patients with elevated IL-6 levels.[100]

As shown in Table 1.5, the results obtained targeting IL-1 were even more disappointing.

Table 1.5: Clinical trials using the IL-1 receptor antagonist[75,95] (and references cited therein)

Year	No. of patients	Inhibitor	Outcome
1994	99	IL-1 receptor antagonist	Dose-related reduction in APACHE* score
1994	893	IL-1 receptor antagonist	No reduction in 28-day mortality
1995	26	IL-1 receptor antagonist	Reduction of mediator release
1997	696	IL-1 receptor antagonist	No improvement in survival

* APACHE scores are a measure of acute severity of illness. It includes acute (A) physiology (P), age (A) and chronic (C) health (H) evaluation (E).

The IL-1 receptor antagonist (IL-1ra) is a protein that is released later than the release of the initial pro-inflammatory cytokines. This protein shares a 22% degree of sequence homology with IL-1, but it is inactive, and acts therefore as an endogenous competitive inhibitor of IL-1.[101] The amount of IL-1ra released is considerably greater than that of IL-6, indicating the important regulatory role of this protein in the counter-inflammatory response. [102] However, none of the clinical trials using IL-1ra demonstrated an improvement in survival (see Table 1.5).

So, if TNF-α and IL-1 are among the most important inflammatory mediators in sepsis, why this approach did not represent any clear improvement in survival in clinical trials?

A frequent explanation is that the administration of cytokine blockers in those clinical trials was simply not quick enough. In other words, patients often come to medical

attention relatively late in the disease, and at that time, neutralizing these early cytokines, may be just too late. Other authors also suggest that often the animal models studied in septicemia do not reproduce the clinical situation in humans. Still today exists debate concerning on the best animal model for the study of sepsis.[103]

Targeting other cytokines: HMGB1 and MIF

In this regard, the identification of two new cytokine-like macrophage products that are released long after LPS stimulation is regarded as a new and promising approach to reduce mortality in sepsis. The first one is high mobility group B1 (HMGB1).[104] The release of this protein seems to be triggered by apoptotic tissue damage, and can itself initiate TLR signaling:[105,106] it induces release of TNF-α, IL-1, IL-6, activation of cell adhesion molecules and NO production. It was observed that after 24h injection of LPS to mice, the concentration of this protein in serum was elevated, much later than the initial peak of TNF-α and IL-1 had declined. In this sense, mice could be rescued from septic shock by administering an antibody to HMGB1, even 2 h after a lethal LPS injection.[107] Subsequently, it was shown that patients with sepsis displayed elevated levels of HMGB1 in serum, and that higher levels were associated with higher risk of mortality. More recently, it has been published that ethyl pyruvate, which inhibits HMGB1 production *in vivo*, improves survival in murine septic models 24 h after the onset of sepsis.[108] These findings convert HMGB-inhibition in a viable option for further clinical trials.

The second macrophage-derived cytokine considered as a potential candidate is macrophage migration inhibitory factor (MIF). MIF was initially described as a product in T cells, and was shown to be produced in the pituitary gland during endotoxemia. MIF is also necessary for TLR4 expression and can override the immunosuppressive effects of glucocorticoids, explaining its broad immunostimulatory activity.[109,110] Mice with a disruption in the gene that encodes MIF are resistant to LPS-induced shock.[111] And inhibition of MIF prevents from septic shock in models of clinical peritonitis.[112] In addition to that, it seems that MIF also mediates shock caused by Gram-positive bacteria, such as the toxic shock syndrome associated to *Staphylococcus aureus*,[113] suggesting that MIF could have a broader spectrum of application in sepsis treatment.

3. The inhibition of other immune and inflammatory mediators

The use of corticosteroids

Corticosteroids are a class of steroid hormones that have a potent anti-inflammatory activity through various mechanisms, including the prevention of dissociation and nuclear translocation of NF-κB and inhibition of the bioactive lipid phospholipase A_2 (PLA_2), an active mediator of inflammation.[114] Cortisol, a corticosteroid hormone, is released by the adrenal gland in response to infection and tissue injury. Malfunction of the adrenal gland and the inability to produce cortisol have been documented in sepsis with adverse effects.[115,116] Therefore, administration of exogenous cortisol could both repair an inadequate response to stress and also reduce inflammation.[117]

The history of the use of corticosteroids extends back to 1963, when high doses of corticosteroids were used to modulate the inflammatory response.[118] But several clinical trials performed subsequently did not show significant improvement in survival in septic patients.[119] Recently, a new approach based on the administration of low-dose of corticosteroids has been proposed. This strategy resulted in a successful Phase III clinical trial.[120] This study showed significant survival improvement in patients with vasopressor-dependent septic shock who showed an abnormal response to adrenocortitropic hormone (ACTH) stimulation (ACTH induces the release of cortisol). This presumed mechanism of action is confirmed by another study that shows how the administration of corticosteroids increases the amount of circulating cortisol.[121] In another study, the treatment of patients with acute respiratory distress syndrome (ARDS) with methylprednisolone was associated with improvement in lung function and therefore reduced mortality.[122] In this case methylprednisolone entailed a reduction in TNF-α and IL-6 concentrations as well as NF-κB activation. Moreover, another study showed that prolonged treatments with this glucocorticoid resolved systemic inflammation and acquired glucocorticoid resistance.[123]

The clinical trials with corticosteroids are interesting because they highlight how both the dose and the timing of the corticosteroid treatment are important for a successful

treatment of sepsis. Two multicentre studies of corticosteroid replacement in septic patients are currently in progress in Europe.[83]

Targeting NO

The effects of NO in septic shock are important and have been already discussed. NO is generated from arginine by nitric oxide synthase (NOS). NOS has different isoforms that can be inhibited, nonspecifically, by $_L$-*N*-monomethyl arginine ($_L$-NMMA)[124] and *N*-omega-nitro-$_L$-arginine methyl ester ($_L$-NAME).[125] Alternatively, one of the products activated by NO, guanylate cyclase, can be inhibited by methylene blue.[126,127] NO is a potent vasodilator, and its inhibition using the previous molecules has resulted in an increase in blood pressure, and therefore a decrease in the need of vasopressors. However, several side-effects have been observed such as the reduction in cardiac output and the increase in pulmonary artery pressure. These effects might be, in part, responsible of the increased mortality of septic patients in a recent Phase III clinical trial using L-NMMA.[128]

Other strategies

The number of other mediators of inflammation that have been targeted is too extensive to be listed and new promising targets and clinical trial appear annually. A short description of the most relevant is summarized next.

1) **The complement system**: The complement system constitutes a series of serum proteins that helps clearing pathogens from an organism. These proteins interact in a cascade of activation steps and belong to the innate immune system. These proteins, when stimulated, cleave other proteins to release cytokines and display a great number of pro-inflammatory effects. Excessive production of one of these proteins, C5a, occurs early in sepsis leading to unregulated cytokine release, tissue damage and organ failure.[5] Recent work suggests that blocking C5a or its receptor C5aR with antibodies might have a therapeutic effect.[129,130] In fact, blocking C5a has been successful in patients with ischemia and heart complications due to inflammation.[131] These agents therefore represent a potential approach to treat sepsis.

2) **Bioactive lipid mediators**: Concomitantly with the release of pro-inflammatory cytokine occurs the generation of bioactive lipids from cell membrane complements. Enzymes of the phospholipase A_2 (PLA_2) family catalyze the conversion of cell membrane phospholipids to arachidonic acid (a precursor for the synthesis of prostaglandins and other molecules involved in inflammation) and platelet activating factor (PAF).[132]

The expression of PLA_2G2A, an isozyme of the PLA_2 family, is induced by LPS and several cytokines.[133] Elevated levels of circulating PLA_2 have been found in critically ill septic patients and are predictive of multiple organ failure.[134] Several small molecules that inhibit PLA_2G2A have been developed,[135] but a Phase II clinical trial of a PLA2 inhibitor (Ly315920) did not represent any improvement in survival.[136]

PAF has also been targeted for inhibition. Receptors for PAF are expressed on platelets and cells of the innate immune system. Interaction of PAF with its receptor activates multiple intracellular pathways that contribute to acute inflammatory injury. Two strategies have been used to neutralize PAF. First, using antagonists of the PAF receptor: BN 52021 (Glinkolide B), TCV-309 and BB-882 (Lexipafant). Unfortunately, pooled data from six clinical trials that enrolled 1,279 patients did not show a statistically significant reduction in mortality[83 (and references cited therein)]. The second strategy involved the administration of PAF acetylhydrolase (an enzyme that inactivates PAF). This treatment resulted in a significant improvement in the 28-day mortality in an unpublished Phase II clinical trial. However, a larger Phase III trial with 1,250 patients was discontinued for futility.[137]

4. The inhibition of members of the coagulation cascade

The coagulation system has been an especially important target for the treatment of sepsis because of the serious implications it has in the development of abnormalities in tissue perfusion and the consequent organ failure. Also, the coagulation and inflammation systems have a close relationship. Hence, anticoagulant therapies also represent, to some extent, anti-inflammatory treatment. To date, three anticoagulant proteins, that are downregulated in sepsis, have been studied in clinical trials, with

uneven results: antithrombin (also known as antithrombin III; AT-III), tissue factor pathway inhibitor (TFPI) and protein C (see pages 20-21, Figures 1.7 and 1.8).

Administration of AT-III and TFPI

AT-III inactivates thrombin by forming AT-thrombin complexes that are cleared by the liver. It also inactivates several coagulation factors such as Factor Xa. Furthermore, it possesses anti-inflammatory properties that are independent of its anticoagulant effects. For instance, inhibiting thrombin blocks NF-κB activation and NO release.[138] For these reasons, AT-III supplementation has been evaluated in a small number of clinical trials. Initial trials with 122 patients showed a 23% reduction in the 28-day mortality;[139] however, in a larger Phase III trial (2314 patients) no substantial improvement in survival was observed.[140] These discouraging results were also obtained for TFPI. TFPI is a serine protease inhibitor that exerts its anticoagulant effects by inhibiting the complex TF-factor VIIa in the presence of Factor Xa. An initial, unpublished Phase II study with 210 patients showed a trend towards a reduced mortality in TFPI-treated patients.[141] However, these effects were not reproduced in a larger (>2000 patients) Phase III trial.[5]

Drotrecogin alfa (activated): Xigris®

Despite these failures, one therapy did work. As previously commented, activated protein C (aPC) has several anti-coagulant effects (inactivates Factors Va and VIIa, and inhibits PAI-1 activity). But it has also other important properties: it decreases apoptosis,[142] adhesion of leukocytes,[143] and cytokine production,[144] all key features in the immunopathogenesis of sepsis. During human sepsis, there is considerably consumption of proteins C and S, as well of downregulation of thrombomodulin, what results in lower levels of aPC. In various animal models of sepsis and clinical Phases I and II, aPC-treatment resulted in improved survival.[145] In 2001, a recombinant human form of aPC (*rh*aPC), named Xigris, *drotrecogin alfa (activated)*, by Eli Lilly & Co. (Indianapolis, IN) was used in clinical trials: Worldwide Evaluation in Severe Sepsis, PROWESS.[146] This trial was terminated earlier because a significant improvement in the mortality rate was reached according to practice guidelines. Overall, the risk of death was reduced a 6.1% (30.8 % of 28-day mortality for the placebo group, compared

with 24.7% for the drotrecogin alfa group). A reduced activation of the coagulation pathway and a decrease in the cytokine production were documented. A detail study revealed that in fact, only patients at high risk of death (two or more failing organs or APACHE II score ≥ 25) showed a substantially improved mortality of 13% (44% placebo, 31% drotrecogin alfa). The data suggested that *rh*aPC treatment in patients with a lower risk of death lacked efficacy or could even be harmful.

On the basis of these results, in November 2001, the Food and Drug Administration (FDA) approved drotrecogin alfa for the treatment of sepsis in the U.S. (in the EU the drug was approved in 2002). The approval of aPC was controversial, with half of the FDA panel voting to require a confirmatory trial due to methodologic and other important problems with the PROWESS study.[147] The registration of a new drug, with a new mode of action, usually requires a second verifying trial. However, in the case of *rh*aPC, the FDA took an unusual but pragmatic decision: the drug was registered for use in the subgroup with the most severe disease. Associated with this limited approval, the FDA asked Lilly to perform additional testing in selected subgroups (septic patients with a lower risk of death). As a result of that, a second, larger, clinical trial named Administration of Drotrecogin Alfa in Early Severe Sepsis (ADDRESS) was done. The initial requirement from the FDA was to enroll 11350 patients, but only 2613 patients were included since the study was prematurely terminated for futility. This study demonstrated that patients at low risk for death had no improvement in survival and had a significantly increased risk of bleeding if treated with drotrecogin alfa.[148] The absence of a beneficial treatment effect, coupled with an increased incidence of serious bleeding complications indicated that drotrecogin alfa should not be used in patients with severe sepsis who are at low risk for death, such as those with single organ-failure or APACHE II < 25. In addition, in this study, even for patients with a high risk of death that would match the present description of drotrecogin alfa, not positive effect was observed. This data compromised the robustness of the PROWESS study, and raised awareness and questioning of the use of Xigris.[147,149]

Overall, it is clear that although the use of aPC may improve survival in critically-ill severe septic patients, is not a panacea for all patients. In deed, a recent study, the Resolution of Organ Failure in Pediatric Patients with Severe Sepsis (RESOLVE) had

to be terminated earlier due to an absence of therapeutic effect in pediatric patients and serious risks.[150] The risk of severe bleeding is viewed as one of the principal drawbacks. This issue and the cost-effectiveness of this drug constitute a matter of intense discussion and debate in the literature.[147,149,151,152,153] In particular, Eichacker, Natanson and Danner, in a very interesting and polemic article, accused Lilly of having initiate false reports of a shortage of the drug to improve sales as part of a marketing strategy. Also the authors claimed that Lilly had funded several campaigns, such as the Surviving Sepsis Campaign to promote guidelines for sepsis management that would serve their own financial goals. The authors therefore questioned the arbitrariness of guidelines adopted by several medical societies/campaigns funded by companies.[147]

There is still a final question: why aPC treatment resulted in improved outcomes in clinical trials, whereas AT-III and TFPI did not? This could be attributed to a broader spectrum of anti-inflammatory mechanisms for aPC. However, definitive data for such a theory does not exist to date.

5. The reversion of the immunosuppresion state and the inhibition of apoptosis

Immunostimulatory molecules

Sepsis has always been described as a syndrome resulting from an overwhelming an excessive activation of the immune system, resulting in an uncontrolled inflammatory response. However, several aspects of the immune responsiveness are reduced in sepsis, and septic patients become hyporesponsive to pro-inflammatory stimuli when compared to normal patients. This state of immune suppression may be dangerous for the patient (i.e. risk of new infections) and therefore several strategies have tried to reverse it.

The growth factors granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factors (G-CSF) stimulate the production of monocytes and neutrophils. In a clinical trial using G-SCF to treat 701 patients with pneumonia and severe sepsis, there was no improvement in survival.[154] In another, smaller study with 58 patients, GM-SCF administration did not improve survival either, but did decrease the length of hospitalization and improved other clinical parameters.[71] Today, there are

concerns that promoting neutrophil production could aggravate organ injury. Whether this strategy may be beneficial or not remains unclear.

Interferon-γ (IFN-γ) is a cytokine produced by immune cells that activates macrophages. The use of IFN-γ to reverse immune suppression cleared sepsis in eight of nine patients;[72] however its use in a larger clinical trial with 416 trauma patients did not reduce infections or overall mortality, though it reduced deaths due to infections.[155] The clinical utility of IFN-γ remains unproven.[156]

Apoptosis

As discussed earlier, in the course of sepsis there is evidence that lymphocytes undergo rapid apoptosis, whereas neutrophils show delayed apoptosis. The former may be the reason for immunosuppression in sepsis; and the latter, results in enhanced tissue damage. In experimental models of sepsis and inflammation, over-expression of anti-apoptotic proteins such as B-cell lymphoma-2 (Bcl-2) and inhibition of caspases have resulted in reduction of lymphocyte apoptosis and decrease in mortality of animals.[69,157] These results warrant further studies with anti-apoptotic agents in clinical sepsis. In this sense, VX-799, a new caspase inhibitor, was well tolerated in animals and will be soon tested in a clinical setting.[5]

6. Non-pharmacological approaches

Non-pharmacological approaches to the treatment of sepsis have recently shown utility. This is the case of what has been called *early, goal-directed therapy*.[158] In this study carried out in 2001 with 263 patients, 6 h of goal-directed therapy resulted in a 16% mortality reduction in patients with severe sepsis and septic shock. The mortality was decreased at 28 and 60 days, as well as the duration of hospitalization. This approach involves aggressive fluid resuscitation to balance oxygen delivery with oxygen demand before the admission to the ICU. The mechanisms of the benefit are believed to be the reversal of tissue hypoxia and a decrease in inflammation and coagulation defects.[48] Also, the tight control of blood glucose levels within normal limits resulted in improved survival and reduced organ dysfunction.[159] However, a

recent study using insulin to correct glucose levels did not represent any improvement in survival.[160]

And finally, if everything fails, a 2001 paper in BMJ suggested that prayer can alter the clinical course of sepsis.[161]

The story so far: summary of treatments and reasons of failure

The following table (Table 1.6) summarizes the treatments described in this introduction and their result in clinical trials. The table is deliberately not exhaustive and a number of other strategies (not appeared in the text) may be currently at different stages of evaluation. The purpose of this introduction was to give a clear and comprehensible review of the most important and representative pathways targeted for the treatment of sepsis, rather than an exhaustive and meticulous report of all the strategies found in the literature.

Overall, sepsis represents a clinical syndrome whose pathophysiology reflects the activation of an innate host response to infection. The apparent simplicity of this definition involves a complex process that still today challenges researchers and clinicians. More than 70 clinical trials addressed to more than 200 potential mediators of inflammation have been reported. Despite the frustrating degree of failure, one new therapy has made it to the market, *drotrecogin alfa (activated)* and the use of corticosteroids has found a successful clinical application. In addition to that, since 2001, consensus guidelines for the management of sepsis have been reported directed to emergency care to early stages of sepsis (0 to 6 h) and treatment for patients in later stages who require critical care.[162] These include early, goal-directed therapy,[158] plus lung-protective ventilation,[163] broad-spectrum antibiotics and possibly activated protein C administration (for severe cases).[146]

Table 1.6: Summary of targets, mediators of sepsis, potential treatments ad results in clinical trials.[48]

General target	Mediator	Treatment	Results of clinical trials
Bacterial components	Bacteria	Antibiotics	Not evaluated
	LPS	LPS antiserum	Negative
		LPS-binding proteins	Negative
	Lipid A	Monoclonal antibodies	Negative
Innate immunity	TLR4	TLR4 antagonists	Currently in clinical phase
	Macrophages	GM-CSF	Negative
		IFN-g	Negative
	Neutrophils	G-CSF	Negative
Pro-inflammatory cytokines	TNF-α	Soluble TNF receptors	Negative
		Monoclonal antibody	Mixed results
	IL-1	IL-1 receptor antagonist	Negative
	IL-6	IL-6 antagonist	Not evaluated
	HMGB1	Monoclonal antibody	To be evaluated
		Ethyl pyruvate	To be evaluated
	MIF	Inhibitor/antibody	To be evaluated
Inflammation	Various	Corticosteroids	Positive
	NO	$_{L}$-NMMA	Negative
	Protein C5a	Antibodies	To be evaluated
	PLA_2	Ly315920	Negative
	PAF	PAF antagonists	Negative
		PAF acetylhydrolase	Negative
Coagulation cascade	AT-III	AT-III administration	Negative
	TFPI	TFPI administration	Negative
	aPC	Recombinant human aPC	Positive
Apoptosis	Lymphocytes	Caspase inhibitor	To be evaluated
Hypoxia	Various	Early, goal-directed therapy	Positive

Not evaluated: the drug failed to reach clinical trials in pre-clinical models

Negative: denotes that no statistically significant improvement in survival has been observed

Currently in clinical trials: the drug is being tested at the time of writing this work

Mixed results: different studies show different results; though, overall the therapeutic effect is considered negative

To be evaluated: the drug has succeed in pre-clinical models, and may be evaluated in clinical trials

Positive: denotes that statistically significant improvement in survival has been observed

Reasons of failure

The history of clinical trials reveals that even extremely successful results from animal models of sepsis have not been translated into clinical efficacy in humans. The reasons for failure are many:

1) *Inadequacies of simple animal models*. One common problem is that animal models of sepsis do not necessarily reproduce the clinical situation in humans. For instance, murine models of septicemia often are obtained after lethal doses of LPS. The overwhelming release of pro-inflammatory cytokines observed in this model differs greatly than the clinical situation of sepsis in humans, where substantial differences are found in the cytokine profile. Recent reviews explore the disconnection between animal models of sepsis and human sepsis.[103,164]

2) *Absence or loss of the agent's biological activity*. Related to the first point, it is not unusual that the apparent biological activity of a certain agent (e.g. a recombinant protein) *in vitro* or in a simple animal model, is not reproducible in human sepsis. A rapid clearance or a rapid metabolic inactivation may be plausible reasons.

3) *Redundancy of the immunogenic cascade.* Interfering with one single inflammatory mediator, such as TNF-α, IL-1, NO, etc. is unlikely to alter the complex course of the sepsis syndrome.

4) *Inappropriate dose, duration or timing of therapy*. For most therapies used in clinical trials the optimal dose and duration of therapy are unknown. This constitutes one of the main issues in the treatment of sepsis. A standard practice in ICU is to define a dose to obtain a clinical efficacy (for example, to study the dose of insulin that will achieve a normal blood-sugar level in a diabetic patient). However titration of therapy in sepsis trials has not been possible. Another important limitation is that the optimal time to initiate therapy is unknown. This is of paramount importance. Late administration of a therapy that targets an early mediator, such as a pro-inflammatory cytokine, might be ineffectual at modifying the course of the disease when progression is no longer dependent on the target of therapy. This will be the case for example of LPS-neutralizers or TNF-α inhibitors. Conversely, when therapy is administered too early,

the target may just not be present. aPC supplementation is another good example. It can be beneficial if administered at late stages of sepsis, but inefficient or even harmful at early stages of sepsis.

5) *Heterogeneity of the target population*. The fact that sepsis is defined as a clinical syndrome and that diagnosis is based basically on symptoms (fever, tachycardia, leukocytosis…) very unspecific presented by a very heterogeneous patient population, represents a major challenge in sepsis treatment. Indeed, several experts claim that the failure of many clinical trials was mainly caused by wrong criteria in patient enrollment.[5,75] For example, LPS may contribute to Gram-negative sepsis but not to sepsis due to Gram-positive bacteria. Therefore, interventions aimed at neutralizing LPS may not be appropriate in all cases. In this sense, if the therapy seeks the evaluation on anti-endotoxin agents, patients should not have organ dysfunction at study entry. Another study showed that anti-TNF treatment can be more effective in patients with elevated levels of IL-6.[100] In this case, TNF inhibition could be of therapeutic value for this subgroup of patients, regardless of lacking beneficial effect in other septic patients. The degree of organ dysfunction should also be taken into account. Patients with the greater degrees in organ dysfunction are good candidates for aPC treatment, while others without organ dysfunction may not benefit from this treatment. In this regard, novel anticoagulant therapies should be addressed to patients with coagulopathy rather than to all-comers patients. Also, it has to be taken into account that heterogeneous patient populations may have different underlying diseases, which in turn have their own intrinsic mortality rates.

6) *The unavailability of diagnostic and prognostic markers*. The time lag between the actual onset of the inflammatory response, a proper clinical diagnosis and the initiation of a suitable therapy is too long. This is mainly caused by the current unavailability of tools for an effective diagnosis and prognosis. Several attempts have been made to develop laboratory techniques to detect septicemia. However, to date, all attempts failed in terms of methodological problems, variability, sensibility and lack of clinical utility. Therefore, clinicians have neither a laboratory tool for making an unequivocal diagnosis nor a prognosis marker to identify those patients who might benefit from a given treatment. This point has been raised by Bone who stated “We should spend more time

learning how to achieve an accurate diagnosis and less time searching for a magic bullet".[65]

6) *Limitation of outcome measure*. Finally, the current measure of success used in sepsis research is the mortality at 28 days. Although survival is unquestionably important for patients, the measure of mortality underestimates other therapeutic effects of the treatment.[165] What is more, from a clinical point of view, mortality is not particularly informative because it is insensitive to small clinical changes that could, if properly detected, redefine therapeutic treatments for a given patient.

Our strategy…

A thorough revision of the literature reveals a series of key concepts in the pathology of sepsis:

i) sepsis is an extremely complex syndrome,
ii) composed by a huge number of immune and inflammatory mediators
iii) these mediators are involved in a great number of pathways, which are intimately correlated and that often complement each other
iv) the inhibition of one single mediator is unlikely to provide a beneficial effect
v) there is a point in the progression of sepsis where irreversible clinical effects occur
vi) therefore, it might be better preventing rather than treating sepsis complications

For all these reasons it seems that halting the ongoing stimulation of the inflammatory response at the early beginning would seem to be more effective than targeting any individual mediator. If there is a point of no return in sepsis, progression of events should be stopped before. If there is an uncontrolled release of inflammatory mediators, which are difficult to deal with, the source of this release should be inhibited.

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1. 2 OBJECTIVES

The aim of the present Doctoral Thesis is:

1. To design, synthesize and evaluate the biological activity of LPS-neutralizing peptides derived from known anti-LPS proteins as future therapeutic agents for the prevention and treatment of sepsis.

2. To design, synthesize and evaluate the biological activity of peptide-based molecules derived from the described LPS-neutralizing peptides, with enhanced LPS-neutralizing capacity and improved pharmaceutical properties.

3. To provide useful insights into the molecular mechanisms of LPS-neutralization.

2. ANTI-LPS PEPTIDES DERIVED FROM LPS-BINDING PROTEINS

2. 1 INTRODUCTION

LPS-binding molecules

As previously explained, LPS constitutes one of the most important bacterial mediators in the pathogenesis of sepsis. However, the effect of LPS does not necessarily need to be harmful. In fact, low amounts of LPS are beneficial for the host, since they stimulate its resistance to infection. At the molecular level, a prerequisite for the induction of harmful as well as beneficial host responses depends on the interaction of LPS with LPS-binding molecules. The association of LPS to these molecules determines whether LPS will lead to beneficial or harmful effects.

It should be noted that LPS, on the bacterial cell wall and in host biological fluids and cells, is never free but constantly attached to LPS-binding proteins. These proteins may have different roles:

i) they can transport LPS,
ii) recognize LPS and deliver a signal, acting therefore as sensors,
iii) or block LPS neutralizing its biological effects

Understanding how these molecular processes work is an important goal directed to the identification and development of promising LPS-neutralizing molecules.[1]

There are a considerable number of molecules that bind to LPS. Indeed, LPS itself is the very first LPS-binding molecule. Because of their amphipathic character, LPS molecules associate together in aqueous media to form aggregates.[2]

A first group of molecules able to recognize LPS are lectins. Lectins are sugar-binding proteins that recognize sugar moieties with high specificity. Lectins therefore can recognize the O-specific polysaccharide chain or the core region of particular LPSs.[3,4,5] However, these proteins cannot be classified as true LPS-binding molecules, since they only bind to a restricted group of LPS, those carrying a specific carbohydrate determinant.

A second group of molecules that interact with LPS is composed by enzymes involved in its degradation. This is the case of the lysosomal phosphatase involved in LPS catabolism[6] or the granule acyloxyacyl hydrolase that removes the secondary acyl chains of LPS.[7]

However, the most important LPS-binding proteins are those belonging to a third group of proteins that interact with the toxic part of LPS, lipid A. Moreover, these proteins constitute the subject of research of the present chapter. Therefore, in the following pages they will be introduced in detail. They will be classified according to their different origins and special attention will be paid on their modes of action in binding LPS. Given the great number of molecules able to bind LPS (over 500 peptides have been reported to participate in innate immunity in multicellular organisms),[8] we will only concentrate on proteins that have been the source of anti-endotoxic peptides.[9,10,11]

2.1.1 PROTEINS AND PEPTIDES THAT BIND TO LIPID A

1. Bacterial LPS-binding molecules (FhuA, Polymyxin B)

Bacterial proteins that are able to interact with LPS may have two distinct origins. They can be constitutive proteins of Gram-negative bacteria, required for their survival. Or, they can be produced by other microorganisms (i.e. Gram-positive bacteria) aiming to kill Gram-negative bacteria.

FhuA

One of these products from Gram-negative bacteria is FhuA. This protein is found on the outer membrane of *Escherichia coli* and belongs to a family of proteins that mediates the active transport of ferric siderophores (iron chelating compounds secreted by microorganisms). FhuA has been shown to bind LPS by both electrostatic interactions via eight positively charged residues and via numerous van der Waals contacts between hydrophobic side-chains of FhuA and acyl chains of LPS (Figure 2.1).[12]

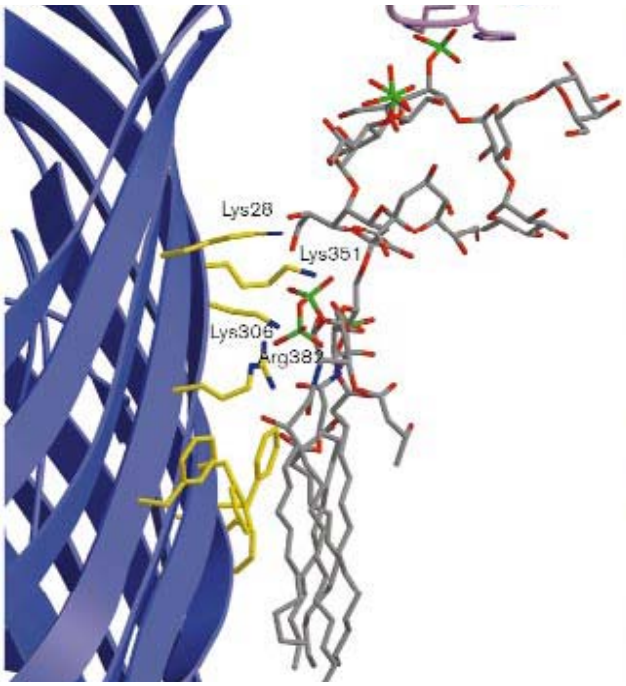

Figure 2.1: Close-up view of the crystal structure of the FhuA-LPS complex. Selected side-chain residues that comprise the LPS-binding motif are colored yellow. From ref [12].

Polymyxin B (PMB)

A second class of bacterial LPS-binding molecules are antibiotic-type molecules produced by some Gram-positive bacteria. These microorganisms appeared earlier in evolution than Gram-negative bacteria. As soon as the first Gram-positive bacteria had to compete with the first Gram-negative bacteria, they probably selected the most effective weapons to fight against the new competitors, and the new molecule LPS constituted an attractive target. A good example of these sorts of weapons is polymyxin B (PMB). PMB is a cyclic antibiotic peptide that contains five positive charges and an *N*-linked fatty acid tail (Figure 2.2 and Table 2.1). NMR and molecular studies of PMB in solution have shown how the addition of LPS induces formation of a defined conformation of PMB which segregates hydrophobic and positively charged residues.[13] PMB binds to the LPS of Gram-negative bacteria, changes the packing order of LPS and increases the permeability of the outer membrane to different molecules, including PMB itself (what is known as self-promoted uptake).[14,15]

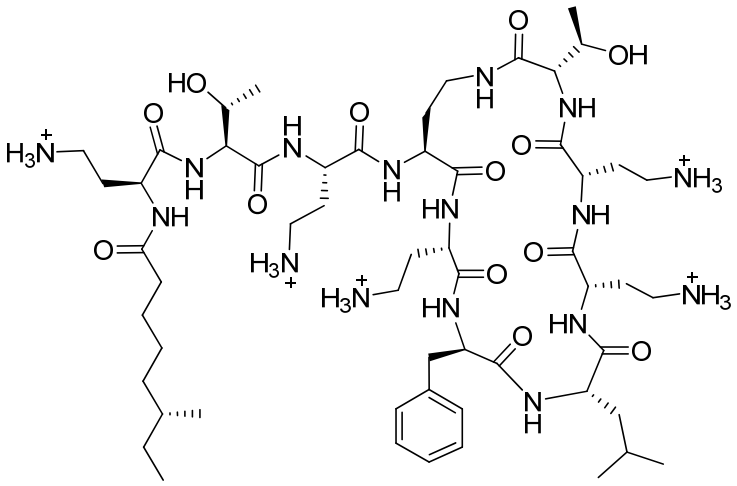

Figure 2.2: Chemical structure of the antibiotic peptide PMB. This cyclic peptide contains five basic diaminobutyric acids and a methyl octanoate acyl chain.

The binding of PMB to LPS has been studied with great detail and consist on two steps: a first bimolecular stage that involves electrostatic interactions between charged parts of the molecules; and then, a rate determining monomolecular stage which represents insertion of acyl chain of PMB into the lipid layer (Figure 2.3).[13,16] This second stage has been proposed to be crucial for LPS-neutralization. In fact, PMB nonapeptide (i.e.

PMB lacking the acyl chain) showed a comparable affinity in binding LPS but a poor ability to neutralize it.[17] Additional studies also support the notion that hydrophobic interactions between the acyl chains of PMB and the lipid chains of LPS play a major role in LPS-neutralization.[18, 19] Association of PMB with lipid A disrupts its supramolecular structure[20] and what has been termed the "endotoxic conformation"[21] which is also believed to be important for the biological effects of LPS (see section 2.1.3 for details).

Figure 2.3: PMB docked to lipid A. PMB is represented in black and lipid A in dotted grey line. The cyclic peptide interacts with lipid A via its charged residues. The acyl tail promotes hydrophobic interaction with LPS acyl chains. From ref [1], adapted from ref [13].

Unfortunately, PMB displays neurotoxicity and nephrotoxicity, in large part due to its slow degradation *in vivo*,[22] and it is only suitable for topical applications. However, PMB has re-emerged in clinical practice as the last resource treatment of nosocomial infections caused by multidrug-resistant Gram-negative bacteria.[23]

Table 2.1. Sequences of peptides reported to bind and neutralize LPS. Partially adapted from references [9, 10] and completed with references cited in the text.

Peptide	Sequence*	Reference
PMB**	mo-K'TK'K'K'fLK'K'T	13
MBI-27	KWKLFKKIGIGAVLKVLTTGLPALIS	30
MBI-28	KWKLFKKIGIGAVLKVLTTGLPALKLTK	30
magainin 2	GIGKFLHSAGKFGKAFVGEIMKS	36
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	39
indolicidin	ILPWKWPWWPWRR	45
LALF-14c	GCKPTFRRLKWKYKCG	52
LALF-22c	GCHYRIKPTFRRLKWKYKFWCG	54
LBP_{91-105}	WKVRKSFFKLQGSFD	64
LBP_{91-108}	WKVRKSFFKLQGSFDVSV	64
LBP_{86-99}	RVQGRWKVRKSFFK	65
$SAP_{186-200}$	QALNYEIRGYVIIKP	80
BU3***	*HIKELQV*KWKAQKRFLKMS*IIVKLNDGRELSLD*	98
LF-33	GRRRRSVQWCAVSQPEATKCFQWQRNMRKVRGP	105
$CAP37_{20-44}$	NQGRHFCGGALIHARFVMTAASCFQ	112

* Underlined letters denote cyclization

** mo: methyl octanoate; K': diaminobutyric acid; f: D-Phe

*** Residues in italics represent β-turn initiation sequences

2. Antimicrobial peptides produced by insects, amphibians and mammals

Similarly to Gram-positive bacteria, others species later in evolution, developed also mechanisms to interact and neutralize LPS.[24] Some examples of molecules produced by insects are **cecropin** in *Drosophila* hemolymph,[25,26] **sarcotoxin IA** from flesh fly,[27] **melittin** in bee venom[28] and **attacin** in silkmoth,[29] which all bind to LPS. Gough and co-workers designed two α-helical peptides derived from cecropin and melittin (MBI-27 and MBI-28, see Table 2.1) with an endotoxin neutralizing activity comparable to that of PMB.[30] These peptides displayed antiendotoxic as well as

antimicrobial activity *in vitro* and *in vivo* in animal models. A number of LPS-neutralizing peptides have been isolated from the skin of amphibians. This is the case of **temporins**, a large (more than 50 members) family of small, amphipathic α-helical antimicrobial peptides.[31] Of these, Temporin L has been studied for its potential to suppress the endotoxic effect of LPS.[32] In addition, this peptide synergizes in killing Gram-negative bacteria and neutralizing endotoxins when is combined with temporins A and B.[33] Another example is the peptide **magainin 2** (Table 2.1), which contains a basic an amphipathic α-helix motif with LPS-binding capacity. This motif is also found in the LPS binding domain of cecropin (Figure 2.4). This peptide was first studied in the context of its antimicrobial activity. When it interacts with the outer membrane of Gram-negative bacteria its primary target is LPS,[34] where it causes a concentration-dependent disordering of the LPS fatty acyl chains.[35] Upon membrane insertion magainin forms an α-helical structure.[36]

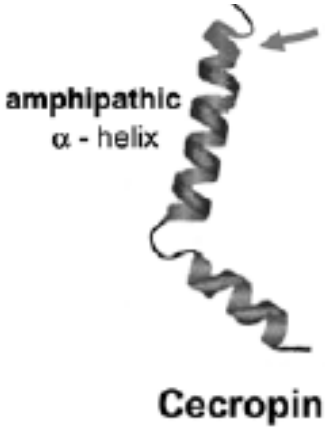

Figure 2.4: Ribbon diagram of the protein cecropin. The LPS-binding domain is indicated by an arrow. This domain contains a basic motif (GKWKAQKRFLKM) followed by an amphipathic α-helix. A similar LPS-binding domain is found in magainin. From ref [1].

Human neutrophils contain two distinct kinds of antimicrobial peptides, **defensins** (β-sheet peptides with three or four disulfide bridges) and **cathelicidins** (α-helical peptides). Human defensins (HNP-1 to HNP-4) have been shown to interact with LPS, although less efficiently than other LPS-neutralizing peptides.[37] This binding capacity is due to exposed cationic and non-polar residues oriented at different faces of the molecule.[38] The human cathelicidin **CAP18** is another antimicrobial protein released during phagocytosis. This protein carries a 37-residues α-helical peptide termed LL-37

(see Table 2.1), which has antimicrobial and anti-LPS binding activities.[39] In recent studies, this peptide has shown to block the toxic effects of LPS both *in vitro*[40] and *in vivo*.[41] The CD spectra of this protein showed an unordered state in the absence of lipid A; but the addition of lipid A increased the helical content.[42] The major impetus for forming this helical structure is to present charged or hydrophobic patches and stripes in the surface of the protein, which help LL-37 to intercalate into lipid matrices composed of LPS.[43] This α-helical structure is shown in Figure 2.5.

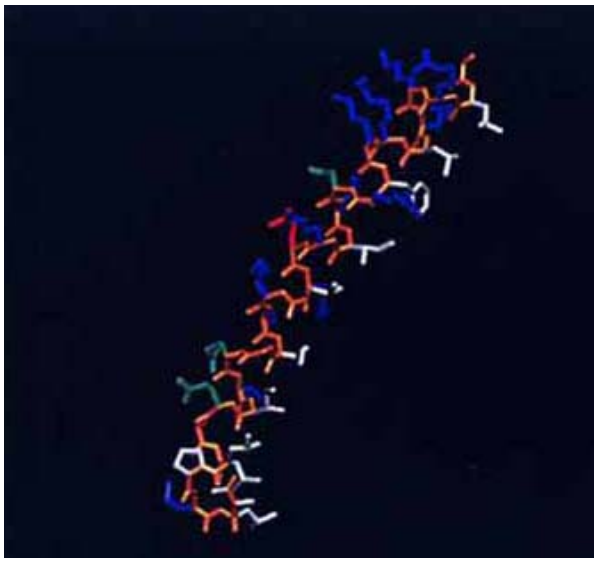

Figure 2.5: Representative 3D structure of the α-helical peptide LL-37 derived from CAP18. Hydrophobic residues are colored in white; basic in blue; and the peptide backbone in gold. From ref [10].

Many other neutrophil cationic proteins have been described to show LPS-binding capacity.[44] One of these groups is constituted by Pro-rich peptides. This is the case of **indolicidin**, a 13 amino acid Trp/Pro-rich peptide (Table 2.1) present in bovine neutrophils that binds LPS efficiently (K_d = 45.2 μM).[45] It has recently been reported that indolicidin and LL-37 synergistically suppressed LPS-induced production of TNF-α from macrophage cell lines.[46]

Correlation between LPS-neutralizing and antibacterial activity

LPS-neutralizing activity is often associated with antibacterial activity, particularly against Gram-negative bacteria. In many cases peptides that neutralize LPS

have been originally studied in the context of their antimicrobial activity. These activities do not necessarily correlate in all cases.[24] However, most LPS-neutralizing peptides are bactericidal, whereas many antimicrobial peptides are inefficient in neutralizing LPS. The rationale behind this behaviour is the following. Endotoxin-neutralizing peptides contain the chemical features necessary for LPS-binding, and Gram-negative bacteria present this molecule in their outer membrane. Therefore, interaction of peptides with LPS, implies in many cases the capacity to interact with Gram-negative membranes, what eventually may lead for example to membrane disruption or perturbation. In contrast, not all antimicrobial peptides will neutralize LPS, since there are various mechanisms of antibacterial activity (i.e. antimicrobial peptides that form neutral α-helix structures).

A paradox frequently observed in the pathology of sepsis is the observation that the administration of antibiotic drugs may result in the increased release of LPS from the outer membrane of the infecting bacteria, therefore negatively affecting the outcome of the pathological condition.[47] For this reason, some authors consider that peptides that combine antimicrobial and LPS-neutralizing activity may be promising agents of therapeutic value for the treatment of sepsis.[48] In contrast, other authors suggest that dissociation of antibacterial and LPS-neutralizing activities is important in order to design LPS-sequestering agents of low toxicity.[49]

3. Proteins from the coagulation system: LALF

Limulus anti-LPS factor (LALF)

Invertebrates early developed an innate immune system that responds to potential pathogens and their products. A very ancient arthropod is the horseshoe crab *Limulus polyphemus*. In *Limulus* hemolymph is found an LPS-binding protein, the *Limulus* anti-LPS factor (LALF). LALF is probably one of the proteins that have been more extensively studied and modified by several research groups. LALF is a small (11.8 kDa) basic protein that inhibits the LPS-mediated cascade.[50] Its structure consists of three α-helices packed against a four stranded β-sheet, with an LPS-binding site in an extended amphipathic loop (residues 31-52).[51] (Figure 2.6 A) This loop alternates

positively charged and hydrophobic residues that, by virtue of the extended β-conformation, point in opposite directions, thus conferring amphipathicity to the whole domain. The β-hairpin loop is stabilized by a disulfide bridge between Cys 31 and 52. (Figure 2.6 B). Interestingly this LPS-binding domain has also been found in proteins such as LBP and BPI (see below, section 2.1.2). Linear peptides from this region bind LPS weakly, but if they are cyclized by a disulfide bond their LPS-neutralization become comparable to that of PMB.[52] These studies determined the minimal LPS-binding domain as a 14-amino acid cyclic peptide named LALF-14c, comprising residues 36-47 (Table 2.1).[52]

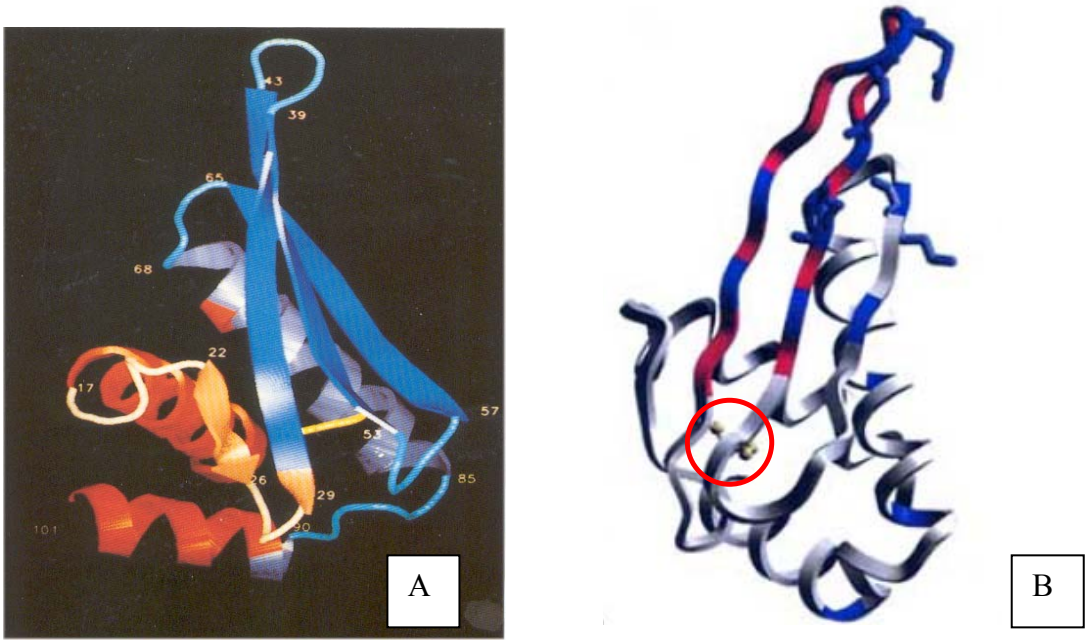

Figure 2.6: A. Crystal structure of LALF at 1.5 Å. From ref [51]. **B**. Detail of the β-hairpin loop (31-52) colored in red. The disulfide bridge Cys31-Cys52 is circled and shown as ball-and-stick model. Residues Arg40, Arg41, Lys47 and Lys64 are shown as stick models. Adapted from ref [11].

Further studies have been pursued in order to better understand the mechanisms of endotoxin neutralization, the biological activity and the structure of LALF and cyclic LALF-derived peptides. Using biophysical techniques Andrä *et al* studied the mechanism of interaction between LALF and LPS.[53] They observed that in parallel to binding to the lipid A, LALF converted the aggregate structure of lipid A from a cubic into a multilamellar one (i.e from the "endotoxic" conformation to an inactive conformation. See section 2.1.3 for a complete discussion). It caused also an overcompensation of the LPS negatively charged groups. In another study, the peptide

LALF-22c (residues 31-52, Table 1) that covers the full LPS-binding domain (see Figure 2.6 B) showed a high inhibition of LPS-induced TNF-α release in murine macrophages.[54] The same peptide was examined by Vallespi *et al*. They found, in an experimental model of Gram-negative peritoneal sepsis, that the administration of LALF-22c reduced the systemic levels of TNF-α, improved the outcome of organ damage and increased the survival of infected mice. Therefore demonstrating the anti-inflammatory properties of this peptide and its potential use in the prophylaxis of sepsis.[55] Interestingly, the same group also used LALF-22c in a mouse model of Gram-positive sepsis, observing that it ameliorated the sepsis-induced effects in the lung and liver. This treatment also increased the survival of mice in a dose and time-dependent manner.[56] From a structural point of view, the structure of the cyclic peptide LALF-14c when bound to LPS was investigated with NMR and molecular modeling.[57] The NMR results showed that, although LALF-14c adopts a β-sheet-like structure in the full-size protein, it has no regular secondary structure in water, but also not when bound to LPS. However, docking calculations showed that the β-sheet-like structure is not a prerequisite for binding of LALF-14c to LPS. Instead, its LPS bound conformation is highly amphipathic and binds to LPS with an electrostatic as well as hydrophobic component. Docking calculations showed that the peptide binds to LPS-phosphates via residue pairs Arg40/Arg41 and Lys43/Lys45 (Figure 2.7).

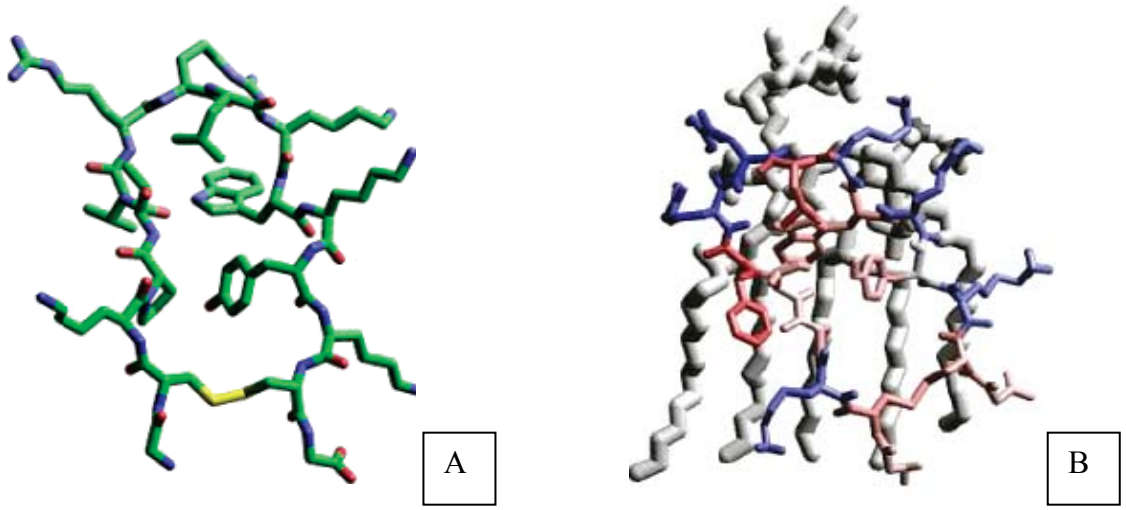

Figure 2.7: A. Representative structure of LALF14c bound to LPS as determined by the etNOESY experiment. Basic residues are drawn in blue, hydrophobic residues in red, and the disulfide bond in yellow-colored. **B.** Structure of the LALF14c-LPS complex calculated by AutoDock. Residues are colored as for **A**. Both adapted from ref [57].

However, other recent studies suggested that the presence of a nascent amphipathic β-hairpin-like structure in cyclic peptides derived from LALF-14c was necessary for their LPS-neutralizing activity.[58]

4. Lipid transport proteins: PLTP, LBP and sCD14

Phospolipid transfer protein (PLTP)

PLTP has been reported to bind LPS and to transfer it from Gram-negative bacterial membranes[59] or LPS aggregates[60] to the circulating high-density lipoprotein (HDL). After its transfer to HDLs, LPS is no longer recognized by macrophages[61] and is cleared by phagocytic cells bearing HDL receptors.[62] Hence, PLTP and HDLs collaborate to sequester LPS from the circulation.

LPS-binding protein (LBP)

Probably the most well-known and studied protein with LPS-binding capacity is the lipopolysaccharide-binding protein (LBP). As previously described the initial activation of the innate immune cells depends on the binding of LPS to LBP and subsequent transfer to the CD14 receptor. This 60-kDa glycoprotein, which is mainly produced by hepatocytes, interacts with the lipid A region with an affinity of 10^{-9} M.[63] The LPS-binding domain of this protein is contained within its *N*-terminal region, and synthetic peptides that mimic this portion also have LPS-binding capacity.[54] In this sense, two overlapping peptides have been described, LBP_{91-108} and LBP_{91-105} (Table 2.1), which specifically bound lipid A with high affinity and inhibited LPS-induced TNF-α release both *in vitro* and *in vivo*.[64] The minimal LPS-binding domain however, was identified in a peptide that comprises residues 86-99 (Table 2.1). Ala scan studies of this peptide showed that residues Trp91 and Lys92 were essential for binding.[65] Recently, the structure of this peptide when bound to LPS has been elucidated by NMR and docking calculations.[66] This information was used to design a peptide that displayed more than 50% increase in LPS inhibition *in vitro*.[66]

LBP displays a dual role depending on its concentration: at low concentrations it intercalates into cell membranes, binds to LPS aggregates and enhances LPS-induced responses. In contrast, at high concentrations, it intercalates into LPS aggregates and inhibits LPS-induced stimulation.[67] The role of LPS aggregates in immune system activation will be discussed later on in this introduction. A second function of LBP, already commented, is the ability to increase the interaction of LPS with soluble CD14 (sCD14) by forming a stable ternary system.[68] This process requires that LBP binds to sCD14. This function is mediated by the *C*-terminal moiety of LBP.[69] This complex can then transport LPS to cells, which will respond to picomolar concentrations of LPS; or it can interact with circulating lipoproteins to detoxify LPS.[70]

LBP shows a high degree of sequence homology with another LPS-binding protein, BPI. Detailed structures of their *N*- and *C*-terminal domains are shown along with BPI description.

CD14

In this regard, sCD14 is another important protein in LPS recognition. In fact, it binds LPS with a dissociation constant of 74 nM.[71] The *N*-terminus of sCD14 contains four LPS-binding domains. The fourth (residues 53-63) is the region of highest amphipathicity, and is analogous to the amphipathic loops of LALF (residues 31-52), LBP (residues 86-104) and BPI (residues 86-104) that bind lipid A.[72] The common structural motifs for LPS-binding will also be discussed later.

5. Other circulating proteins: HDL, serum amyloid P

Plasma lipoproteins

LPS binds to all major plasma lipoproteins: HDLs, low-density lipoproteins (LDLs), very low density lipoproteins (VLDLs) and chylomicrons.[73] The interaction between LPS and HDLs has been studied and is mediated via the phosphates and the diglucosamine backbone of lipid A.[74] Moreover, it has been shown that the apolipoprotein apoE, a constituent of lipoproteins, contains an heparin-binding sequence[75] and can bind LPS, possibly by a hydrophilic domain involving Arg

residues.[76] As discussed earlier, binding of LPS to circulating lipoproteins often leads to the secretion and elimination of LPS.

Serum amyloid P (SAP)

Another interesting candidate for LPS-inhibition is the serum amyloid P (SAP). SAP is a multispecific serum glycoprotein that binds heparin, various sulfated carbohydrates and LPS among other molecules.[77] However, it was not until 1998 that De Haas and co-workers reported the binding of SAP to LPS.[78] It was shown that SAP bound to LPS via its lipid A, and had different affinities toward different LPS types (i.e. rough- and smooth-LPS). Using LPS-coated chips, the binding affinity of SAP for LPS was measured to be 3.9 nM.[79] Using a panel of overlapping 15-mer synthetic peptides, three LPS-binding sites were identified for SAP (residues 27-39, 61-75 and 186-200). The corresponding synthetic peptides derived from these sequences exhibited LPS-neutralizing activity. Moreover, the 15-mer SAP-derived peptide, $SAP_{186-200}$ (Table 2.1), showed protection against LPS-induced septic shock in mice,[80] indicating a potential use of this peptide in human Gram-negative sepsis.

Nevertheless, it is difficult to speculate on the physiological role of SAP binding to LPS *in vivo*.[81] In this regard, it seems that SAP plays a regulatory role in innate immunity similar to LBP or sCD14. Besides binding LPS, it also can bind to Gram-negative bacteria with an "antiopsonic" effect, which results in reduced phagocytosis and killing of bacteria.[82] This dual role has been examined in recent investigations.[83,84,85]

6. Proteins produced by neutrophils: BPI, lactoferrin, CAP37

Bactericidal permeability-increasing protein (BPI)

BPI is a 55 kDa cationic antimicrobial protein that is present in the azurophilic granules of leukocytes and on the surface oh human mucosal epithelia.[86] BPI is highly bactericidal, mainly against Gram-negative bacteria. It has both heparin- and LPS-binding capacity and holds a ~44% degree of homology with LBP.[87] However, in

contrast to LBP, BPI binds to LPS, increases the sizes of the aggregate[88] and prevents its further interaction with other molecules, thus effectively neutralizing LPS.

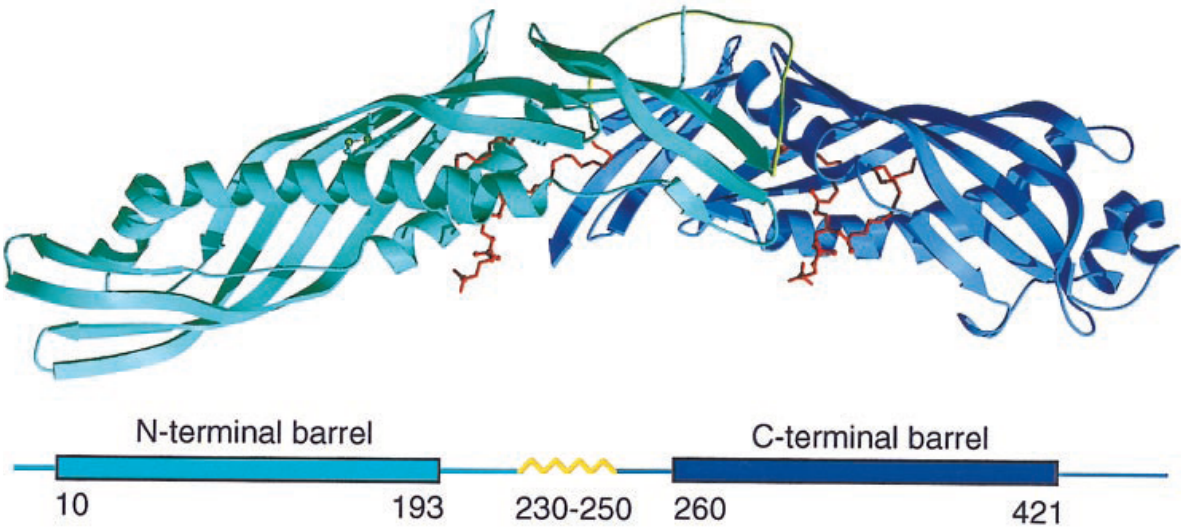

Figure 2.8: A ribbon diagram of human BPI. The *N*-terminal domain (on the left) is cyan, the *C*-terminal domain is blue, and the Pro-rich linker is in light green. The two bound phospholipids are shown as ball-and-stick in red. From ref [90].

The three dimensional structure of BPI was determined (Figure 2.8).[89] It was found to be a boomerang-shaped molecule of two *N*- and *C*-terminal domains of similar size that are connected by a Pro-rich linker of 21 residues (amino acids 230 to 250). The two domains form three structural units: two barrels at each end of the protein and a central β-sheet at the interface between the barrels. In turn, each barrel (residues 10 to 193 and 260 to 421) contains three common structural elements: a short α-helix, a five stranded anti-parallel β-sheet and a long helix. At the *N*-terminal domain, the long helix is connected to the β-sheet by a disulfide bond between Cys135 and Cys175. In the crystal structure two phospholipids are bound in hydrophobic pockets situated between the *N*- and *C*-terminal barrels and the central β-sheet. The acyl chains insert approximately 15 Å into the interior of the protein and are surrounded by apolar side chains. This discovery was important since it suggested a possible site of interaction between BPI and LPS. Moreover, the phospholipids bound (phosphatidylcholine) share some structural similarity with LPS. Including negatively charged phosphate groups and, most notable, acyl chains. Since BPI binds LPS, and a lipid is bound in pockets of BPI, it seemed reasonable that acyl chains of LPS bind in apolar pockets.[90]

The sequence alignment of proteins LBP and BPI (44% sequence identity) shows that structurally important residues are conserved in the two proteins. This is the case of the two Cys 135 and 175, which are critical to the function of BPI. Overall differences in charge and electrostatic potential between BPI and LBP suggest that the bactericidal activity of BPI is closely related to the high positive charge of its *N*-terminal domain.[89,91] As described for LBP, the *N*-terminal domain of BPI (rBPI21) is also responsible for its LPS-binding capacity. In fact, the isolated *N*-terminal domain of BPI is a potent LPS-neutralizer[92] with therapeutic indication in meningococcal sepsis.[93] The *C*-terminal domain, in contrast, dictates the route and host responses to complexes they form with endotoxin.[94] For instance, the *C*-terminal domain of LBP is necessary for transferring LPS to CD14.[69]

The *N*-terminal domain of BPI has been the subject of a number of studies. Three regions (residues 17-45, 65-99 and 142-169) have been proposed to cooperate in the binding of LPS.[95] These regions are highly basic and promote electrostatic interactions with negatively charged groups of LPS. In addition, residues 82-106 showed sequence similarity with the amphipathic LPS-binding loop of LALF, and have been predicted to form an amphipathic β-hairpin similar to that seen in the LALF structure.[96] Some small synthetic peptides have been synthesized based on these amino acid sequences. For instance, a 15-mer peptide (residues 85-99) was shown to be bactericidal,[95] and a synthetic peptide derived from residues 148-161 neutralized endotoxins *in vitro* and *in vivo*.[97] An interesting example is the peptide BU3, which displayed a great LPS-neutralizing activity.[98] This hybrid peptide contains a portion of BPI's LPS-binding domain and is flanked by amino acids known to initiate β-turns (Table 2.1). This example illustrates how the biological activity of anti-LPS peptides may be optimized via chemical manipulation of their structure.

Lactoferrin (Lf)

Another thoroughly-studied protein is lactoferrin (Lf). Lf is a 80 kDa multispecific protein that binds LPS among many other molecules such as iron, heparin and DNA.[99] Similarly to BPI, it is released from neutrophil granules and mucosal epithelial cells in response to inflammatory stimuli.[100] It has antimicrobial activity by

means of reducing the iron available for the microorganisms during growth, and destabilizing of the outer membranes of Gram-negative bacteria.[101] Lf directly binds to lipid A and decreases its endotoxicity.[102,103] Two LPS binding regions have been identified for Lf: a loop region that spans residues 28-34 and an *N*-terminal stretch of only four Arg residues (2-5).[99] A synthetic octadecapeptide corresponding to residues 20-37 inhibit Lf-LPS interaction, thereby confirming the importance of this region.[104] LF-33, a human Lf-derived 33-mer peptide (Table 2.1) dramatically reduced the lethality of murine models of endotoxemia, demonstrating its potential use for the treatment of LPS-induced septic shock.[105]

Proteolytic digestion of Lf yields an antimicrobial peptide fragment called **lactoferricin** (Lfcin), which corresponds to the helix portion of Lf, in the region of LPS-binding.[106] Addition of a 12-C acyl chain to human fragment of Lf, which had weak antimicrobial activity, enhanced its LPS neutralizing activity two orders of magnitude.[107] It also increased its antibacterial activity, most significantly against Gram-positive bacteria. This strategy was then successfully applied to a Lfcin fragment leading to enhanced endotoxin neutralization.[108] These results highlight the importance of hydrophobic interactions in the neutralization of LPS and suggest that alkyl chain insertion to anti-LPS peptides might be a valuable strategy to achieve greater endotoxin-neutralizing activities.[109]

CAP37

CAP37 is a 37kDa cationic antimicrobial protein, also known as heparin-binding protein (HBP), produced by human neutrophils granules.[110] Once released from neutrophils, it binds to endothelial cell surface proteoglycans and contributes to the progression of inflammation. Apart from its proteoglycan/heparin-binding site, this multifunctional protein has a high-affinity binding site for lipid A.[111] This domain consists of a phosphate binding hydrophilic pocket (Asn20, Gln21 and Arg23) and a hydrophobic pocket (Phe25, Cys26, Cys42 and Phe43) that accommodates the fatty acyl chains of lipid A.[111] A synthetic peptide containing these two regions, residues 20-44 (Table 2.1), has been shown to bind lipid A and mimic the antibiotic action of the whole protein.[112] The disulfide bridge between Cys26 and Cys42 turned out to be necessary for biological studies. LPS/lipid A preparations strongly inhibited the antibiotic action

of CAP37, suggesting that antibiotic and lipid A binding domains are the same. However, it has also been reported that, like LBP, CAP37 might enhance LPS-induced TNF-α release from monocytes, and act therefore as a sensor of the LPS response.

2.1.2 COMMON STRUCTURAL MOTIFS FOR LPS-BINDING

Several investigators searched for common structural motifs in LPS-binding proteins. The first studies involved proteins LBP and BPI. As previously explained, these proteins have a 44% sequence homology.[87] The interaction of these proteins with LPS seems to be a complex multistep process that involves, on the one hand, positively charged residues in a region of the *N*-terminal domain that interact with the phosphate groups of lipid A; and, on the other hand, a lipid-binding pocket that can accommodate the acyl chains of lipid A, though with little specificity. Whether there were other LPS-binding proteins with sequence homology to LBP/BPI was examined. This was the case of LALF. Since this protein did not display any degree of sequence homology with these proteins, no obvious region for LPS-ligation could be originally assigned. However, the publication of the crystal structure of LALF revealed the presence of a positively charged amphipathic loop that interacts specifically with lipid A.[52] This binding domain was also identified in LBP and BPI.[64] Synthetic peptides derived from these regions were synthesized, namely $LALF_{28-54}$, LBP_{82-108} and BPI_{82-108}. All three peptides significantly inhibited LPS-induced TNF-α production in macrophages.[113] More recently, a synthetic amphipathic peptide derived from these sequences exhibited anti-endotoxic activity.[114]

In a very interesting study,[115] Frecer *et al*. compared the sequences of a number of LPS-binding peptides and proteins. As a result of their observations, they suggested that the more favorable amino acid sequence for specific lipid A binding is the amphipathic cationic pattern BHPHB (B, basic; H, hydrophobic; P, polar). This pattern is found in LBP (sequence RVQGR, residues 111-115), BPI (sequence KISGK, residues 113-117), LALF (sequence RLKWK, residues 39-43) and TLR4 (sequence KLTLR, residues 190-194). The best minimum binding sequence proposed was KFSFK. In another study, Koshiba *et al*. identified an alternative pattern, well conserved in these LPS-binding

proteins, a tri-peptide sequence motif, arranged in the order of H/A-BH (H, hydrophobic; A, aromatic; B, basic).[116]

However, these sequences do not always account for LPS-binding. In some proteins, the basic residues that interact with the phosphate groups of lipid A are often discontinuous but spatially proximal β-sheets or α-helices. Hence, a second model was proposed by Ferguson *et al*.[12] The authors searched in protein data bases proteins containing residues in a three-dimensional arrangement similar to that of the LPS-binding residues of FhuA. They identified four residues (K-K-R-K) that were common to several known anti-LPS binding proteins such as BPI, Lf or LALF. They observed that these residues form hydrogen bonds with the two phosphate groups of lipid A.

Overall, we can conclude that the requirements for LPS-binding are not too strict (for a discussion see below section 2.1.3). In fact, it has been shown that small amino acid sequences, containing both basic and hydrophobic residues, are sufficient for an effective lipid A ligation. At this point, one could argue why are LPS-proteins necessary in biological processes if the same LPS-binding functions can be displayed by derived, much shorter LPS-neutralizing peptides. In this regard, it is important to note that LPS-binding proteins always have a further role. Some may transport LPS within the organism, others might inactivate it (blockers) and a third group will opsonize LPS or activate it (sensors). Whereas in some cases blockers can be very short peptides (i.e. PmB), sensors must have a more complex structure since they must also deliver a recognition signal (i.e. LBP, TLR4). It is also noteworthy that the majority of LPS-binding proteins contain multiple LPS-binding sites that often work cooperatively. For example, there are two binding sites described for Lf,[99] three for BPI[95] and SAP,[80] and four for CD14.[72] In turn, this has allowed these LPS-binding sites being usually very short and not very specific to LPS, thereby proving that the association of several binding sites of moderate affinity is an efficient way to confer to a whole protein the ability to recognize LPS more specifically.

2.1.3 ENDOTOXIN NEUTRALIZATION

Peptides that bind to lipid A: True LPS-neutralizers?

There are multiple factors responsible for an optimal binding of peptide structures to lipid A, including the amphipathic and cationic features of the primary structure, the size of the structure and the peptide conformation. Cyclization has also proved to be useful in some peptides. A summary of these principles are consistent with optimal peptide sequences containing ten to twelve amino acid residues, rearranged in cyclic conformation, having an index ratio of cationic/hydrophobic residues equal or greater than one. Strict structural or stereochemical requisites are probably not critical in LPS-binding molecules. For example, the binding domain of LALF is a β-sheet-like amphipathic loop.[51] Peptides derived from this region such as LALF-14c, which do not form a β-sheet structure, retain the activity of the molecule, as long as they are cationic, amphipathic and cyclic.[57] Moreover, the binding of peptides to lipid A appears to be relatively independent of their amino acid sequence suggesting that the interactions are not sensitive to the conformations of the non-bound peptides.

However, at this point it is important to emphasize the difference between LPS-binding and neutralization. Binding affinity, *per se*, is an unsatisfactory predictor of endotoxin-neutralizing activity, since, as discussed in this introduction, molecules that bind LPS may either opsonize or neutralize the toxin. There are no rules to *a priori* determine whether a given peptide structure will be an LPS-binder or neutralizer. Nevertheless, the binding process of peptides to lipid A might give us some hints. As introduced in the text, this process usually has two main steps: a preliminary charge-to charge interaction between the cationic amino acids of the peptide and the phosphate groups of lipid A; and a final hydrophobically-stabilized binding between the hydrophobic residues of the peptide and the acyl chains of the lipid A. This further hydrophobic accommodation usually yields stable complexes. It seems that in some cases, peptides that are able to interact only electrostatically are LPS-binders, whereas peptides that further promote hydrophobic interactions might be true LPS-neutralizers.

Therefore, basicity is an important chemical feature for LPS-binding, but it is not sufficient for LPS-neutralization. For example, many small molecules are able to bind to LPS with high affinity; however, some of them neutralize LPS *in vivo* poorly.[117] One of the reasons, common also to many antimicrobial peptides, is that when the interaction is electrostatically driven it is significantly diminished in the physiological milieu. On the other hand, peptides bearing hydrophobic amino acids promote hydrophobic interactions that stabilize the complex peptide-LPS. Thus, hydrophobicity could be crucial for LPS-neutralization. This is the case of PMB or the Lf peptide, which after insertion of an acyl chain in its structure displays a 2-fold enhancement in its LPS-neutralizing activity.[107]

However, even if a peptide displays a good endotoxin-neutralizing activity *in vitro*, it is often very unlikely that this biological activity will correlate *in vivo*. For instance, on the basis of an *in vitro* parameter like the affinity constant value of a peptide for lipid A, no safe predictions can be done on the efficiency of the resulting detoxification activity *in vivo*. The main reason for this observation relies on several additional parameters to be considered *in vivo*, like the proteolytic stability of the peptide in biological fluids; the balance of the peptide distribution between blood and target organs; the capacity of the peptide to effectively compete with other LPS-binding proteins and receptors; and the clearance-period of the peptide from the bloodstream. All these factors, affect the final concentration and efficiency of a given peptide in the neutralization and detoxification of LPS *in vivo*. This explains why, despite a molar stoichiometry of binding *in vitro* between an LPS-neutralizing peptide and lipid A, it is often necessary to provide a significant excess of the peptide in the *in vivo* experiments. A common solution to overcome these limitations is the use of protease-resistant peptides, which would allow longer circulating half-time lives in the bloodstream. However, this strategy is not exempt from drawbacks. This is the case of PMB. As discussed above, this cyclic peptide is toxic.[22] There are two reasons that explain its toxicity. First, its lack of biodegradability due to its peptide structure resistant to serine proteases; and then, the interactions of this drug with the epithelium of the kidneys (probably mediated by its aliphatic alkyl chain), which prevent from being eliminated in the urine. Therefore an enhanced proteolytic stability or a chemical modification that enhances the endotoxin-neutralizing activity of a given peptide might sometimes result in undesired *in vivo* side-

effects. This is probably the reason why, to date; none of the peptides discussed in this introduction has been approved for clinical application with anti-endotoxin indication.

Model of endotoxin neutralization (the active conformation of LPS)

To understand the inhibition of LPS-induced activation of immune cells by LPS-neutralizing peptides there is another important issue to be taken into account: Which is the biologically active conformation of LPS?

According to a recent review,[118] there is no unequivocal answer to this question. The interaction of LPS with immune cells leads to the intercalation of the endotoxin into the cell membrane, an event which is presumably mediated by LBP, and probably also CD14.[119] But whether this intercalation is mediated by LPS-monomers or aggregates is subject of different interpretations. In earlier reports in 1994, Takayama and co-workers[120] found that rough Re-LPS form *Eschirichia coli* was more active in the monomeric than in the aggregated form. However, these authors used biological assays that differ from the usual cytokine assay. In contrast, Shnyra *et al.*[121] found higher activity for the aggregated than for the monomeric form of both rough and smooth LPS. However, in this case also the comparability to newer findings may be difficult due to differences in the bioassays used and the kind and purity of the endotoxins. In more recent studies using highly purified LPS and lipid A as well as synthetic lipid A it was found that cytokine induction was induced only by aggregated forms of LPS and not by monomers.[122,123,124] From these and other models, a model of endotoxin activation can be developed (Figure 2.8). This model proposes a non-lamellar cubic inverted aggregate structure of the lipid A as the bioactive conformation of LPS. This aggregate structure corresponds to a conical shape of the single molecules. Membrane-bound molecules such as CD14, but also the membrane form of LBP, mLBP,[125] bind to the LPS aggregate leading to a membrane intercalation of parts of the aggregates in immune cells. These exert a mechanical stress on membrane receptor proteins such as TLR4/MD2, which with the participation of a K+ channel (MaxiK) might elicit the intracellular signal cascade (see Figure 2.9).[126]

In contrast, in the presence of LPS-neutralizing peptides, a reagreggation process takes place in a way that lipid A is converted into a multilamellar structure. [127,128,129,130] The authors of this model reason that since most LPS-neutralizing peptides strongly reduced the interaction between LPS and LBP (aggregate dissociation prevents LPS from binding to LBP)[130], the interaction of LPS with the immune cells may be inhibited. And even if the complex peptide-LPS could target cell membranes by the action of binding proteins, the multilamellar structure of LPS would not lead to a mechanical disturbance of the immune cells membrane, and therefore the immune cascade would not be triggered.[118]

Although this model is supported by many experimental findings (many often from the same laboratories that propose the model) there are a number of alternative and plausible explanations. In this regard, the group of Weiss characterized the complex LPS-MD2 and observed that a monomeric LPS binds to a single receptor molecule.[131] Thus indicating that the monomeric form of LPS is actually what elicits the immune response.[132] To assess which mechanism holds true is not easy, and probably both proposed activation mechanisms may play a role.

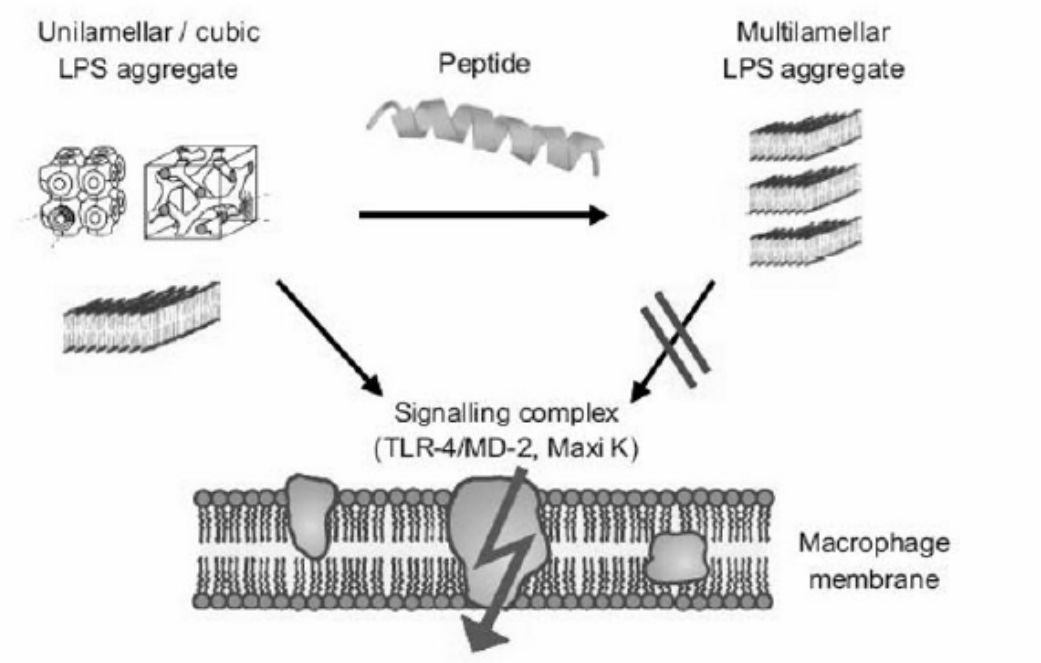

Figure 2.9: Model of the inhibition of LPS-induced response proposed by the group of Brandenburg. The intercalation of LPS aggregates (unilamellar/cubic structure) into the membrane, induced by proteins such as LBP and CD14, triggers the immunogenic cascade via TLR4/MD2 membrane receptors. Cationic LPS-neutralizing peptides induce a multilamellar LPS structure which does not activate the signal. From ref [103].

2.1.4 OBJECTIVES

The objectives of the present chapter are:

1. To study and modify the active sequence of the LPS-neutralizing protein LALF in order to identify optimal minimized LPS-binding sequences and to obtain peptides with enhanced LPS-neutralizing activities.

2. To study the effect of acyl chain incorporation into the active sequences of three LPS-neutralizing proteins: LALF, BPI and SAP.

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2.2 RESULTS AND DISCUSSION

"Design of a minimized cyclic tetrapeptide that neutralizes bacterial endotoxins"

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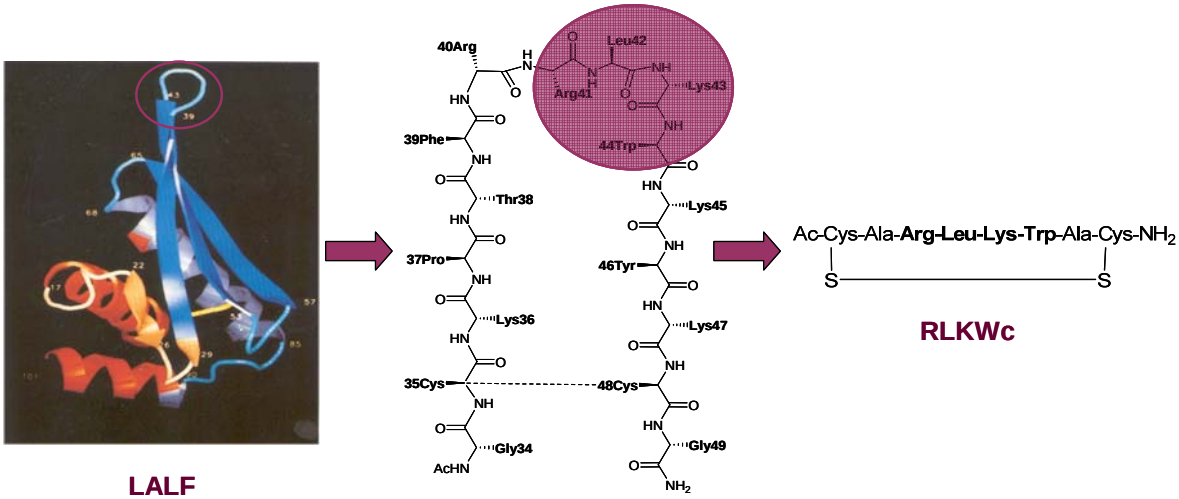
* These two authors contributed equally to the present study

RESUM

LALF-14c és un pèptid cíclic que mimetitza el gir amfipàtic d'unió al LPS de la proteïna LALF. Aquest pèptid representa el mínim domini d'unió al LPS descrit per LALF i té una capacitat per neutralitzar endotoxines bacterianes comparable a la de la PMB. En la següent publicació, vam examinar la seqüència de LALF-14c mitjançant un "mapeig" d'hexapèptids i un "scan" d'alanines. Aquests estudis van proporcionar informació valuosa sobre els aminoàcids més rellevants per a l'activitat biològica. En aquest sentit, es va determinar la seqüència d'un tetrapèptid com el domini d'unió mínima de LALF-14c al LPS. La inserció d'aquesta seqüència en un pèptid cíclic amb cisteines va donar lloc a un nou pèptid cíclic, el pèptid RLKWc, el qual va exhibir la mateixa activitat biològica que el pèptid original. Aquests resultats, per tant, demostren que el domini d'unió al LPS de la proteïna LALF, proposat fins aleshores, era susceptible de ser minimitzat.



Contribucions a aquest treball:

- La Dra. Puig Mora va realitzar els estudis de "scan" d'alanines i els assaigs d'activitat biològica per a tots els compostos descrits. També va contribuir en la preparació del manuscrit.

- En Carlos va realitzar la síntesi i la caracterització dels hexapèptids lineals, els seus anàlegs cíclics i el pèptid RLKWc. A més va contribuir activament en la preparació del manuscrit.

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JOURNAL OF PEPTIDE SCIENCE

Design of a minimized cyclic tetrapeptide that neutralizes bacterial endotoxins

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Abstract: Septic shock is a leading cause of mortality in intensive care patients, and no specific drugs are as yet available for its treatment. Therefore, new leads are required in order to increase the number of active molecules that may develop into efficacious and safe LPS-neutralizing molecules during pre-clinical stages. We used peptides, derived from the binding regions of known LPS-binding proteins, as scaffolds to introduce modifications at the amino acid level. Structure–activity relationship studies have shown that these modifications generate highly active peptides. Thus, from a bioactive peptide with an initial 16 amino acid residues, a tetrapeptide sequence was determined. After inserting this sequence in a Cys cyclic peptide, it showed the same biological activity as the parent peptide. This sequence could provide the basis for the design of small molecules with LPS-binding properties. Copyright © 2006 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: endotoxin; septic shock; sepsis; LALF; peptide mapping; lipid A; disulfide bridge formation; molecular recognition; solid phase; peptides

INTRODUCTION

Sepsis, a systemic inflammatory response to infection, can lead to multiple organ failure known as *septic shock*, the first cause of mortality in intensive care units [1]. Recognition of the bacterial LPS by immune system cells is detected on the basis of the pathology [2]. LPS is a pathogen-associated molecular pattern (PAMP) present in the outer leaflet of Gram-negative bacteria [3]. Continuous exposure to LPS in mammalian bloodstream induces the deregulation of inflammatory cytokine release, thereby leading to the pathological condition. The cascade of events is initiated by the recognition and binding of LPS to circulating LPS-binding proteins. Among other proteins, the LPS-binding protein (LBP) binds to LPS and transfers it to the CD14 receptor [4,5]. Although LBP and CD14 are at the top of the cell responsive pathway to LPS, a cell membrane receptor must interact with the complex in order to transduce the signal into the cell. TLR2 and TLR4 receptors, members of the toll-like receptors family (TLR), participate in the transduction of the LPS signal to the cell nucleus; an event that initiates the transcription of cytokine genes [6].

Research efforts have been directed towards the characterization of all the members involved in cascade recognition events and the full elucidation of the LPS-signalling pathway in order to define pharmacological targets. However, although inhibitors of TNF-α factor and other inflammatory mediators have been targeted for inhibition, to date this approach has not increased the survival of patients with septic shock [7,8]. Therefore, increased interest has been devoted to the inhibition of early events of the process. Compounds that could neutralize LPS or its toxic part, the lipid A moiety, may provide a potential source of useful lead compounds of pharmacological relevance [8].

Recent developments in identifying novel strategies to overcome endotoxic shock involve LPS-neutralizing peptides. Of these, special interest has been focused on Limulus anti-LPS factor (LALF), a small (101 amino acids) basic protein that binds and neutralizes LPS with high affinity [9]. From the analysis of the crystal structure of recombinant LALF (rLALF) [9], it was proposed that an amphipathic loop that spans residues 31 to 52 is the true LPS-binding domain [9]. In particular, the minimal LPS-binding domain is a 14-amino acid cyclic peptide (residues 36–47 – named LALF-14c), which binds LPS with an activity comparable to the high-affinity endotoxin-binding peptide polymyxin B (PMB), whereas its linear counterpart has a lower activity [10].

Herein, we report on the minimization of the LPS-binding domain of LALF-14c. We focused on

Abbreviations: BPI, bactericidal/permeability-increasing protein; DPLA, 1-4′-diphosphoryl lipid A; EDT, 1,2-ethanedithiol; LALF, *Limulus* anti-LPS factor, LAL, *Limulus* amebocyte lysate; LPS, lipopolysaccharide; LBP, lipopolysaccharide-binding protein; TBME; *tert*-butyl methyl ether; TIPS, triisopropylsilane

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hexapeptide mapping and alanine scanning of LALF-14c, which together provided data on the most relevant amino acids for biological activity.

MATERIAL AND METHODS

Materials and HPLC

Rink amide MBHA resin (0.66 mmol/g) and protected Fmoc-L-amino acids were purchased from Iris Biotech GmbH (Marktredwitz, Germany), Luxembourg Industries (Tel-Aviv, Israel), Neosystem (Strasbourg, France), Calbiochem-Novabiochem AG (Laüfelfingen, Switzerland) and Bachem AG (Bubendorf, Switzerland). Diisopropylcarbodiimide (DIC) was obtained from Fluka Chemika (Buchs, Switzerland), HOAt from GL Biochem (Shanghai, China), PyBOP from Calbiochem-Novabiochem AG and *N*,*N*-diisopropylethylamine (DIEA) from Albatros Chem. Inc. (Montreal, Canada). Solvents for peptide synthesis and RP-HPLC equipment were obtained from Scharlau (Barcelona, Spain). Trifluoroacetic acid (TFA) was supplied by KaliChemie (Bad Wimpfen, Germany). Other chemicals used were obtained from Aldrich (Milwaukee, WI, USA) and were of the highest purity commercially available. Buffers and solutions used in the *in vitro* LPS-neutralizing assays were endotoxin-free. Endotoxin-free water and LPSs from *Escherichia coli* 0111:B4 were from BioWhittaker (Rockland Maine, Walkersville, USA). HPLC was performed using a Waters Alliance 2695 (Waters, MA, USA) chromatography system with a photodiode array (PDA 995) detector, a reverse-phase Symmetry C_{18} (4.6 × 150 mm) 5-μm column and linear gradient MeCN with 0.036% TFA into H_2O with 0.045% TFA. The system was run at a flow rate of 1.0 ml/min. HPLC-MS was performed using a Waters Alliance 2796 with a UV–Vis detector 2487 and ESI-MS Micromass ZQ (Waters) chromatography system, a reversed-phase Symmetry 300 C_{18} (3.9 × 150 mm) 5-μm column, and H_2O with 0.1% formic acid and MeCN with 0.07% formic acid as mobile phases. Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (PE Biosystems, Foster City, CA, USA).

Solid-phase Synthesis

Linear hexapeptides and their cyclic derivatives were synthesized using the Fmoc solid-phase strategy performed manually in polypropylene syringes fitted with polyethylene porous disks. Side chains of Fmoc amino acids were protected as follows: Tyr and Thr were protected with the *tert*-butyl group (tBu), Lys and Trp with the *tert*-butyloxycarbonyl group (Boc), Cys with the trityl group (Trt) and Arg with the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl group (Pbf). Solvents and soluble reagents were removed by suction. Washings between deprotection, couplings and subsequent deprotection steps were carried out with DMF and DCM using 10 ml of solvent per gram of resin each time. The Fmoc group was removed by treatment with piperidine–DMF (1 : 4) for 20 min. All syntheses were performed on Rink Amide MBHA resin (300 mg) by a Fmoc solid-phase strategy. Couplings of all Fmoc-aa-OH (4 equiv.) were performed with DIC (4 equiv.) and HOAt (4 equiv.) in DMF for 2 h at room temperature. Recouplings were done either with HATU (4 equiv.) and DIEA (8 equiv.) in DMF for 30 min at 25 °C or with PyBOP (4 equiv.), HOAt (4 equiv.) and DIEA (12 equiv.) for 2 h at room temperature. The resin was washed with DMF and DCM after each coupling. Couplings were monitored using the Kaiser [11] or de Clercq [12] method. After each coupling, the capping steps were performed with HOAc–DIEA–DMF (4 : 2 : 94). For the deprotection of side-chain groups and concomitant cleavage of the peptide from the support, the resin was washed with DCM (3 × 1 min), dried and treated with a TFA–H_2O–TIS (95 : 2.5 : 2.5) mixture for a range of times depending on the peptide sequence (1 to 2 h). When necessary, a thiol-containing cleavage mixture, TFA–H_2O–TIS–EDT (95 : 2 : 2 : 1), was used instead. TFA was then removed by evaporation with nitrogen, and peptides were precipitated with cold anhydrous TBME, dissolved in H_2O–MeCN (distinct mixtures used) and then lyophilized. The crude peptides were purified either by semi-preparative or preparative HPLC. The alanine scanning–derived peptides were synthesized using an automatic peptide synthesizer ABI 433A (Applied Biosystem) and a *fast*Fmoc solid-phase strategy on polystyrene aminomethyl Rink Amide AM resin (RAM) resin (0.76 meq/g, Rapp polymer).

LALF-14c and the alanine scanning derived–peptides were cyclized upon dissolution in a HOAc–DMSO–H_2O (1 : 3 : 16) solution at a concentration of 0.5 mg/ml. A neutral pH was achieved after treatment with ammonium carbonate. The solution was then stirred at room temperature for 24 h [13,14]. Alternatively, LALF07c, LALF08c and LALF09c peptides were dissolved with H_2O–MeCN (1 : 1) in a round-bottom flask at a concentration of 0.5 mg/ml. The pH was then adjusted to 9 with a 20% solution of NH_3. The solution was stirred at room temperature for one or two days to allow air oxidation. Cyclization was easily monitored either by Ellman's test [15] and/or by RP-HPLC.

LPS-neutralizing Activity

The chromogenic LAL test assay [16] was used following the manufacturer's instructions (BioWhittaker). LAL contains a clottable enzyme that is activated in the presence of non-neutralized LPS [17] and is an extremely sensitive indicator of the presence of endotoxin. LPS-activated enzyme catalyses the release of *p*-nitroaniline (pNA) from the colourless chromogenic substrate Ac-Ile-Glu-Ala-Arg-pNA. The pNA released was measured photometrically at 405 nm in a Rosys Anthos 2010 microtiter plate reader (Tecnomara AG, Zurich, Switzerland). LPS-neutralization assays were performed at a fixed concentration of LPS (100 pg/ml) and using a range of peptide concentrations in a PBS saline buffer (137 mM NaCl, 2,7 mM KCl, 4,3 mM Na_2HPO_4, 1,4 mM KH_2PO_4 pH 7.0) in a 96-well microtiter plate. Peptides were pre-incubated with LPS for 45 min at 37 °C. The colorimetric reaction was started by adding LAL (50 μl) for an incubation period of 6 min, followed by the addition of the chromogenic substrate (100 μl) and incubation for 10 min. Finally, the reaction was stopped by the addition of acetic acid up to 25% of the total reaction volume.

RESULTS AND DISCUSSION

Hexapeptide Mapping Analysis of LALF-14c

LALF-14c has characteristics similar to PMB in terms of structure and charge. PMB is an amphipathic,

positively charged cyclic oligopeptide, which belongs to a family of antibiotics that binds lipid A with high affinity [18]. LALF-14c adopts a positively charged amphipathic hairpin loop with a β-turn stabilized by a disulfide bridge (Figure 1). In fact, Cys^{35} and Cys^{48}, which are not present in the natural protein, were added at the *N*- and *C* terminus to obtain the cyclic peptide that stabilizes the secondary structure [10].

Previous studies to reduce the length of the active peptides derived from the LPS-binding domain of LALF were performed with peptides 10-amino acids long and no improvement in LPS-binding activity was achieved [10]. In an attempt to identify the minimal LALF peptide sequence required for LPS inhibition, we mapped the LALF14c region with a series of overlapping linear hexapeptides (Table 1). The resulting seven hexapeptides comprised the whole LPS-binding site of LALF-14c. Peptide characterization is shown in Table 2. The linear peptides were dissolved in PBS saline buffer and their concentration was measured spectrophotometrically. The LPS-binding activity of each peptide was measured at 200 μM in the presence of 100 pg/ml of LPS (Figure 2). Although all the hexapeptides showed a reduced LPS-neutralizing activity compared to LALF-14c, all except LALF-09 displayed biological activity. These results indicate that particular peptides containing sequence residues Arg^{41},

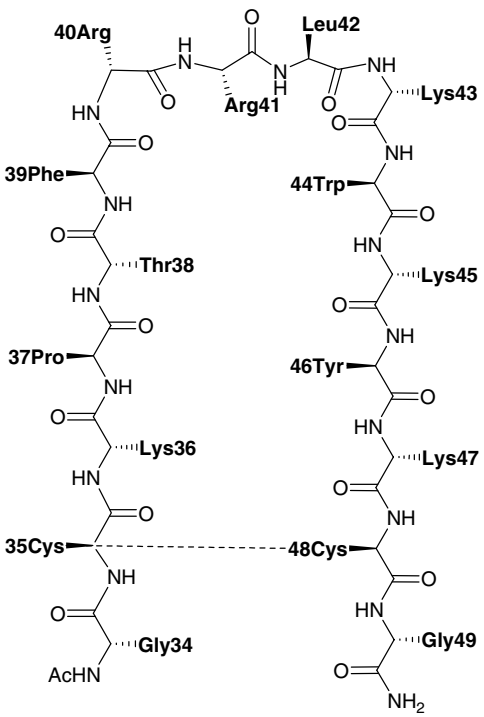

Figure 1 Schematic representation of the amphipathic β-hairpin of LALF14c. Amino acids are represented with the three-letter code. Wedged bonds and hashed wedged bonds indicate the relative position of side chains. A dashed bond represents the disulfide bridge between the two Cys introduced.

Table 1 Design of synthetic hexapeptides mapping the LALF14 domain

Name	Sequence[b]
LALF[a]	ECHYRIKPTFRRLKWKYKGKFWCP
LALF-14c	G-(CKPTFRRLKWKYKC)-G
LALF-03	KPTFRR
LALF-04	PTFRRL
LALF-05	TFRRLK
LALF-06	FRRLKW
LALF-07	RRLKWK
LALF-08	RLKWKY
LALF-09	LKWKYK

[a] LALF stands for the proposed LPS-binding domain of the LALF protein [10].
[b] All the peptides are acetylated and amydated at the *N*- and *C*-terminus, respectively. Sequence in parenthesis indicates cyclized.

Table 2 Characterization of Linear Hexapeptides

Name	t_R/min (HPLC)	Calculated mass	Experimental mass (MALDI)		
			M + H	M + Na	M + K
LALF-03	4.2	844.50	845.54	867.43	—
LALF-04	5.5	829.49	830.58	852.55	868.33
LALF-05	4.9	860.53	861.55	883.54	899.50
LALF-06	5.9	945.57	946.60	—	—
LALF-07	4.5	926.59	928.16	950.14	966.12
LALF-08	5.0	933.55	934.70	—	—
LALF-09	5.0	905.55	906.71	928.70	944.68

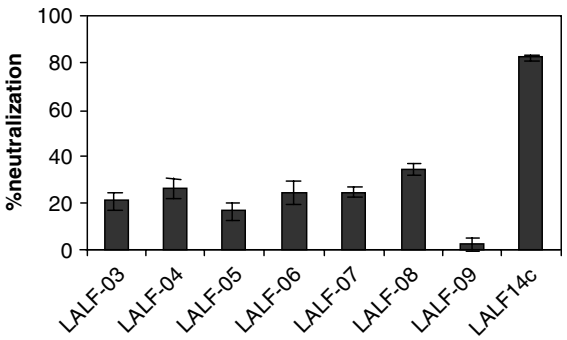

Figure 2 Inhibition of LPS-inducing gelling of chromogenic *Limulus* amebocyte lysate by LALF14c synthetic derived linear peptides or by control peptide (LALF14c) (200 μM) using 100 pg/ml of LPS. The assay was performed as described under 'Material and Methods'. The LPS-binding activity is performed in three independent assays, and the data is represented with ±SD.

Leu^{42}, Lys^{43} and Trp^{44} were slightly more active, and the absence of Arg^{41} in peptide LALF-09, together with the decrease observed in activity, shows that this residue may have a relevant role in the biological activity.

Table 3 Alanine Scanning in LALF14 Domain

Name	Sequence
LALFK47Ac	AC-GCKPTFRRLKWKY**A**CG-NH2
LALFY46Ac	AC-GCKPTFRRLKWK**A**KCG-NH2
LALFK45Ac	AC-GCKPTFRRLKW**A**YKCG-NH2
LALFW44Ac	AC-GCKPTFRRLK**A**KYKCG-NH2
LALFK43Ac	AC-GCKPTFRRL**A**WKYKCG-NH2
LALFR41Ac	AC-GCKPTFR**A**LKWKYKCG-NH2
LALFR40Ac	AC-GCKPTF**A**RLKWKYKCG-NH2
LALFF39Ac	AC-GCKPT**A**RRLKWKYKCG-NH2

Alanine Scanning of LALF-14c Sequence

As mentioned earlier, anti-LPS peptides are amphipathic and positively charged [19,20]. The sequence of LALF-14 is rich in basic amino acids (lysine and arginine) and in hydrophobic residues (phenylalanine, tryptophan and tyrosine). Results from our laboratories indicate that the amino acids located at the *C*-terminus of the β-hairpin loop of LALF-14c (Figure 1) are of relevance for biological activity (Mora *et al.*, to be published). To analyse the contribution of each amino acid from this particular sequence, we performed an alanine scan (Table 3). The peptides were synthesized with *N*- and *C*-terminal cysteines in order to obtain cyclic peptides. Peptide characterization is shown in Table 4. The LPS-binding activity of each peptide was measured at a range of peptide concentrations using 100 pg/ml of LPS, and the IC_{50} of each peptide was then evaluated (Figure 3). The substitution of the aromatic residues Trp^{44} and Tyr^{46} induced a dramatic decrease in biological activity. Surprisingly, the alanine substitution of basic residues was not deleterious to the activity. Given the proposed structure for LALF-14c [10], residues Trp^{44} and Tyr^{46} were found to contribute to the partial hydrophobic inner face of the β-hairpin strand and to the stabilization of the structure by both H-bonding and aromatic interactions. These contributions may therefore occur early in the folding process of the active structure and may contribute to the orientation of the positively charged amino acids at the loop and *C*-terminal regions, thereby favouring productive peptide–LPS binding. These results are in agreement with previous studies on the LPS-binding domain of related proteins [21]. In a very recent study, it has been proposed that the presence of the pair Arg^{40}-Arg^{41} plays an important role for the structure stabilization of LALF-14c bound to LPS complex [22]. These observations are consistent with our findings in the hexapeptide mapping (see above), where the peptide LALF-09, which holds no arginine residues, showed a reduced LPS-neutralizing activity. However, the output of the alanine-scanning substitution indicates that a single point mutation was easily overcome and did not affect LPS-neutralizing activity.

Table 4 Alanine-scanning Characterization

Name	t_R/min (HPLC)	Calculated mass	Experimental mass (MALDI)		
			M + H	M + Na	M + K
LALFK47Ac	12.0	1953.58	1955.50	—	—
LALFY46Ac	11.5	1919.86	1920.50	—	—
LALFK45Ac	12.2	1954.86	1954.38	—	—
LALFW44Ac	10.9	1895.79	1897.35	—	—
LALFK43Ac	12.2	1953.00	1955.38	—	—
LALFR41Ac	12.2	1924.87	1926.37	—	—
LALFR40Ac	12.2	1927.93	1927.37	—	—
LALFF39Ac	11.0	1934.71	1935.37	—	—

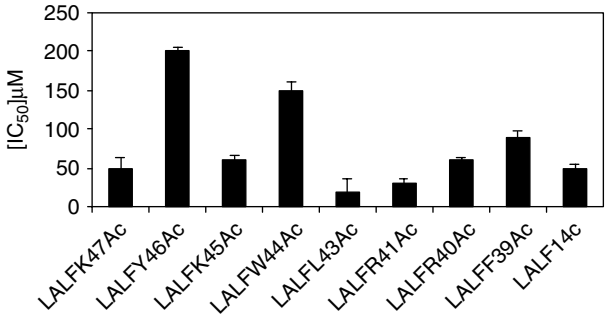

Figure 3 The inhibition of the different peptides determined using a chromogenic *Limulus* amebocyte lysate assay. The inhibition activity of LPS by peptides derived from the alanine scanning is represented as the IC_{50}. IC_{50} is the concentration necessary to neutralize *in vitro* 50% of LPS as determined by a serial dilution assay (12, 5, 25, 50, and 100 μM) using 100 pg/ml of LPS. The determined IC_{50} is the average of three independent assays, and the data is represented with ±SD.

Design of Minimized LALF14-based LPS-neutralizing Cyclic Peptides

Peptides LALF-07 and LALF-08, which cover the *C*-terminus of LALF-14-containing aromatic residues and arginines, were chosen for cyclization. In addition, peptide LALF-09 was also included in the following procedures as a putative control peptide to analyse the role of arginine residues in the biological activity of the minimized cyclic peptides. Cysteines were added at the terminal of the linear peptides LALF-07, LALF-08 and LALF-09 in order to oxidize the peptides via a disulfide bridge formation (Table 5). To facilitate the formation of the cyclic hexapeptides, we performed a distinct procedure to that used for the synthesis of LALF-14c and its alanine scanning–derived peptides (Air oxidation assisted by DMSO at neutral pH). Cys

Table 5 Design of Cyclic Hexapeptides

Name	Sequence
LALF-14c	G-(CKPTFRRLKWKYKC)-G
LALF-07	RRLKWK
LALF-08	RLKWKY
LALF-09	LKWKYK
LALF-07c	(C-RRLKWK-C)
LALF-08c	(C-RLKWKY-C)
LALF-09c	(C-LKWKYK-C)

Table 6 Characterization of Cyclic Hexapeptides

Name	t_R/min (HPLC)	Calculated mass	Experimental mass (MALDI)		
			M + H	M + Na	M + K
LALF-07c	7.6	1130.60	1131.71	1153.70	1169.68
LALF-08c	8.7	1137.56	1138.81	1160.78	1176.77
LALF-09c	8.1	1109.55	1110.71	1132.68	1148.66

was protected with the acid-labile trityl (Trt) protecting group, which is easily removed by the acidic conditions used in the final cleavage step. However, tritylation of several functional groups was observed in some crude samples, indicating that an optimized selection of scavengers should be critical at this point. Crude peptides were purified before cyclization. After all these manipulations, a small amount of cyclic peptide was observed in all cases. Peptides were dissolved in aqueous NH_3 solution at pH 9 and monitored by the Ellman's test and RP-HPLC. Reactions were followed for 3 days; a conversion higher than 90% was consistently obtained after 24–30 h of reaction. Finally, the reaction mixture was purified to afford the desired cyclic peptides. Although both methods work well, the second one (absence of DMSO) allows easier control of the reaction by HPLC and facilitates the work-up. On the other hand, a careful neutralization is required in this second method because liofilization at high pH leads to the scrambling (intermolecular reactions between thiol groups) as shown by the complexity of the HPLC. The characterization of cyclic peptides is shown in Table 6.

The LPS-binding activity of each peptide was measured at 100 μM using 100 pg/ml of LPS (Figure 4). Compared to the biological activity of the original peptides (see Figure 1), the cyclic peptides showed increased activity, indicating that both the amino acid sequence and a restricted conformation are of special relevance for LPS-binding. These results also highlight the importance of the amino acids located at the *C*-terminus of the loop region of the LALF-14c sequence for LPS-neutralizing activity (Figure 5). LALF-08c and

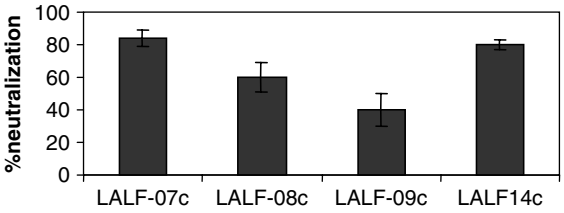

Figure 4 Anti-LPS activity of cyclic peptides determined using chromogenic *Limulus* amebocyte lysate assay described previously. Cyclic peptides were assayed at a fixed concentration (100 μM) and LPS (100 pg/ml). The neutralization activity of each peptide is compared with that of LALF14c (100 μM), the control peptide. The LPS-binding activity represented for each cyclic peptide is the average value obtained in three independent assays, and the data is represented with ±SD.

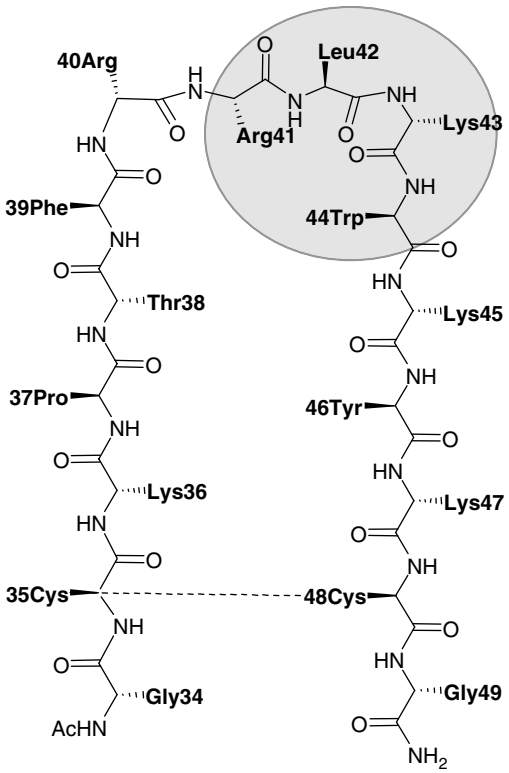

Figure 5 Schematic drawing of the loop structure of rLALF. The directions of the amino acid (three-letter code) side chains are indicated. Solid bonds indicate side chains pointing out of the plane of the diagram and dashed bonds into the plane. Cysteines were introduced pairwise instead of the authentic amino acid residues at positions indicated with a dotted line representing the resulting disulfide bridge. Amino acids inside the circle are postulated as the most relevant for the LPS-binding capability. They are contained in the sequence of the most active hexapeptides (LALF-07c, LALF-08c and RLKWc).

LALF-07c, which contain the four amino acids of the Arg-Leu-Lys-Trp sequence that were proposed as critical residues for the activity of the linear peptides, showed improved LPS-binding activity over LALF-09c,

which contains only three of the four amino acids of the same sequence. Moreover, LALF-07c showed higher activity than LALF-08c. The extra arginine residue in the former might enhance the LPS-binding capacity, as reported in previous studies [22].

The peptide LALF-07c, which showed LPS-neutralizing activity similar to that of LALF-14c, was selected for further analysis and an IC_{50} value of 60 μM was obtained, indicating that the peptide proposed earlier is a minimal LPS-binding domain of LALF, i.e. the peptide LALF-14c was susceptible to further minimization. To confirm the importance of the Arg-Leu-Lys-Trp sequence, we designed a new cyclic peptide with these four active residues (peptide named RLKWc). We added two alanines at the *N*-terminal and *C*-terminus of the RLKW sequence to keep the hexapeptide length. Two cysteines were also added to the *N*-terminus and *C*-terminus to obtain the cyclic peptide. The peptide RLKWc showed the same LPS-binding affinity at 100 μM peptide concentration as LALF14c, and IC_{50} values of 30 and 40 μM were observed for peptides RLKWc and LALF-14c, respectively.

In conclusion, our results indicate that a detailed analysis of bioactive peptides could render a minimization of the biologically active sequences, which could be of interest for further optimization studies, such as those addressing the design of small organic molecules. In fact, from an initial bioactive peptide (LALF-14c) with 16 amino acid residues and after a detailed analysis of sequence–activity relationships, we obtained a tetrapeptide sequence, which when inserted in a Cys-aided cyclic peptide, displayed the same biological activity.

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COMPLEMENTARY EXPERIMENTAL DATA FOR:

Design of a minimized cyclic tetrapeptide that neutralizes bacterial endotoxins

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Synthesis and cyclization of RLKWc: [CA-RLKW-AC]*

Synthesis

Fmoc-Rink Amide MBHA resin (100 mg, 0.45 mmol/g) was placed in a 10 mL-polypropylene syringe fitted with a polyethylene filter disk. After Fmoc removal, Fmoc-amino acids were coupled with DIC (4 equiv) and HOAt (4 equiv) in DMF for 2 h. Side chains of Fmoc-amino acids were protected as follows: Lys and Trp were protected with the *tert*-butyloxycarbonyl group (Boc), Cys with the trityl group (Trt) and Arg with the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl group (Pbf). For the deprotection of side-chain groups and concomitant cleavage of the peptide from the solid support, the resin was washed with CH_2Cl_2 (3 × 1 min), dried and treated with a TFA–H_2O–TIS (95:2.5:2.5) mixture for 1.5 h. TFA was then removed by evaporation with nitrogen, and the peptide was precipitated with cold anhydrous TBME, dissolved in H_2O–MeCN (1:1) and then lyophilized to afford the linear precursor (29 mg, 68%).

Cyclization

Next, the linear peptide (8.9 mg, 0.009 mmol) was dissolved with H_2O–MeCN (1:1) at a concentration of 0.5 mg/ml in a round-bottom flask. The pH was then adjusted to 8-9 with a 20% solution of NH_3. The solution was stirred at room temperature for 24 h to allow air oxidation. Cyclization was easily monitored either by Ellman's test and/or by RP-HPLC. The solution was then acidified with a 4 % AcOH aqueous solution and lyophilized to yield the cyclic peptide **RLKWc** (5.2 mg, 58 %).

Purification and characterization[†]

RLKWc was finally purified by HPLC (linear gradient from 0 to 50 % MeCN over 30 min, flow rate 3 mL/min) to obtain 1.6 mg (purification yield 31 %). Characterization of **RLKWc**: HPLC (t_R = 5.42 min, from 0 to 50 % CH_3CN over 8 min, purity >99 %), MALDI-TOF (*m/z* calcd. for $C_{41}H_{66}N_{14}O_8S_2$ 946.46, found 947.26 $[M+H]^+$, 969.23 $[M+Na]^+$).

[*] Brackets denote cyclization via disulfide bridge

[†] For semi-preparative HPLC was used a Symmetry C18 column (30 x 150 mm, 5-µm) at a flow rate of 20.0 mL/min. Analytical HPLC was conducted on a Sunfire C18 column (4.6 x 100 mm, 3.5-µm) at a flow rate of 1.0 mL/min. Chromatography systems are explained in detail in the Material and Methods section

“Pro-insertion on LALF-14c: modulation of its LPS-neutralizing activity”

Unpublished results

INTRODUCTION

The early inhibition of LPS has been proposed as a promising strategy to prevent the harmful over-stimulation of the immune system that occurs in the pathology of sepsis. In this regard, we have previously introduced LPS-neutralizing peptides as a powerful tool for the discovery of potent and efficient LPS-neutralizers. An interesting candidate for these purposes is the cationic protein LALF.[1] This 11.8-kDa basic protein inhibits the LPS-mediated cascade in the horseshoe crab *Limulus polyphemus* and has been the subject of a great number of studies that have addressed the identification of small peptides with LPS-neutralizing activity. One of these studies proposed the cyclic peptide LALF-14c as the minimal LPS-binding domain of the protein.[2] This peptide (residues 36-47) mimics the amphipathic β-hairpin loop (residues 31-52) present in the protein[3] and has LPS-neutralizing activity comparable to that of PMB.[2]

However, whether the peptide LALF-14c adopts the same β-hairpin structure found in the natural protein or not, has yet to be unequivocally elucidated. NMR and molecular modeling studies performed by Pristovsek *et al*. showed that LALF-14c does not have a regular secondary structure in water or when bound to LPS.[4] This observation thus suggests that a β-sheet-like conformation is not a prerequisite for LPS-binding, as long as the amphipathic conformation is maintained. In contrast, Pérez-Payá's group has recently published a study where they demonstrate that the presence of a "nascent" secondary structure (i.e. β-hairpin) is related to the biological activity of LPS-neutralizing peptides.[5] In this study, on the basis of DC and NMR observations, the authors proposed that LALF-14c adopts an amphipathic β-hairpin structure (Figure 1).

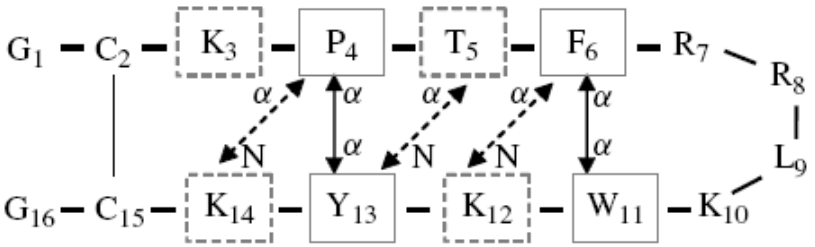


Figure 1: Schematic representation of the β-hairpin proposed to be adopted by LALF-14c. Arrows indicate long-range contacts detected by NMR: continuous arrows represent Hα-Hα NOEs and discontinuous arrows Hα-NH NOEs. From ref [5].

Next, they evaluated the effect of the insertion of D-Pro at the loop region (amino acids Arg_{40} Arg_{41} Leu_{42} Lys_{43}) in terms of structure and biological activity. The rationale of this design was based on previous work that reported that β-sheet hairpins containing type I or II turns are stabilized by the presence of a D-amino acid (D-Pro) and Gly at the i+1 and i+2 positions of the loop.[6] Hence the authors reasoned that the stabilizing effect of D-Pro in different β-turns could be applied to LALF-14c to induce changes in its secondary structure and in its activity. The schematic representation of the resulting peptides is shown in Figure 2.

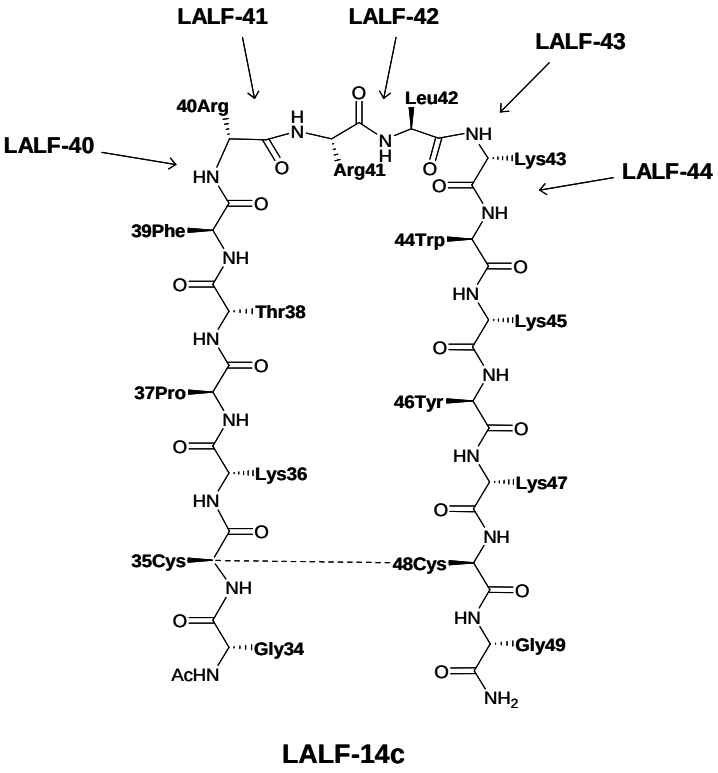


Figure 2: Schematic representation of D-Pro insertions (indicated by an arrow). D-Pro was inserted before the residues that cover the β-turn: Arg40, Arg41, Leu42, Lys43 and Trp44. The resulting peptides were named according to these positions.

The authors analyzed these peptides and concluded that the insertion of D-Pro induced a position-dependent partial destabilization of the turn. The β-hairpin distortion was more accentuated for peptides deriving from D-Pro insertion at the *N*-end of the β-turn, i.e. peptides LALF-40, -41 and -42. The LPS-neutralizing activity of these peptides was also abruptly reduced. In contrast, peptides LALF-43 and -44 retained the original activity. Since these peptides presented a lower extent of structure distortion, the

authors concluded that only peptides with a certain degree of a β-hairpin-like secondary structure display LPS-neutralizing activity. Furthermore, LALF-44 significantly inhibited the release of TNF-α in a murine model of endotoxaemia *in vivo*.[5]

As a continuation of this work and in collaboration with that research group, we addressed the effect of further modifications of the structure of LALF-14c. We introduced three distinct motifs based on Pro residues known to induce β-turns to its sequence and replaced the disulfide bridge by an amide bond. Here we present the synthesis of these new LALF-44 analogues and their biological activity.

RESULTS AND DISCUSSION

Design of LALF-44 analogues

In the case of LALF-44, the introduction of a D-Pro after Lys_{43} induced a two-residue turn (Lys_{43}-D-Pro) that led to a much distorted β-hairpin (compared to LALF-14c) where Tyr_{46} and Trp_{44} interacted with Thr_{38} and Leu_{42}.[5] The incorporation of both L- and D-Pro into cyclic peptides seems to be the most promising way to induce β-turn structures.[7] This capacity is due to the cyclic conformation of this amino acid, which favors a *cis*-amide bond configuration. The introduction of D-amino acids in cyclic peptides has also been described to be useful to induce these kinds of structures.[8,9] Another structure-inducing element is the pair D-amino acid-L-Pro. This sequence is present as a β-turn motif in several cyclic β-sheet peptides.[10]

Hence, we designed three peptides with distinct β-turn-inducer elements in order to evaluate the effect of these modifications on the final biological activity of peptides. These peptides contained an L-Pro residue after Lys_{43} (**L-LALF-44**), a D-Pro after Lys_{43} (**D-LALF-44**) or an L-Pro proceeded by Lys_{43} in the D-configuration (**k-LALF-44)**.

In addition, a further modification of these peptides was considered, and the disulfide bridge was substituted by an amide bond. For this purpose, Cys were replaced by diaminopropionic acid (Dap) and Glu at the *C*- and *N*-terminus respectively. Glu was

preferred to Asp because of the great tendency of the latter to induce aspartimide formation.[11] The sequences of the designed peptides designed are shown in Table 1.

Table 1: Sequences of the LPS-neutralizing peptides LALF-14c and LALF-44, and the derivatives designed.

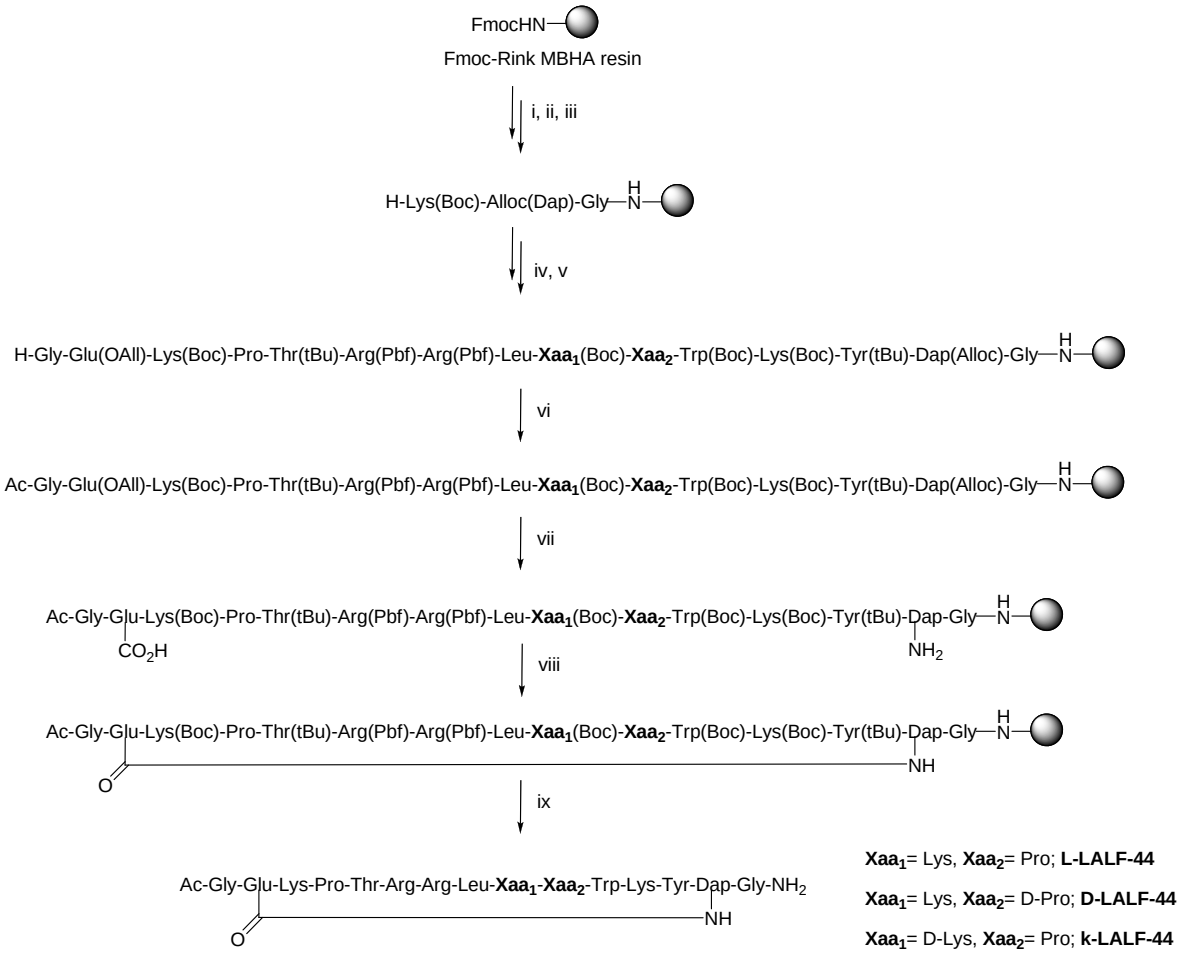
Peptide	Sequence[a]
LALF-14c	G(C-K-P-T-F-R-R-L-K-W-K-Y-K-C)G
LALF-44	G(C-K-P-T-F-R-R-L-K-p-W-K-Y-K-C)G
L-LALF-44	G(E-K-P-T-F-R-R-L-K-P-W-K-Y-K-Dap)G
D-LALF-44	G(E-K-P-T-F-R-R-L-K-p-W-K-Y-K-Dap)G
k-LALF-44	G(E-K-P-T-F-R-R-L-k-P-W-K-Y-K-Dap)G

[a] Brackets denote cyclization via either disulfide bridge or amide bond. Lower-case letters represent D-amino acids (in green). The insertion of L-Pro is shown in blue. Residues that substitute Cys are shown in red: glutamic acid (E) and diaminopropionic acid (Dap).

Synthesis of LALF-44 analogues

The first consideration to take into account for the synthesis of the analogues was to decide on cyclization in solution or in solid-phase. Although there are no strict limitations for the use of one approach or the other, generally, for side-chain to side-chain cyclizations, on-resin ring closure is preferred.[12] Fmoc-Rink MBHA resin was chosen as solid support and an orthogonal protection scheme using Fmoc/tBu/Allyl protecting groups was selected (see Scheme 1). Hence, Fmoc-Gly-OH was manually inserted into the resin using DIC and HOAt as coupling method. Next, Fmoc-Dap(Alloc)-OH was subequivalently (in defect) coupled in order to reduce the loading of the resin, since various comparative studies have confirmed that the amount of unwanted cyclodimers, cyclooligomers and other by-products obtained in the cyclization step, is higher with resins of high loading capacity.[13,14] The peptidyl-resin was acetylated and Fmoc-Lys(Boc) was incorporated with DIC in the presence of HOAt. Subsequent peptide chain elongation was carried out automatically on a peptide synthesizer using TBTU/HOBt as coupling reagents. When the peptide assembly was completed, the *N*-terminal amino group was acetylated and Allyl-protected side-chains were orthogonally deprotected with catalytic palladium treatments. Side-chain to side-chain on-resin cyclization was accomplished after treatment with PyBOP, HOAt and

DIEA for 24 to 48 h, as indicated by colorimetric methods and HPLC analysis. The peptides were finally cleaved, purified and characterized as described in the Experimental Section.



Scheme 1: General procedure for the synthesis of LALF-44 derivatives: (i) piperidine–DMF (1:4); (ii) Fmoc-AA-OH, DIC, HOAt, DMF; repeat [i,ii] 2 times; (iii) piperidine–DMF (1:4); (iv) Fmoc-AA-OH, TBTU, HOBt, DMF; repeat [iii, iv] 14 times; (v) piperidine–DMF (1:4); (vi) Ac_2O–DIEA–DMF (1:2:7); (vii) $PhSiH_3$, $Pd(PPh_3)_4$; (viii) PyBOP, HOAt, DIEA, DMF; (ix) TFA–H_2O–TIS (95:2.5:2.5).

LPS-neutralizing activity of LALF-44 analogues

The anti-LPS activity of the three peptides was assayed using the chromogenic LAL assay[15] (see the Experimental Section for details). The three peptides displayed high values of LPS-neutralization at 100 μM (data not shown), similar to the parent peptides

LALF-14c and LALF44; hence, they were subjected to serial dilutions and their IC_{50} values (i. e. the concentration necessary to neutralize 50% of LPS *in vitro*) were calculated (Table 2).

Table 2: LPS-neutralizing activity (IC_{50}) of LALF-44 analogues

Peptide	IC_{50} (μM)[a]
LALF-14c	40
LALF-44	60
L-LALF-44	6
D-LALF-44	15
k-LALF-44	8

[a] The inhibition of the compounds was determined using the chromogenic LAL assay. The inhibitory activity is represented as IC_{50}. Standard deviation of the data was lower than 10%. The assay was performed as described in the Experimental section.

Overall the modifications introduced in the LALF-14c sequence resulted in peptides with enhanced LPS-neutralizing activities. These biological activities were 3- to 10-fold greater than for the original parent peptides. The introduction of L-Pro (**L-LALF-44**) and D-Lys-L-Pro (**k-LALF-44**) gave the most active compounds. In the case that these activities are correlated with the tendency to adopt nascent secondary structures, that would imply that the introduction of these elements is a preferred option to induce β-turns than the insertion of D-Pro. Nevertheless, structural studies aimed to identify the degree of structuration of these peptides should be performed in order to support this notion. In addition, the substitution of a disulfide bridge by an amide bond seems to be a viable strategy to modify anti-LPS peptides. This is illustrated specially in the case of **D-LALF-44**. This analogue is identical in sequence to LALF-44, the only difference being the presence of an amide bond in lieu of the disulfide bond. This modification rendered a peptide with a 4-fold increase in biological activity over than of LALF-44. To the best of our knowledge, this is the first example of peptides derived from the protein LALF that are cyclized by an amide bond and show significant anti-endotoxin potency. We believe that this modification could also be applied to other LALF-derived peptides as a promising new strategy to identify LPS-inhibitors. In addition, an amide bond confers a non-pH dependent bond with higher stability in biological media.

CONCLUDING REMARKS

Here we have presented three novel LPS-neutralizing peptides based on the introduction of modifications in the peptide sequence of LALF-14c. Both the presence of elements that induce β-turns and the cyclization via an amide bond proved excellent strategies to improve the LPS-neutralizing capacity of LALF-derived peptides. However, to better understand the mechanisms of action of these peptides and to assess the influence of secondary structures in their biological activities, structural studies should be performed. The efficacy of these compounds to neutralize LPS should also be confirmed in cellular systems and *in vivo* models of septicemia.

EXPERIMENTAL SECTION

Material and general methods

Materials and instrumentation: Fmoc-Rink amide MBHA resin and protected Fmoc-amino acids were purchased from Iris Biotech GmbH (Marktredwitz, Germany). Coupling reagents, solvents for peptide synthesis and other reagents were purchased from commercial suppliers at the highest purity available and used without further purification. Analytical HPLC was performed using a Waters Alliance 2695 (Waters, MA, USA) chromatography system with a PDA 995 detector, a reverse-phase Symmetry C_{18} column (4.6 x 150 mm, 5-μm) column and linear gradients of MeCN with 0.036% TFA into H_2O with 0.045% TFA. The system was run at a flow rate of 1.0 mL/min over 15 min. Semi-preparative HPLC was carried out on a Waters chromatography system with a dual absorbance detector 2487, a reverse-phase Symmetry C_{18} column (30 x 150 mm, 5-μm) column and linear gradients of MeCN with 0.05% TFA into H_2O with 0.1% TFA. The system was run at a flow rate of 20.0 mL/min over 30 min. HPLC-MS was performed using a Waters Alliance 2796 with a dual absorbance detector 2487 and ESI-MS Micromass ZQ (Waters) chromatography system, a reverse-phase Symmetry 300 C_{18} (3.9 x 150 mm, 5-μm column) and H_2O with 0.1% formic acid and MeCN with 0.07% formic acid as mobile phases. Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (Applied Biosystems, Foster City, CA, USA) using ACH matrix.

Solid-phase peptide synthesis: Manual solid-phase peptide synthesis was performed in polypropylene syringes, each fitted with a polyethylene porous disk, using the Fmoc/^{t}Bu strategy. Solvents and soluble reagents were removed by suction. Washings between deprotection, couplings and subsequent deprotection steps were carried out with DMF and DCM using 10 mL of solvent/g of resin each time. The Fmoc group was removed by treatment with piperidine-DMF (1:4, v/v) and acetylation steps were performed with Ac_2O–DIEA–DMF (1:2:7). Couplings and washes were performed at 25 ºC. Couplings were monitored using Kaiser, de Clercq or chloranil methods.[16] Automatic peptide synthesis was carried out using an automatic peptide synthesizer ABI 433A (Applied Biosystem) following standard Fmoc chemistry and a FastMoc protocol.

Loading calculation: Nα-Fmoc deprotection with piperidine gives the fulvene-piperidine adduct, which can be quantitatively determined by spectrophotometric measurements at 290 nm (ε_{290nm} = 5800 $L \cdot mol^{-1} \cdot cm^{-1}$).

Synthesis of LALF-44c-derivatives

General procedure: LALF-44-derivatives were synthesized each in 10 mL-polypropylene syringes using Fmoc-Rink Amide MBHA resin (300 mg, 0.66 mmol/g). After Fmoc removal, Fmoc-Gly-OH (4 equiv) was coupled using DIC (4 equiv) and HOAt (4 equiv) in DMF for 2 h. The resin was then acetylated and the Fmoc group removed. Loading calculation revealed a resin loading of 0.55 mmol/g. Next, Fmoc-Dap(Alloc)-OH (0.6 equiv) was subequivalently added to reduce the loading of the resin using the same DIC (0.6 equiv)/HOAt (0.6 equiv) coupling system for 2 h. The incomplete coupling was corroborated with a positive Kaiser test. After acetylation, the third amino acid Fmoc-Lys(Boc)-OH (4 equiv) was inserted in the presence of DIC (4 equiv) and HOAt (4 equiv) for 2 h. After these manipulations, the loading was re-calculated, giving values of 0.19-0.21 mmol/g. Subsequent peptide elongations were conducted using an automatic peptide synthesizer, on a 0.1-mmol scale with a 10-fold excess of Fmoc-protected amino acids and 0.45 M TBTU in the presence of HOBt in DMF as coupling reagents. The syntheses were carried out with a 15-min deprotection step and a 35-min coupling. After the assembly was completed, the peptide was acetylated and the allyl protecting groups were removed with 3 x 15 min treatments of

$PhSiH_3$ (20 equiv) and $Pd(PPh_3)_4$ (0.2 equiv) in DCM. After these treatments the resin was washed with sodium dithiodiethylcarbamate (0.02 M in DMF, 3 x 15 min) to remove any remaining palladium traces of the resin. The efficiency of this deprotection step was monitored by HPLC. Next, peptides were cyclized on-resin using PyBOP (4 equiv), HOAt (4 equiv) and DIEA (12 equiv) in DMF for 24 to 48 h depending on the peptide. Cyclization completion was analyzed by Kaiser and/or HPLC methods. Finally, for the deprotection of side-chain groups and concomitant cleavage of the peptide from the support, the resin was washed with DCM (3 x 1 min), dried, and treated with TFA-H_2O-TIS-EDT (95:2:2:1) for 2 h. TFA was then removed by evaporation with nitrogen, and the peptides were precipitated with cold anhydrous TBME, dissolved in H_2O-MeCN (1:1) and then lyophilized. The cyclic crude peptides were purified by semi-preparative HPLC (see conditions used below) up to optimal purities. Compounds were characterized by HPLC and mass spectrometry.

L-LALF-44: The crude cyclic peptide was purified by semi-preparative HPLC: linear gradient from 10 to 40 % MeCN over 30 min, flow rate 20 mL/min. Characterization: HPLC (from 0 to 50 % MeCN over 15 min, t_R = 8.35 min, 94 %), MALDI-TOF (*m/z* calcd. for $C_{99}H_{154}N_{30}O_{21}$ 2099.19; found, 2100.39 $[M+H]^+$, 2122.02 $[M+Na]^+$).

D-LALF-44: The crude cyclic peptide was purified by semi-preparative HPLC: linear gradient from 15 to 30 % MeCN over 30 min, flow rate 20 mL/min. Characterization: HPLC (from 0 to 50 % MeCN over 15 min, t_R = 8.17 min, 98 %), MALDI-TOF (*m/z* calcd. for $C_{99}H_{154}N_{30}O_{21}$ 2099.19; found, 2099.68 $[M+H]^+$, 2122.62 $[M+Na]^+$, 2137.62 $[M+K]^+$).

k-LALF-44: The crude cyclic peptide was purified by semi-preparative HPLC: linear gradient from 15 to 30 % MeCN over 30 min, flow rate 20 mL/min. Characterization: HPLC (from 0 to 50 % MeCN over 15 min, t_R = 8.46 min, 99 %), MALDI-TOF (*m/z* calcd. for $C_{99}H_{154}N_{30}O_{21}$ 2099.19; found, 2100.62 $[M+H]^+$, 2122.61 $[M+Na]^+$, 2138.31 $[M+K]^+$).

LPS-neutralizing activity

All solutions used in the LPS-neutralizing activity assay were tested to ensure they were endotoxin-free and material was sterilized by heating for 3 h at 180 ºC. LPS from *E. Coli* 055:B5 and Polymyxin B were purchased from Sigma. LPS-neutralizing activity was measured using the chromogenic *Limulus* Amebocyte Lysate (LAL) test,[15] following the manufacturer's instructions (Cambrex). LAL reagent contains a clottable protein that is activated in the presence of non-neutralized LPS and is an extremely sensitive indicator of the presence of endotoxin. When activated, this enzyme catalyses the release of p-nitroaniline (pNA) from the colorless chromogenic substrate Ac-Ile-Glu-Ala-Arg-pNA. The pNA released can be measured photometrically at 405 nm. Compounds were initially incubated at 100 μM with LPS (100 pg/mL) in a 96-well microtiter for 60 min at 37 ºC. Polymyxin B (10 μg/mL) was used as positive control. LAL (12.5 μL) was added to start the reaction at 37 ºC. After 10 min, non-neutralized LPS was detected after a 5-8 min incubation with the chromogenic substrate (25 μL). Acetic acid (25 % v/v final concentration) was added to stop the reaction and the absorbance was monitored at 405 nm in a Multiskan Ascent microtiter plate reader (ThermoLabsystems). The IC_{50} values (the concentration necessary to neutralize 50% of LPS *in vitro*) for each peptide were determined by a serial dilution assay using 100 pg/mL of LPS and a range of compound concentrations (50 to 0.001 μM). All assays were run in triplicate, and the curves were automatically adjusted by non-linear regression using "Prism 4" (GraphPad) software.

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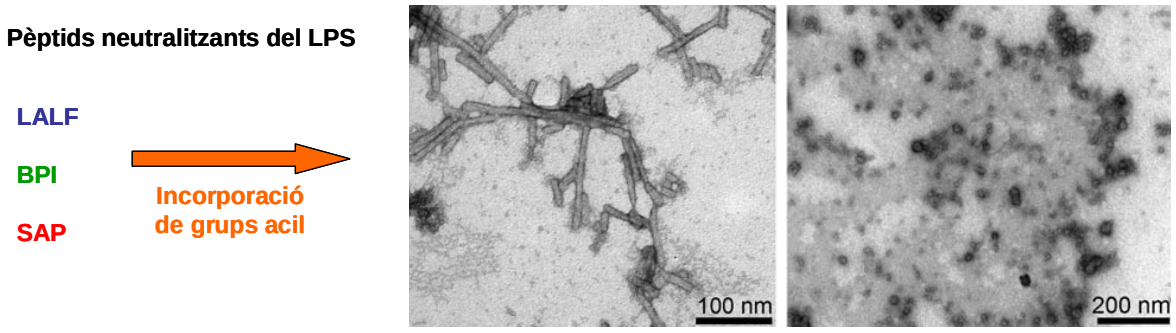
"Nanostructure formation enhances the activity of LPS-neutralizing peptides"
Carlos Mas-Moruno, Laura Cascales, Luis J. Cruz, Puig Mora, Enrique Pérez-Payá, Fernando Albericio
ChemMedChem, **2008**, vol. *3*(11), pp. 1748-1755

RESUM

En el següent treball es presenta la preparació de diferents pèptids acilats a l'extrem *N*-terminal amb activitat anti-LPS derivats de les proteïnes LALF, BPI i SAP. En tots els casos, la presència d'àcids grassos de cadena llarga va comportar un gran augment en la capacitat d'aquests pèptids per neutralitzar el LPS. L'anàlisi estructural d'aquests pèptids per microscopia de transmissió electrònica (TEM), va demostrar que la inserció de cadenes alifàtiques en aquests pèptids promou la formació de nano-estructures de tipus micelar o fibrilar. D'aquesta manera es va poder establir una correlació entre la capacitat que tenen els pèptids per estructurar-se i la seva activitat biològica.



Formació de nano-estructures i activitat millorada

Contribucions a aquest treball:

- En Carlos va realitzar el disseny, la síntesi i la caracterització de tots compostos descrits. També va col·laborar ens els assaigs d'activitat biològica i va portar a terme els estudis de microscopia de transmissió electrònica. Va elaborar la major part del manuscrit.

- La Laura Cascales va realitzar els assaigs d'activitat biològica i els experiments de toxicitat en cèl·lules. També va contribuir en la preparació del manuscrit.

CHEMMEDCHEM

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Nanostructure Formation Enhances the Activity of LPS-Neutralizing Peptides

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Peptides that interact with lipopolysaccharide (LPS) can provide the basis for the development of new antisepsis agents. In this work, several LPS-neutralizing acyl peptides derived from LALF, BPI, and SAP were prepared, structurally characterized, and biologically evaluated. In all cases, peptides with long acyl chains showed greater LPS-neutralizing activities than the original acetylated peptides. Structural analysis of these peptides revealed that N-acylation with long acyl chains promotes the formation of micellar or fibril-like nanostructures, thus proving a correlation between anti-LPS activity and nanostructure formation. The results of this study provide useful structural insight for the future design of new acyl peptides that strongly bind LPS and therefore act as antisepsis drugs. Furthermore, this nanostructure–biological activity correlation can be translated into other therapeutic areas.

Introduction

Sepsis, including its acute state, septic shock, is the first cause of mortality in intensive care units[1] and is among the leading causes of mortality worldwide. In 2003, septicemia was the 10th foremost cause of death in the US.[2] Sepsis and other infectious diseases are produced by a bacterial endotoxin, the lipopolysaccharide (LPS), a major component of the cell wall in Gram-negative bacteria.[3] LPS recognition by immune system cells is detected on the basis of the pathology.[4] Although low amounts of LPS can be beneficial for the host immune system, continuous exposure to LPS in the mammalian bloodstream induces deregulation of the release of inflammatory cytokines, thereby leading to the pathological condition.[5] The immunogenic cascade is initiated by LPS recognition and binding to the circulating LPS binding protein (LBP), which transfers the bacterial endotoxin to the CD14 receptor.[6,7] This complex binds to the transmembrane receptors of the Toll-Like receptor family (TLR) in order to transduce the signal into the cell, an event that initiates the transcription of cytokine genes.[8]

Research efforts have been directed towards the characterization of the LPS signaling pathway in order to define pharmacological targets.[9–14] However, although inhibitors of tumor necrosis factor (TNF) and other inflammatory mediators have been targeted, this approach has not yet increased the survival rates of septic shock patients.[15,16] There is currently only one USFDA-approved therapy that increases survival in adult patients with high-risk severe sepsis: Xigris (activated drotrecogin alfa), which decreases microvascular dysfunction by reducing inflammation and coagulation, and by increasing fibrinolysis.[17] Although drotrecogin alfa improves survival,[18,19] there are concerns regarding some of the effects of this drug, and complementary agents would improve the therapeutic outcome.[20] In this regard, the inhibition of LPS at the beginning of this process is considered a promising approach. Thus, compounds with the capacity to extracellularly neutralize LPS or its toxic portion, the lipid A moiety, may provide a potential source of useful lead compounds of pharmacological relevance. In this sense, a considerable research effort has been devoted to LPS binding proteins and their derived peptides, as described in a detailed review recently published by Chaby.[21] In the study reported herein, we focused on three LPS binding proteins of distinct origin: 1) the *Limulus* anti-LPS factor (LALF), 2) the bactericidal permeability-increasing protein (BPI), and 3) serum amyloid P (SAP).

LALF is a small (101 amino acids) basic protein that inhibits the LPS-mediated coagulation cascade.[22] An amphipathic loop that spans residues 31–52 has been described as the LPS binding site.[23] Further studies determined the minimal LPS binding domain as a 14-amino acid cyclic peptide named LALF-14c, comprising residues 36–47.[24] Recently, we described an even shorter cyclic peptide, RLKWc, containing key residues Arg41-Leu42-Lys43-Trp44 with activity similar to that of LALF-14c.[25] BPI is a 57-kDa cationic protein from human neutrophils that binds to and neutralizes LPS and has high bactericidal activity against Gram-negative bacteria. It shares a high degree of ho-

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mology with LBP[26] and contains three LPS binding sites (residues 17–45, 65–99, and 142–169).[27] Of these regions, a 15-mer synthetic peptide (residues 85–99) was shown to be bactericidal.[27] Interestingly, the region comprising amino acids 86–104 shows homology with the antiparallel β-sheet folding present in LALF and that participates in LPS binding. The two sequences share a common structural motif, with hydrophobic and cationic β-sheet faces, which facilitates LPS interaction.[28] Finally, SAP is a serum multi-specific glycoprotein that binds LPS and other molecules.[29] SAP has three LPS binding sites (residues 27–39, 61–75, 186–200),[30] but only the latter two are truly accessible for interaction on the protein surface. Moreover, the 15-mer SAP-derived peptide (186–200) protects against LPS-induced septic shock in animal models.[30]

The molecular mechanism by which these proteins neutralize LPS derives from their capacity to recognize and bind the endotoxic lipid A moiety of LPS[33,34] (Figure 1). The requirements to exert effective binding are best described for the cyclic antibiotic peptide polymyxin B (PMB),[35] which contains five positive charges and an N-linked acyl chain. In fact, a C_{12} alkyl acylation of the lactoferrin-derived peptide LF11 results in both changes in its conformational properties, and in an enhancement of its LPS binding affinity.[36–38] Herein, the solid-phase synthesis, structural analysis, and biological activity of N-acylated peptides derived from LALF-14c, BPI, and SAP are described.

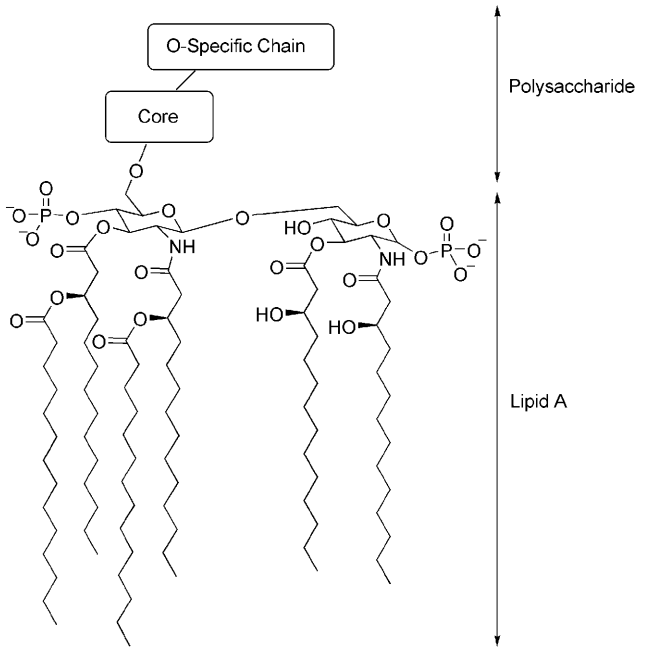


Figure 1. General structure of LPS showing the chemical detail of the lipid A moiety. LPS comprises three covalently linked domains: 1) an O-specific chain, an immunogenic, highly variable repeating polysaccharide that extends into the external medium; 2) an inner core oligosaccharide; and 3) a glycolipid, the so-called lipid A, formed by a 1,4′-bisphosphorylated glucosamine disaccharide that carries several amide- and ester-linked fatty acids.[31,32] The lipid A structure is conserved in all species of Gram-negative bacteria.

Results and Discussion

N-Acylated peptides derived from LALF-14c

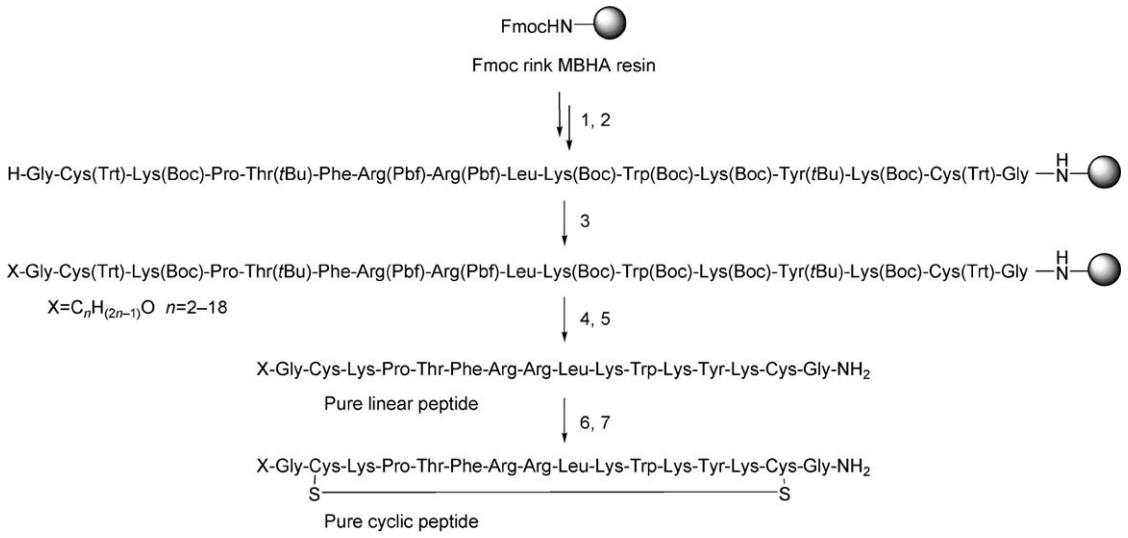
The optimal chain length for LPS interaction was studied after conjugation of LALF-14c (H-G[CKPTFRRLKWKYKC]G-NH_2)[1] with distinct aliphatic acids: acetic acid (C_2), butyric acid (C_4), caproic acid (C_6), octanoic acid (C_8), decanoic acid (C_{10}), lauric acid (C_{12}), myristic acid (C_{14}), palmitic acid (C_{16}), stearic acid (C_{18}), and linoleic acid (C_{18} unsaturated). Although the synthesis of acetylated LALF-14c (C_2-LALF-14c) has already been described[39] and can be easily achieved upon cyclization with dimethyl sulfoxide (DMSO), the synthesis of N-acylated derivatives, particularly those with a greater number of carbon units, was more challenging. The solubility of the peptides decreases with increasing chain length. These peptides were prone to aggregation, and were therefore difficult to purify. To overcome this limitation, a new strategy was developed (see Scheme 1): peptides were synthesized on solid phase following an Fmoc/*t*Bu strategy. Cysteine residues were added at the N and C termini to obtain the cyclic peptide that stabilizes the secondary structure.[24] Acyl chains were then introduced onto the free amino group at the N terminus using PyBOP/HOAt/DIEA as coupling reagents. The linear peptides were cleaved from the solid support, purified by semipreparative RP-HPLC (linear peptides at this point do not present solubility problems) and then cycled. When cyclization was completed, the crude products were treated with HP-20 Diaion resin, which, after an easy workup, yielded the cyclic peptides with excellent purities and without further purification. This methodology can be applied to various peptides and is a straightforward procedure to obtain cyclic peptides with low aqueous solubility.

The resulting peptides were characterized by HPLC and mass spectrometry and assayed for anti-LPS activity using the chromogenic *Limulus* amebocyte lysate assay (LAL)[40] (Table 1). C_{18}-LALF-14c and C_{18}-unsat-LALF-14c were discarded for this assay because the former was insoluble in all the aqueous media assayed, and the latter underwent hydration of its saturated bonds under acidic cleavage conditions.

The initial increase in acyl chain length from C_2-LALF-14c to C_4- and C_6-LALF-14c had a minor effect on LPS-neutralizing activity. However, this activity clearly improved for the peptides with longer acyl chains, that is, the C_8-LALF-14c to C_{16}-LALF-14c series. In particular, C_{16}-LALF-14c showed a 10-fold increase in LPS-neutralizing activity over the original C_2-LALF-14c peptide.

Further biological analyses were performed using whole-cell systems. As mentioned above, the pro-inflammatory cytokine TNF is one of the earliest and most important mediators of the LPS-induced immune response.[41] Before testing whether these compounds inhibit the release of TNF in LPS-challenged macrophages, the nonspecific toxicity of the compounds was investigated with MTT assays in RAW 264.7 macrophages. C_{12}-, C_{14}-, and C_{16}-LALF-14c were slightly toxic toward these cells (Figure 2). However, the toxicity profile of C_2-, C_4-, C_6-, C_8-, and

[1] *Brackets [ ] indicate where the peptide is cyclized by a disulfide bridge.*



Scheme 1. General procedure for the synthesis of N-acylated LALF-14c-derived peptides: 1) piperidine/DMF (1:4); 2) Fmoc-AA-OH, HOAt, DIC; repeat 15 times; 3) fatty acid, PyBOP, HOAt, DIEA; 4) TFA/H_2O/TIS (95:2.5:2.5); 5) semipreparative HPLC purification; 6) HOAc/DMSO/H_2O (1:3:16), $(NH_4)_2CO_3$; 7) treatment with Diaion HP-20.

C_{10}-LALF-14c could be described as tolerable for this cell system. In fact, C_8- and C_{10}-LALF-14c showed a greater capacity to inhibit LPS-induced TNF production than the parent C_2-LALF-14c peptide (Figure 3). These results, except for those peptides that exceeded the toxicity limits, correlate well with the activities found in the in vitro assay, thereby suggesting that the neutralization of LPS affects cell signaling and could be a valuable approach to control the undesired release of TNF and other cytokines.

Table 1. LPS-neutralizing activity of LALF-14c-derived peptides

Peptide	IC_{50} [μM]	SD
C_2-LALF-14c	37	1.2
C_4-LALF-14c	51	1.1
C_6-LALF-14c	38	1.1
C_8-LALF-14c	15	1.1
C_{10}-LALF-14c	8	1.1
C_{12}-LALF-14c	4	1.1
C_{14}-LALF-14c	3	1.5
C_{16}-LALF-14c	3	1.1

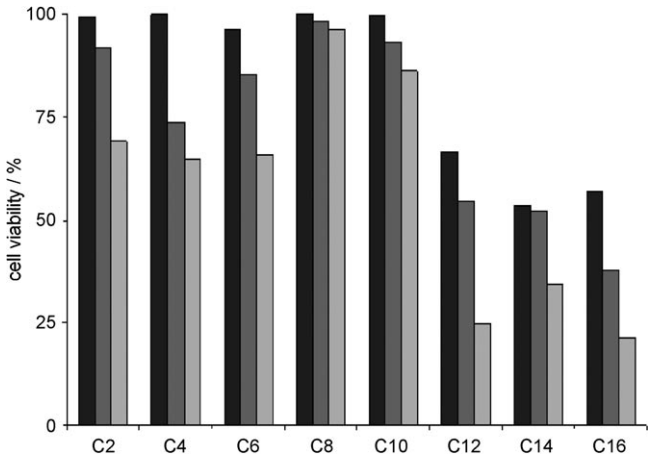


Figure 2. Cell viability is shown at a range of peptide concentrations: 10 (black bars), 5 (gray bars), and 1 μM (light gray bars). Cell viability was evaluated in RAW 264.7 cells by MTT assay, as described in the Experimental Section.

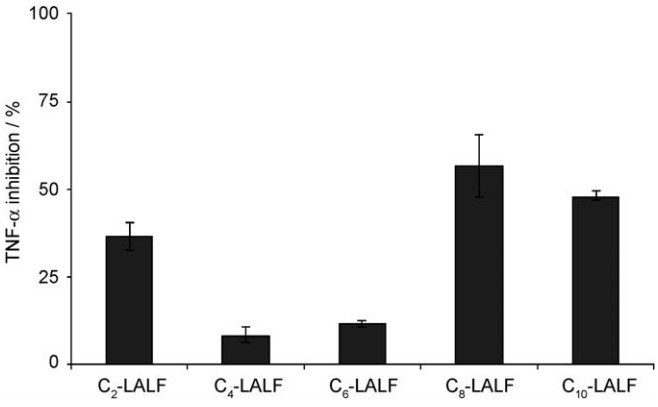


Figure 3. The inhibition of LPS-induced TNF in RAW 264.7 cells is shown. Peptides were tested at a concentration of 10 μM. Only TNF inhibition of nontoxic peptides at this concentration is shown.

Structural studies on LALF-14c-derived peptides

The above results suggested that the acyl moiety plays an important role in the conformation of the peptide, which can affect the biological activity of the peptides. Thus, a comparative analysis of the conformational behavior of C_2- and C_{16}-LALF-14c was carried out by transmission electron microscopy (TEM). The peptides were dissolved in phosphate-buffered saline (PBS) at a concentration range of 0.05–5 mg mL^{-1} and stained with 2% uranyl acetate. TEM images (see Figure 4) showed that C_{16}-LALF-14c, but not C_2-LALF-14c, formed

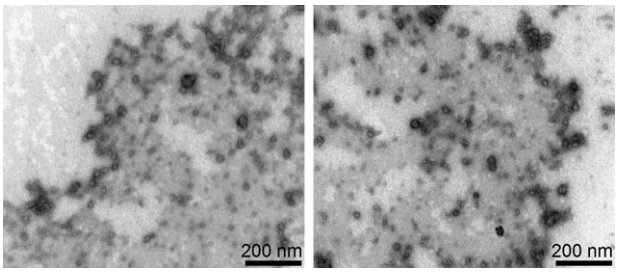


Figure 4. TEM images of C_{16}-LALF-14c. Two representative micrographs are shown. Peptides were dissolved in PBS at 5 mg mL^{-1} and treated as explained in the Experimental Section. All samples were prepared in triplicate, and images obtained were reproducible in all grids.

micelles at 5 mg mL^{-1} (i.e. 2 μM for C_{16}-LALF-14c). This result is consistent with previous studies in which peptide amphiphiles with acyl tails (i.e. C_{16}) are described to form spherical micelles.[42] The diameter of these micelles varied from 15 to 20 nm, and several of these micelles accumulated into aggregates of multiple monomers. Nanoparticle formation strongly depended on peptide concentration. At concentrations <5 mg mL^{-1} the relative abundance of these structures decreased, but they were still observable at 0.05 mg mL^{-1} (images not shown). The extent of these aggregation effects was studied in the rest of the LALF-14c-derived peptides at concentrations of 0.5 mg mL^{-1} in order to determine the minimal acyl chain length required for nanostructure formation. Under assay conditions (see Experimental Section), C_2- and C_4-LALF-14c did not form micelles. C_6- to C_{10}-LALF-14c showed a low tendency to form micelles, but it was not until C_{12}-LALF-14c that the abundance of these aggregates was similar to that observed for C_{16}-LALF-14c (images not shown).

Palmitic acid incorporation in BPI- and SAP-derived peptides

Because C_{16}-LALF-14c presented the highest values of LPS neutralization in the LAL assay and also showed the most dramatic effects in terms of aggregation and nanostructure formation, we were particularly interested in verifying whether this phenomenon could be translated to other anti-LPS peptides such as those derived from BPI and SAP.

Active sequences from BPI (residues 85–100: IKISGKWKAQKRFLKM) and SAP (residues 186–200: QALNYEIRGYVIIKP) were synthesized and N-acetylated or palmitoylated on solid phase. The synthesis of BPI-derived peptides did not represent a challenge. However, SAP contains a hydrophobic sequence (Val-Ile-Ile) and standard solid-phase methods ended with deletion peptides that were very difficult to purify. Microwave (MW)-assisted solid-phase synthesis helped to overcome this problem (see Experimental Section for details). After purification and characterization, the IC_{50} values for these peptides as LPS-neutralizers were calculated using the in vitro assay (Figure 5) and compared against those obtained for LALF-14c analogues.

The acetylated analogues of BPI and SAP, with respective IC_{50} values of 16 ± 7.4 and 8 ± 1.7 μM, were more active than the original C_2-LALF-14c. Nevertheless, the activity of these

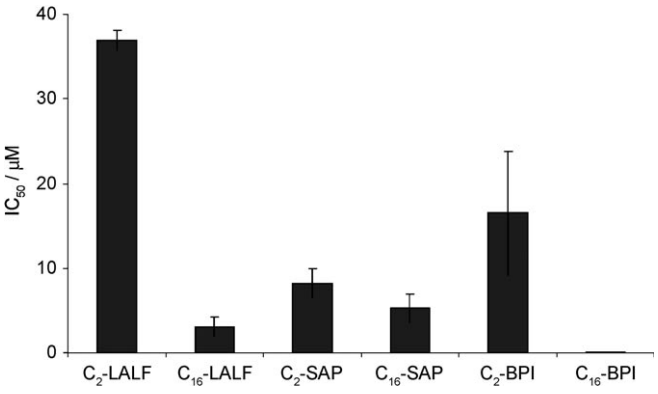


Figure 5. LPS-neutralization activity (IC_{50}) of BPI- and SAP-derived peptides. C_2- and C_{16}-LALF-14c peptides were also included. IC_{50} values are represented as mean values $\pm$SD. The inhibition of the peptides was determined using the chromogenic LAL assay. The assay was performed as described in the Experimental Section.

peptides was again clearly improved with N-terminal palmitoylation. C_{16}-BPI had a greater inhibitory activity (60 nM) than its acetylated analogue, and palmitoylated SAP was almost twofold more active (5.3 ± 1.6 μM) than C_2-SAP. These results are consistent with those obtained for LALF, and highlight the activity enhancement that the fatty acyl chain achieves when it is incorporated to various anti-LPS peptides. The cytotoxicity of these peptides was also evaluated. The results were similar to those obtained for LALF-14c-modified peptides (Figure 6).

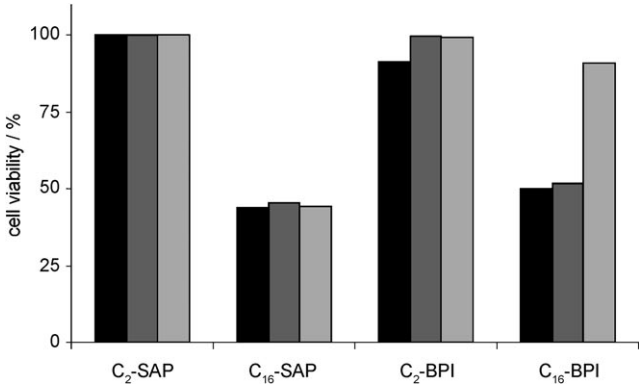


Figure 6. Cytotoxicity of BPI- and SAP-derived peptides. Cell viability is represented at a range of peptide concentrations: 10 (black bars), 5 (gray bars), and 1 μM (light gray bars). Cell viability was evaluated on RAW 264.7 cells by MTT assay as described in the Experimental Section.

Acetylated peptides showed good cell viability, in contrast to palmitoylated derivatives, which displayed some toxicity. C_{16} peptides were hence discarded for TNF inhibition studies in macrophages. However, it is important to note that C_{16}-BPI at 1 μM (concentration above its IC_{50} value) shows low toxicity, and therefore these results demonstrate that promoting the formation of nanostructures on anti-LPS peptides could induce high LPS-neutralizing activity and moderate or total absence of toxicity. The acetylated peptides did not have any relevant activity (data not shown).

The effect of the C_{16} acyl chain on these new peptides was again evaluated by TEM. C_2- and C_{16}-BPI-derived peptides were dissolved in PBS at a concentration of 5 mg mL^{-1}. Alternatively, C_2- and C_{16}-SAP-modified peptides were dissolved in H_2O at 0.5 mg mL^{-1}, because at higher concentrations the palmitoylated derivative was insoluble. Again, no aggregates or defined structures were observed for the acetylated peptides. In contrast, for both C_{16}-BPI and C_{16}-SAP peptides, clear fibril-like nanostructures were detected (Figure 7).

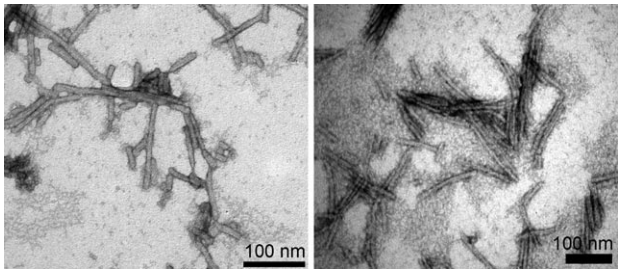


Figure 7. TEM images of C_{16}-BPI and C_{16}-SAP. One representative micrograph is shown for each peptide (C_{16}-BPI: left; C_{16}-SAP: right). C_{16}-BPI was dissolved in PBS at 5 mg mL^{-1}, and C_{16}-SAP was dissolved in H_2O at 0.5 mg mL^{-1}. Peptides were treated as described in the Experimental Section. All samples were prepared in triplicate, and images obtained were reproducible in all grids.

C_{16}-BPI displayed defined tubular structures either alone or associated in multiple fibrils. These fibrils were on average 50–140 nm long and 9–12 nm wide. In contrast, C_{16}-SAP fibrils were more prone to self-associate and aggregate. In this case, fibril length varied from 70 to 130 nm, and width ranged from 10 to 12 nm. These images show the extent of the aggregation properties of these new LALF, BPI, and SAP lipopeptides.

Conclusions

Several N-acylated peptides derived from LALF-14c were synthesized, cyclized, and evaluated for anti-LPS activity. An increase in activity of at least 10-fold was observed for C_{16}-LALF-14c over the parent peptide, C_2-LALF-14c. On the basis of TEM images, these enhanced activities could be associated with the capacity of the peptides to form nanostructures. TEM studies revealed that long fatty acyl chains promote the formation of micellar and fibrilar superstructures. This correlation between biological activity and nanostructure formation has been corroborated with other anti-LPS peptides, BPI, and SAP, which also displayed improved activities. Although some of them show some toxicity in macrophages, these results demonstrate that nanostructure formation can lead to anti-LPS peptides with higher LPS-neutralizing activities at cell-tolerable concentrations. Furthermore, we are currently exploring applications of these peptides at cell-tolerated concentrations as cell-penetrating micellar-based peptides.

Experimental Section

LPS-neutralizing activity: All solutions used in the LPS-neutralizing activity assay were tested to ensure they were endotoxin-free, and material was sterilized by heating for 3 h at 180 °C. LPS from *E. coli* 055:B5 and polymyxin B were purchased from Sigma. LPS-neutralizing activity was measured by using the chromogenic *Limulus* amebocyte lysate (LAL) test[40] following the manufacturer's instructions (Cambrex). LAL reagent contains a clottable protein that is activated in the presence of non-neutralized LPS and is an extremely sensitive indicator of the presence of endotoxin. When activated, this enzyme catalyses the release of *p*-nitroaniline (pNA) from the colorless chromogenic substrate Ac-Ile-Glu-Ala-Arg-pNA. The pNA released was measured photometrically at 405 nm in a Rosys Anthos 2010 microtiter plate reader (Tecnomara AG, Zurich, Switzerland). Peptides were incubated at a range of concentrations (50–0.001 μM) with LPS (100 pg mL^{-1}) in a 96-well microtiter plate for 45 min at 37 °C. Polymyxin B (10 μg mL^{-1}) was used as positive control. LAL (12.5 μL) was added to start the reaction at 37 °C. After 16 min, non-neutralized LPS was detected after 10 min incubation with the chromogenic substrate (25 μL). Acetic acid (25% *v/v* final concentration) was added to stop the reaction, and the absorbance was monitored at 405 nm in a Multiskan Ascent microtiter plate reader (Thermo Labsystems). IC_{50} values (the concentration necessary to neutralize 50% of LPS in vitro) were determined by a serial dilution assay using LPS (100 pg mL^{-1}) and a range of peptide concentrations as mentioned above.

Cell culture: Mouse macrophages (RAW 264.7) were obtained from the ATCC (American Type Culture Collection, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% L-glutamine. The cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2, 95% air. Subcultures of macrophages were prepared every 2–3 days by scraping cells into fresh medium.

MTT cell viability assays: Cell viability was evaluated by means of a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. RAW 264.7 cells were seeded in sterile 96-well microtiter plates at a density of 100 000 cells mL^{-1} in DMEM supplemented with 1% FBS and allowed to settle for 24 h. Peptides at a range of concentrations were added to the plates, and the cells were further incubated for 24 h. After removal of the medium, the precipitated formazan crystals were dissolved in optical grade DMSO (100 μL), and the plates were read at 570 nm using a Wallac 1420 workstation microtiter plate reader.

Evaluation of TNF expression: RAW 264.7 cells were seeded in 96-well plates at a density of 100 000 cells mL^{-1} in DMEM supplemented with 1% FBS and allowed to settle for 24 h. Then, LPS (0.5 ng mL^{-1}) was added in the absence or presence of C_x-LALF-14c at a range of concentrations. After 24 h, supernatants were collected and centrifuged for 10 min at 400 *g* and stored at −20 °C until measurement of cytokine content. TNF in the cell supernatant was determined using a commercial ELISA kit (BD Bioscience), following the manufacturer's protocol. Samples were diluted 10-fold with buffer. Color changes at 450 nm were measured using a microtiter plate reader. Cytokine levels were expressed as pg mL^{-1}. The detection range of the ELISA kit was 0–1000 pg mL^{-1}. The TNF content of each sample was assayed three times.

Transmission electron microscopy (TEM): Samples were stained with a conventional negative staining for TEM using 2% uranyl acetate on Formvar-carbon-coated copper grids. All electron micrographs were obtained with a Jeol JEM 1010 electron microscope (Tokyo, Japan) operating at 80 kV. Images were obtained with a

CCD Megaview III (SIS) camera (Münster, Germany). Three samples were prepared following the procedure, and results obtained by TEM imaging were reproducible.

Chemistry: Rink amide MBHA resin and protected Fmoc-L-amino acids were purchased from Iris Biotech GmbH (Marktredwitz, Germany). Coupling reagents, solvents for peptide synthesis, and other reagents were purchased from commercial suppliers at the highest purity available and used without further purification. Analytical HPLC was performed using a Waters Alliance 2695 chromatography system (Waters, MA, USA) with a PDA 995 detector, a reversed-phase Symmetry C_{18} column (4.6×150 mm, 5 μm), and linear gradients of MeCN with 0.036% trifluoroacetic acid (TFA) into H_2O with 0.045% TFA. The system was run at a flow rate of 1.0 mL min^{-1} over 15 min. Semipreparative HPLC was carried out on a Waters chromatography system with a dual absorbance detector 2487, a reversed-phase Symmetry C_{18} column (30×150 mm, 5 μm) and linear gradients of MeCN with 0.05% TFA into H_2O with 0.1% TFA. The system was run at a flow rate of 20.0 mL min^{-1} over 30 min. HPLC–MS was performed using a Waters Alliance 2796 instrument with a dual absorbance detector 2487 and ESI-MS Micromass ZQ chromatography system (Waters), a reversed-phase Symmetry 300 C_{18} (3.9×150 mm, 5 μm) column, and H_2O with 0.1% formic acid and MeCN with 0.07% formic acid as mobile phases. Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (Applied Biosystems, Foster City, CA, USA) using ACH matrix.

All LALF-14c-derived peptides were manually synthesized using the Fmoc/*t*Bu strategy in polypropylene syringes, each fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Washings between deprotection, couplings, and subsequent deprotection steps were carried out with *N*,*N*-dimethylformamide (DMF) and CH_2Cl_2 using 10 mL solvent per gram of resin each time. The Fmoc group was removed by treatment with piperidine/DMF (1:4 *v*/*v*). Peptide synthesis transformations and washes were performed at 25 °C. BPI was synthesized with an automatic peptide synthesizer ABI 433A (Applied Biosystems) following standard Fmoc chemistry and a FastMoc protocol. SAP was manually synthesized using a Discover SPS Microwave Peptide Synthesizer (CEM Corporation, North Carolina, USA) following the protocols described below.

Synthesis of LALF-14c-derived peptides: Fmoc Rink amide MBHA resin (2.00 g, 0.65 mmol g^{-1}) was placed in a 20-mL polypropylene syringe fitted with a polyethylene filter disk. After Fmoc removal, the coupling reactions were carried out with Fmoc-amino acids (4 equiv), 7-aza-1-hydroxy-1*H*-benzotriazole (HOAt, 4 equiv) and *N*,*N*′-diisopropylcarbodiimide (DIC, 4 equiv) in DMF for 2 h. Couplings were monitored using the Kaiser, de Clercq, or chloranil methods.[43] When necessary, recouplings were done either with *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU, 4 equiv) and *N*,*N*-diisopropylethylamine (DIEA, 8 equiv) for 30 min, or with 1-benzotriazolyloxytris(pyrrolidino)-phosphonium hexafluorophosphate (PyBOP, 4 equiv), HOAt (4 equiv), and DIEA (12 equiv) for 2 h. After completion of the synthesis, the resin was divided, and acylation treatments were carried out individually. Fatty acids (5 equiv) were coupled using PyBOP (5 equiv), HOAt (5 equiv), and DIEA (15 equiv) for 48 h at room temperature. For C_2-LALF, this protocol was substituted with a standard acetylation step with Ac_2O/DIEA/DMF (10:20:70) for 30 min. For the deprotection of side chain groups and concomitant cleavage of the peptide from the support, the resin was washed with CH_2Cl_2 (3×1 min), dried, and treated with TFA/H_2O/TIS/EDT (95:2:2:1) for 2 h (TIS = triisopropylsilane, EDT = 1,2-ethanedithiol). TFA was then removed by evaporation with nitrogen, and the peptides were precipitated with cold anhydrous *tert*-butylmethyl ether (TBME), dissolved in H_2O/MeCN (distinct mixtures used) and then lyophilized. The linear crude peptides were purified by semipreparative HPLC (see conditions used below). After purification, linear peptides were cycled upon dissolution in a solution of HOAc/DMSO/H_2O (1:3:16) at a concentration of 0.5 mg mL^{-1}. Neutral pH was reached after treatment with ammonium carbonate. The solution was then stirred at room temperature for 24 h and monitored by Ellman's test[44] and/or HPLC. When cyclization was finished, the reaction mixture was treated with the aromatic adsorbent Diaion HP-20 (Supelco, Bellefonte, PA, USA) for 12 h. The peptide adsorbed was gently washed with H_2O to remove all DMSO and was then eluted with increasing concentrations of MeCN in aqueous mixtures. Lyophilization yielded the desired peptides, which were then assayed for biological activity without further purification.

Acetyl-LALF-14c (C_2-LALF-14c): The linear peptide (25.7 mg) was purified by semipreparative HPLC: linear gradient from 10 to 50% MeCN over 30 min, flow rate 20 mL min^{-1}. Obtained: 7.3 mg (purification yield 29%). The purified linear peptide (7.3 mg) was cycled as described above. Obtained: 4.9 mg (cyclization yield 67%). The peptide was characterized by HPLC (10→50% MeCN over 15 min, t_R = 6.44 min, 94%) and MALDI-TOF (*m*/*z* calcd for $C_{92}H_{144}N_{28}O_{19}S_2$: 2009.06, found: 2010.61 $[M+H]^+$, 2033.61 $[M+Na]^+$.

Butyryl-LALF-14c (C_4-LALF-14c): The linear peptide (30.8 mg) was purified by semipreparative HPLC: linear gradient from 10 to 50% MeCN over 30 min, flow rate 20 mL min^{-1}. Obtained: 11.3 mg (purification yield 37%). The purified linear peptide (11.3 mg) was cycled as described above. Obtained: 8.7 mg (cyclization yield 77%). The peptide was characterized by HPLC (10→50% MeCN over 15 min, t_R = 6.83 min, 96%) and MALDI-TOF (*m*/*z* calcd for $C_{94}H_{148}N_{28}O_{19}S_2$: 2037.09, found: 2038.10 $[M+H]^+$, 2060.09 $[M+Na]^+$.

Caproyl-LALF-14c (C_6-LALF-14c): The linear peptide (30.3 mg) was purified by semipreparative HPLC: linear gradient from 20 to 35% MeCN over 30 min, flow rate 20 mL min^{-1}. Obtained: 7.4 mg (purification yield 24%). The purified linear peptide (7.4 mg) was cycled as described above. Obtained: 3.5 mg (cyclization yield 47%). The peptide was characterized by HPLC (0→100% MeCN over 15 min, t_R = 8.39 min, 99%) and MALDI-TOF (*m*/*z* calcd for $C_{96}H_{152}N_{28}O_{19}S_2$: 2065.12, found: 2066.16 $[M+H]^+$, 2088.14 $[M+Na]^+$, 2106.13 $[M+K]^+$.

Octanoyl-LALF-14c (C_8-LALF-14c): The linear peptide (28.7 mg) was purified by semipreparative HPLC: linear gradient from 20 to 35% MeCN over 30 min, flow rate 20 mL min^{-1}. Obtained: 6.2 mg (purification yield 22%). The purified linear peptide (6.2 mg) was cycled as described above. Obtained: 6.2 mg (cyclization yield quant.). The peptide was characterized by HPLC (20→35% MeCN over 15 min, t_R = 5.83 min, 91%) and HPLC–MS (*m*/*z* calcd for $C_{98}H_{156}N_{28}O_{19}S_2$: 2093.15, found: 1046.6 $[M+H]^+$/2, 698.3 $[M+H]^+$/3, 523.7 $[M+H]^+$/4.

Decanoyl-LALF-14c (C_{10}-LALF-14c): The linear peptide (31.5 mg) was purified by semipreparative HPLC: linear gradient from 20 to 35% MeCN over 30 min, flow rate 20 mL min^{-1}. Obtained: 5.1 mg (purification yield 16%). The purified linear peptide (5.1 mg) was cycled as described above. Obtained: 3.6 mg (cyclization yield 71%). The peptide was characterized by HPLC (0→100% MeCN over 15 min, t_R = 7.25 min, 99%) and MALDI-TOF (*m*/*z* calcd for $C_{100}H_{160}N_{28}O_{19}S_2$: 2121.19, found: 2122.44 $[M+H]^+$, 2145.44 $[M+Na]^+$.

Lauryl-LALF-14c (C_{12}-LALF-14c): The linear peptide (40.2 mg) was purified by semipreparative HPLC: linear gradient from 20 to 40% MeCN over 30 min, flow rate 20 mL min^{-1}. Obtained: 7.7 mg (purification yield 19%). The purified linear peptide (7.7 mg) was cycled as described above. Obtained: 5.5 mg (cyclization yield 71%). The peptide was characterized by HPLC (0→100% MeCN over 15 min, t_R = 7.69 min, 99%) and MALDI-TOF (*m/z* calcd for $C_{102}H_{164}N_{28}O_{19}S_2$: 2149.22, found: 2150.43 $[M+H]^+$, 2173.43 $[M+Na]^+$, 2188.44 $[M+K]^+$.

Myristyl-LALF-14c (C_{14}-LALF-14c): The linear peptide (32.8 mg) was purified by semipreparative HPLC: linear gradient from 30 to 40% MeCN over 30 min, flow rate 20 mL min^{-1}. Obtained: 5.7 mg (purification yield 17%). The purified linear peptide (5.7 mg) was cycled as described above. Obtained: 3.2 mg (cyclization yield 56%). The peptide was characterized by HPLC (0→100% MeCN over 15 min, t_R = 8.17 min, 88%) and MALDI-TOF (*m/z* calcd for $C_{102}H_{164}N_{28}O_{19}S_2$: 2177.25, found: 2178.47 $[M+H]^+$, 2200.44 $[M+Na]^+$, 2216.44 $[M+K]^+$.

Palmitoyl-LALF-14c (C_{16}-LALF-14c): The linear peptide (33.3 mg) was purified by semipreparative HPLC: linear gradient from 35 to 45% MeCN over 30 min, flow rate 20 mL min^{-1}. Obtained: 9.0 mg (purification yield 27%). The purified linear peptide (9.0 mg) was cycled as described above. Obtained: 5.6 mg (cyclization yield 62%). The peptide was characterized by MALDI-TOF (*m/z* calcd for $C_{106}H_{172}N_{28}O_{19}S_2$: 2205.28, found: 2205.83 $[M+H]^+$, 2227.82 $[M+Na]^+$, 2245.80 $[M+K]^+$.[2]

Synthesis of BPI-derived peptides: The automatic synthesis was conducted on Fmoc Rink amide MBHA resin (154 mg, 0.65 mmol g^{-1}) on a 0.1-mmol scale with a 10-fold excess of Fmoc-protected L-amino acids and *O*-benzotriazol-1-yl-*N*-tetramethyluronium tetrafluoroborate (TBTU, 0.45 M) in the presence of 1-hydroxy-1*H*-benzotriazole (HOBt) in DMF as coupling reagents. The synthesis was carried out with deprotection and coupling steps of 15 and 35 min, respectively. After the assembly was completed, the peptide resin was washed with CH_2Cl_2 and divided in two. The peptide was acetylated or palmitoylated as indicated above. Cleavage of each fraction and subsequent workup was done as explained before. The linear peptides were purified and characterized as follows.

Acetyl-BPI (C_2-BPI): The peptide (18.3 mg) was purified by semipreparative HPLC: linear gradient from 5 to 60% MeCN over 30 min, flow rate 20 mL min^{-1}. Obtained: 10.4 mg (purification yield 57%). The peptide was characterized by HPLC (0→100% MeCN over 15 min, t_R = 6.38 min, 99%) and HPLC–MS (*m/z* calcd for $C_{94}H_{159}N_{27}O_{19}S$: 2002.20, found: 1001.9 $[M+H]^+/2$, 668.2 $[M+H]^+/3$, 501.5 $[M+H]^+/4$.

Palmitoyl-BPI (C_{16}-BPI): The peptide (21.3 mg) was purified by semipreparative HPLC: linear gradient from 20 to 100% MeCN over 30 min, flow rate 20 mL min^{-1}. Obtained: 11.9 mg (purification yield 56%). The peptide was characterized by HPLC (0→100% MeCN over 15 min, t_R = 9.17 min, 98%) and HPLC–MS (*m/z* calcd for $C_{108}H_{187}N_{27}O_{19}S$: 2198.42, found: 1100.0 $[M+H]^+/2$, 733.6 $[M+H]^+/3$, 550.5 $[M+H]^+/4$.

Synthesis of SAP-derived peptides: The synthesis was performed on Fmoc Rink amide MBHA resin (400 mg, 0.70 mmol g^{-1}) using MW conditions. Couplings were carried out with Fmoc-protected L-amino acids (4 equiv), HOAt (4 equiv), and DIC (4 equiv) in DMF for 10 min at 75 °C (MW, 20 W). Recouplings were done by repeating the same conditions or with HATU (4 equiv) and DIEA (8 equiv) in DMF for 10 min at 75 °C (MW, 20 W). The Fmoc group was removed by treatment with piperidine/DMF (1:4 *v/v*) for 5 min at 75 °C (MW, 20 W). After the assembly was completed, the peptide resin was washed with CH_2Cl_2, divided in two, and treated as explained above for BPI.

Acetyl-SAP (C_2-SAP): The peptide (36.8 mg) was purified by semipreparative HPLC: linear gradient from 5 to 95% MeCN over 30 min, flow rate 20 mL min^{-1}. Obtained: 4.2 mg (purification yield 11%). The peptide was characterized by HPLC (5→100% MeCN over 15 min, t_R = 6.74 min, 82%) and MALDI-TOF (*m/z* calcd for $C_{85}H_{136}N_{22}O_{22}$: 1817.02, found: 1818.31 $[M+H]^+$, 1840.31 $[M+Na]^+$, 1856.29 $[M+K]^+$.

Palmitoyl-SAP (C_{16}-SAP): The peptide (19.0 mg) was purified by semipreparative HPLC: linear gradient from 30 to 80% MeCN over 30 min, flow rate 20 mL min^{-1}. Obtained: 1.8 mg (purification yield 10%). The peptide was characterized by HPLC (30→100% MeCN over 15 min, t_R = 7.79 min, 86%) and MALDI-TOF (*m/z* calcd for $C_{99}H_{164}N_{22}O_{22}$: 2013.24, found: 2014.33 $[M+H]^+$, 2036.32 $[M+Na]^+$, 2052.29 $[M+K]^+$.

[2] *In this case, characterization by HPLC was not possible due to the presence of aggregates in the chromatogram profile. After a reducing treatment, these aggregates disappear, and a single reduced peak is obtained, which implies good final purity.*

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"Study of the interaction of a palmitoyl BPI-derivative with cells using Confocal Laser Scanning Microscopy (CLSM)"

Unpublished results

INTRODUCTION

In the previous work, we showed that *N*-acylation of LPS-neutralizing peptides is a useful strategy to improve their anti-LPS activity by means of enhanced hydrophobic interactions with the acyl chains of lipid A.[1] In addition, *N*-acylation promotes the formation of defined micellar or fibrilar nanostructures. However, this strategy is not exempt from limitations, since most palmitoylated derivatives displayed toxicity in RAW macrophages, whereas the acetylated analogues were tolerable for cells.

One plausible explanation for these experimental findings could be the enhanced affinity for cell membranes and improved cellular uptake that has been described for acylated peptides and proteins. In fact, fatty acylation is a natural biochemical process that occurs co- or post-translationally and significantly increases the hydrophobicity of the protein and consequently modulates its physiological functions. Acylated proteins have been shown to play crucial roles in membrane association, subcellular trafficking and signalling and also influence protein–protein interactions and protein stability.[2,3] In this regard, the synthetic acylation of peptides and proteins has been extensively studied as an approach to improve their pharmacological effects. For therapeutic application, the potential to enhance membrane permeability is of greatest interest.

A number of recent studies show how the acylation of proteins and peptides results in higher membrane affinity and improved cell uptake properties while retaining the biological activity of these molecules.[4,5,6,7,8,9,10,11] These studies show for instance how not only positive charges are key features of efficient intracellular uptake, but also the presence of hydrophobic moieties. This is the case of an octaarginine modified with a stearyl group at its *N*-terminus, which displayed two orders of magnitude higher transfection efficiency than octaarginine alone.[7] Another example is the myristoylation of a Pro-rich peptide, which resulted in a 6-fold increase in cellular uptake compared to the original peptide.[10] In addition, a recent review on amphipathic cell-penetrating peptides (CPPs) suggests that the electrostatic interaction of these peptides with cell-membrane polysaccharides is the first step of the internalization process, but that is not sufficient for effective peptide uptake. The authors propose that other features are critical, such as the hydrophobic content, amphipathic character or self-assembly capacity of the peptide.[12]

On the basis of these lines of evidence, we examined the interaction of 5(6)-carboxyfluorescein (CF)-labeled peptides derived from C2- and C16-BPI. Conducted using Confocal Laser Scanning Microscopy (CLSM), these studies showed how these peptides are both able to translocate and interact with cell membranes.

RESULTS AND DISCUSSION

Design of CF-labeled peptides derived from BPI

In our previous work, we described three palmitoylated LPS-neutralizing peptides: C16-LALF-14c, C16-BPI and C16-SAP.[1] For the present study, we selected C16-BPI because it presents several interesting properties over the other peptides: i) it has the highest anti-LPS activity (60 nM); ii) it is linear, and therefore further modifications are synthetically more accessible than for C16-LALF-14c (which is a cyclic peptide); and iii) it has a much better water solubility than C16-SAP, thereby allowing its use in biological systems.

The peptide H-BPI contains several basic residues in its sequence; five Lys and one Arg (see Figure 1). These amino acids contain side-chain amino groups that can be conjugated with CF. However, as positively charged residues have been proposed to be crucial in LPS-binding,[13,14] neutralization of one of these charges could be deleterious for the biological activity of the peptide. Hence, in our design, Fmoc-Lys(Alloc)-OH was inserted at the *N*-terminus. This amino acid is orthogonally protected and allows the specific functionalization of both the α- and the ε-amino groups of the Lys. Since CF is light-sensitive, it seemed reasonable to introduce this molecule at the very last step of the synthesis. Therefore, the α-amino group was selectively deprotected and acylated. Finally, the ε-amino protecting group was removed and CF inserted (see Figure 1).

Figure 1: The sequence of H-BPI is shown. Basic residues are indicated. The introduction of an orthogonally protected Lys allows selective chemical modifications at two positions.

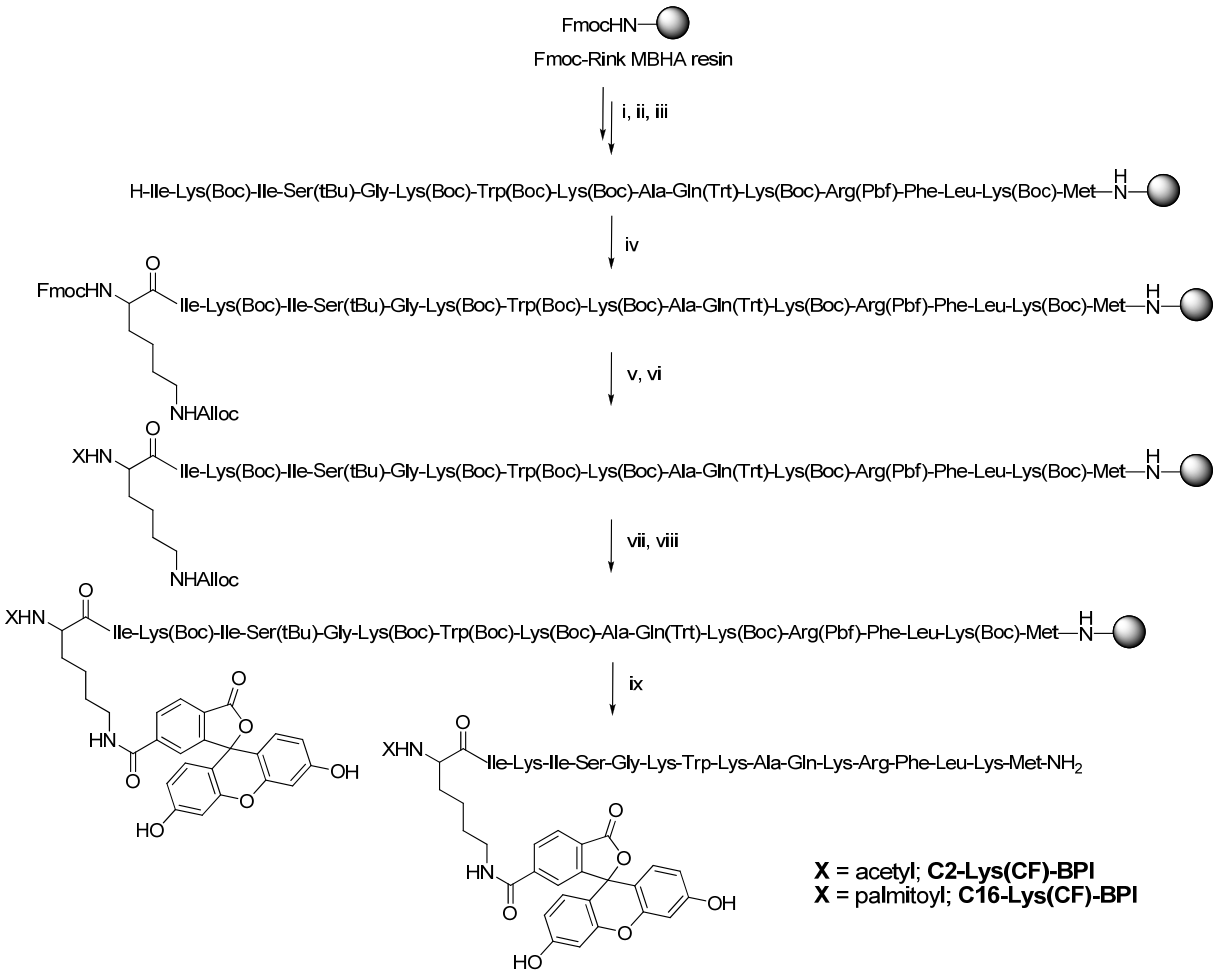
Synthesis of CF-labeled peptides derived from BPI

Thus, the acetylated and palmitoylated BPI-derived peptides were synthesized and labeled with CF to obtain peptides **C2-** and **C16-Lys(CF)-BPI** respectively.

1. First attempts. The first attempt to synthesize BPI analogues was performed using an automatic synthesizer and TBTU/HOBt as the coupling system. The solid support selected was Rink amide MBHA resin (see the Experimental Section for details). This synthesis was prematurely abandoned due to the presence of numerous amino acid deletions in the peptide H-BPI. Next, the synthesis was repeated manually on two resins; a polystyrene-based MBHA resin and a poly(ethylene glycol)-based aminomethyl ChemMatrix resin. In this case, both resins failed to afford the desired peptides, but analysis of the synthesis every three or four couplings by HPLC and MALDI identified a difficult sequence consisting of amino acids Ile-Lys-Ile at the *N*-terminus.

2. Synthesis of C2-Lys(CF)-BPI. From those considerations, the synthesis was repeated automatically on Rink amide MBHA resin using the more potent HATU and HOAt as coupling reagents. This time several recouplings were introduced at the most problematic residues (i.e. Lys and Ile). The synthesis yielded the peptide H-BPI with an acceptable purity. Next Fmoc-Lys(Alloc)-OH was introduced, the Fmoc group was removed and the α-amino group acetylated. Finally, the ε-amino protecting group Alloc was removed and CF was coupled following a described methodology.[15] All these modifications were carried out under N_2 atmosphere to prevent Met-oxidation. The extent of this side-reaction was studied in a small portion of the peptide-resin. Under usual non-inert conditions, the peptide was fully oxidized (see the Experimental

Section). The peptide was finally cleaved with standard TFA-mixtures. The peptide was purified by semi-preparative HPLC up to optimal purities and characterized by HPLC and MALDI. The synthesis of **C2-Lys(CF)-BPI** is shown in Scheme 1.



Scheme 1. General procedure for the synthesis of CF-labeled BPI peptides: (i) piperidine–DMF (1:4); (ii) Fmoc-AA-OH, HATU, HOAt, DIEA; repeat [i,ii] 15 times; (iii) piperidine–DMF (1:4); (iv) Fmoc-Lys(Alloc)-OH, DIC, HOAt, DMF (2 treatments); (v) piperidine–DMF (1:4); (vi) acetylation: Ac_2O–DIEA–DMF (1:2:7); palmitoylation: palmitic acid, PyBOP, HOAt, DIEA, DMF; (vii) $PhSiH_3$, $Pd(PPh_3)_4$; (viii) first treatment: CF, PyAOP, HOAt, DIEA, DMF-CH_2Cl_2 (9:1); second treatment: PyAOP, HOAt, DIC, DMF-CH_2Cl_2 (9:1); (ix) TFA–H_2O–TIS (95:2.5:2.5) for the C2-peptide; TFA-H_2O-TIS-Me_2S-NH_4I (95:2:1:1:1) for the C16-peptide.

3. Synthesis of C16-Lys(CF)-BPI. The synthesis of the palmitoylated analogue was performed following the methodology described above for the acetyl derivative (see Scheme 1 and the Experimental Section for details). After the insertion of Fmoc-Lys(Alloc)-OH, the Fmoc group was removed and palmitic acid was inserted using PyBOP, HOAt and DIEA. In this case, despite working under N_2 atmosphere, Met-

oxidation was detected by MALDI. For this reason, after CF incorporation, the peptide was cleaved using a reductive cleavage cocktail (see the Experimental Section). This treatment yielded the desired peptide free of Met-oxidation. Purification afforded the target peptide with satisfactory purity. The peptide was characterized as described in the Experimental Section.

Cell viability

Before testing the designed analogues in cell systems, we evaluated whether the addition of CF caused modification in the toxicity profile of these peptides. For this purpose the cytotoxicity of **C2-** and **C16-Lys(CF)-BPI** peptides was assayed at 1, 5 and 10 μM concentrations in HeLa cells and RAW 264.7 macrophages by MTT assays. The acetylated analogue was tolerable for HeLa cells at all the concentrations tested. In contrast, the palmitoylated derivative displayed a moderate toxicity in this cell system (Figure 2). The same behavior was observed in RAW macrophages (data not shown). These results are in agreement with our previous published work[1] and indicate that the presence of CF does not interfere in the toxicity of the peptides.

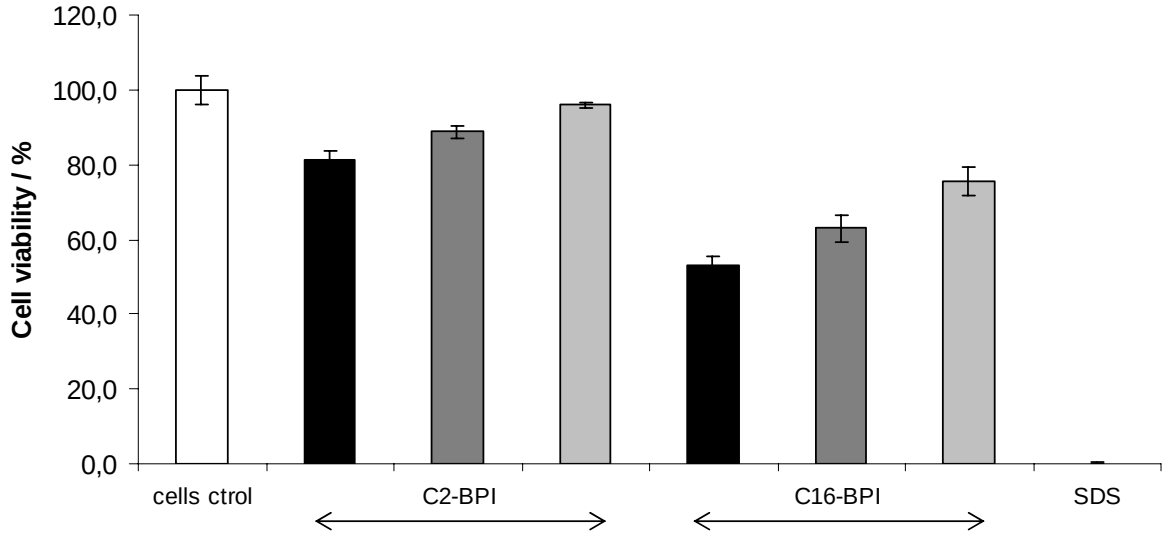


Figure 2: Cell viability is shown at three compound concentrations: 10 (black bar), 5 (dark grey bar) and 1 (light grey bar) μM. Untreated cells (white bar) and SDS have been included as controls. Cell viability was evaluated by MTT assay in HeLa cells, after 24 h of incubation with peptides **C2-** and **C16-Lys(CF)-BPI**. The assay is described in the Experimental Section.

Confocal laser scanning microscopy (CLSM) studies

1. HeLa cells

The capacity of these peptides to either be internalized by cells or to interact with cell membranes was first studied in HeLa cells by means of CLSM. We wanted to examine peptides originally at a 1 μM concentration (i.e. a non-toxic concentration for all the derivatives). However, according to several publications on CPPs and other compounds that are internalized by cells,[16,17,18,19] this is a low concentration to study internalization processes. In these studies higher concentrations were used, ranging from 10 to 50 μM. The time of incubation of CF-peptides with cells is also an important factor. In the studies cited, this time varied from 30 min to 3 h. In our case we were also interested in the effect of peptides after 24 h, because this was the time-point used for the cytotoxicity studies.[1] Hence, two conditions were assayed. HeLa cells were incubated with peptides at 5 and 10 μM for 1.5 h, and also at a much higher concentration, 100 μM, for an extended 24-h period.

When compared to untreated control cells (Figure 3, left), the peptide **C2-Lys(CF)-BPI** did not translocate cell membranes at low concentrations (5 and 10 μM) after 1.5 h of incubation (Figure 3, middle). However, after a 24-h incubation and at 100 μM, internalization by HeLa cells was clearly observed (Figure 3, right). These results also demonstrate that this peptide is totally tolerable for cells even at high concentrations.

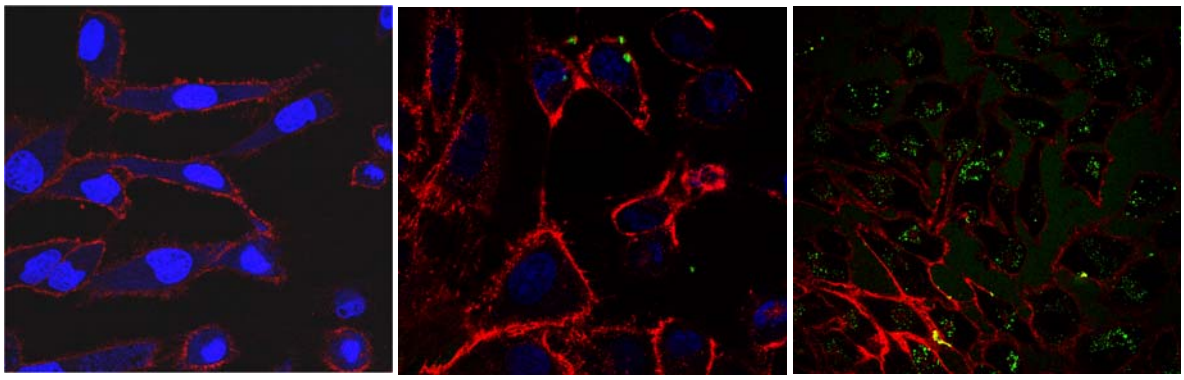


Figure 3: CLSM images of HeLa cells: Control cells (left) were incubated with 10 μM **C2-Lys(CF)-BPI** (green channel) for 1.5 h (middle) or with 100 μM of peptide for 24 h (right). Control cells were treated only with CF. Cytoplasmic membranes (red channel) were stained with WGA-TRITC and nuclei (blue channel) with Draq5 (see the Experimental Section for details).

In contrast, as expected, at 100 μM, **C16-Lys(CF)-BPI** was toxic for the cells. In fact, all cells were killed after a 24-h treatment with this peptide concentration. Nevertheless, after a 1.5-h incubation at 5 and 10 μM, the peptide was observed attached to cell membranes (images not shown). It is important to highlight that under these conditions, the acetylated peptide did not interact with HeLa cells, thereby suggesting a decreased affinity for cell membranes for this peptide. To further analyze the behaviour of **C16-Lys(CF)-BPI** in cellular systems, cells were incubated with this peptide at an intermediate concentration of 25 μM, for 12 h. We reasoned that although at this peptide concentration, the palmitoyl analogue is toxic to cells; the observation of the interaction of the peptide with cells after 12 h of incubation could allow us to obtain useful information on its mechanism of action before all cells die. Results are shown in Figures 4 and 4.

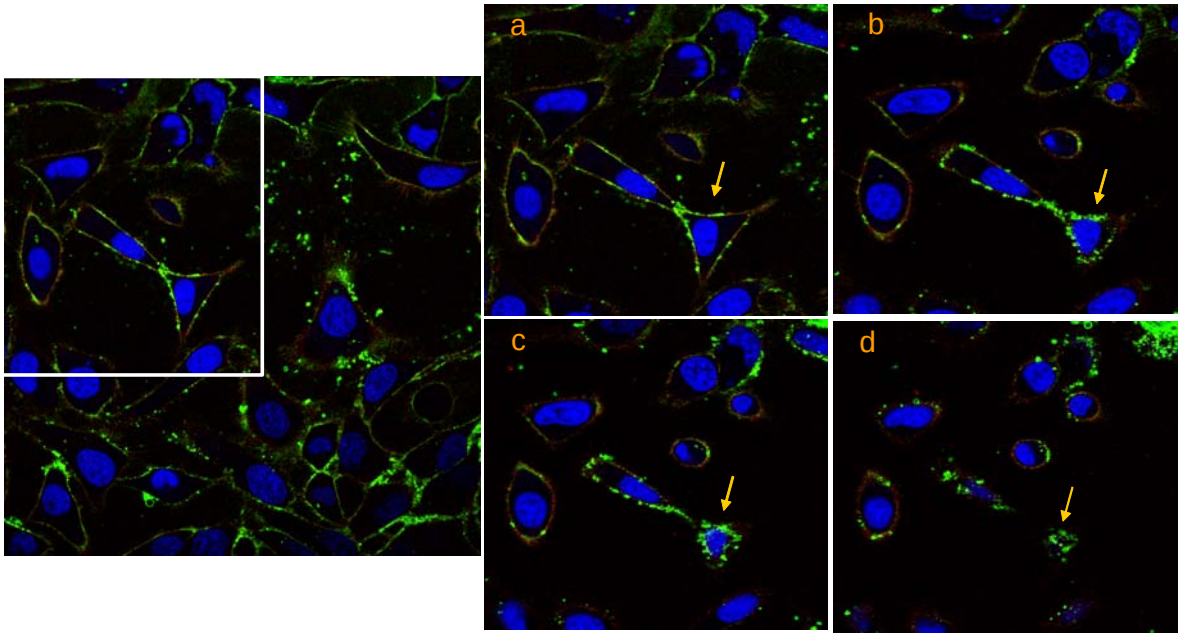


Figure 4: CLSM images of HeLa cells incubated with 25 μM **C16-Lys(CF)-BPI** for 12 h. The detail of one section (left, white square) has been amplified and observed at different sections through the z-axis (right, from a to d).

Figure 4 shows how C16-Lys(CF)-BPI interacts with HeLa cells. A detailed study of one of the sections observed indicates that the peptide binds to the membranes but is not internalized by the cell, as can be deduced from acquisitions of different sections through the *z*-axis (Figure 4, right, images from a to d). Repetitions of these experiments showed similar results, though in some cases the peptide was observed inside the cells.

In addition, cell death was observed at different stages, as indicated by a variety of morphological changes in HeLa cells, such as the formation of blebs (irregular bulges in the plasma membrane), loss of membrane asymmetry, cell shrinkage or release of cellular debris. Some representative images are shown in Figure 5. The normal morphology of untreated cells (Figure 5, left) was altered in the presence of **C16-Lys(CF)-BPI** (Figure 5, middle and right).

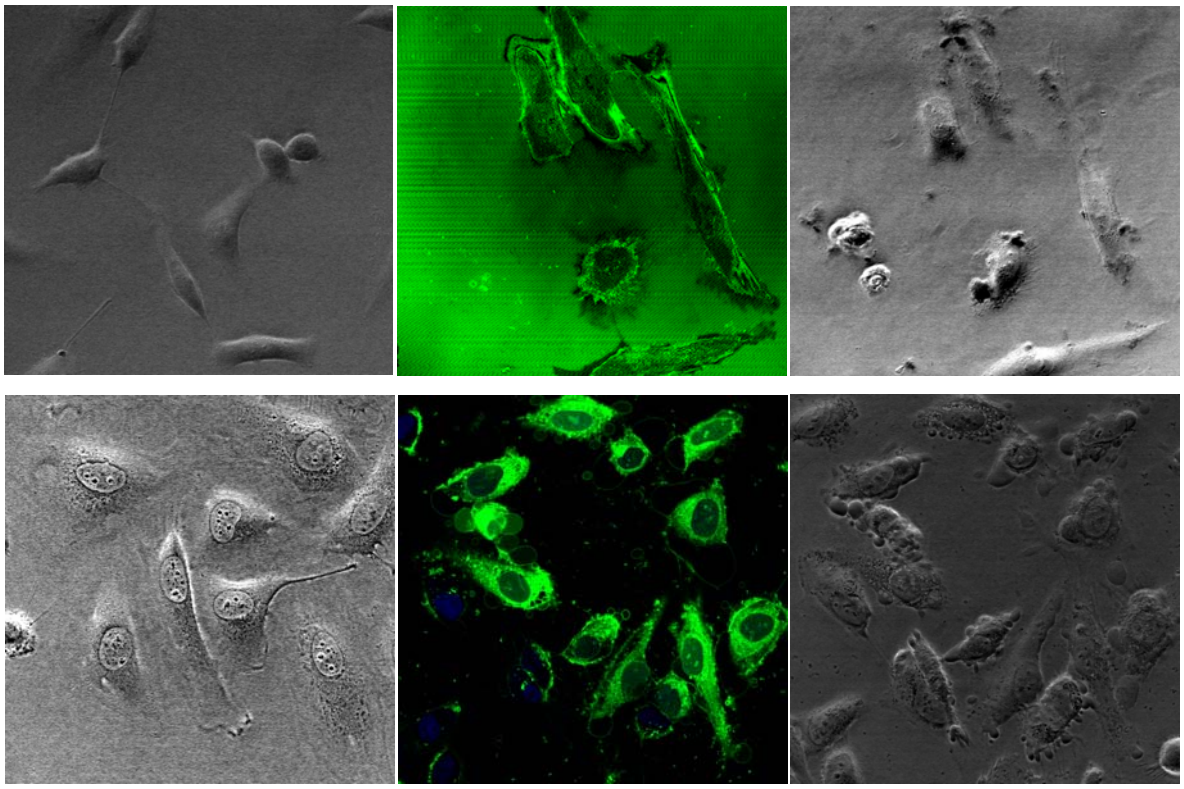


Figure 5. CLSM images of untreated HeLa cells (left) and incubated with 25 μM **C16-Lys(CF)-BPI** for 12 h (middle and right). Transmitted light images of HeLa cells are shown in left and right columns.

2. Murine RAW macrophages

Finally, the same experiments were conducted on murine RAW macrophages. Overall, the observations in these cells were consistent with the previous findings for HeLa cells. Hence, **C2-Lys(CF)-BPI** translocate cell membranes of murine macrophages after a 24-h incubation at 100 μM (Figure 6). In this case internalization was also observed at shorter incubation times such as 12 h (images not shown).

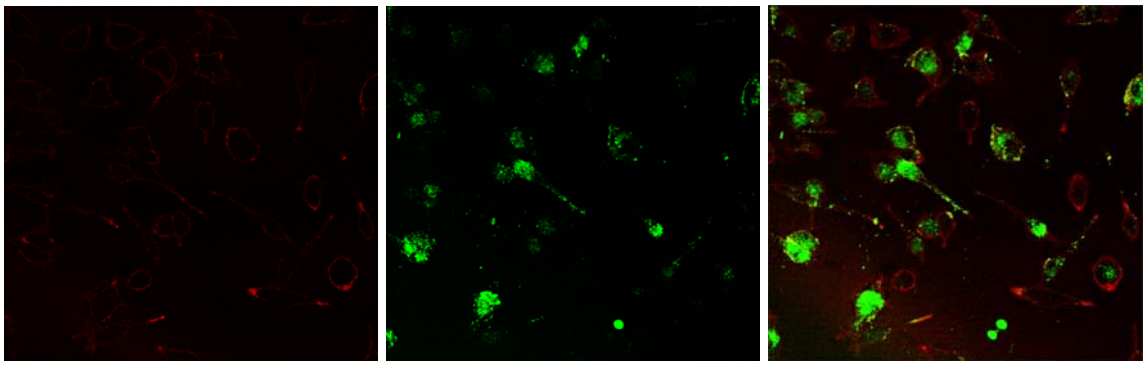


Figure 6: CLSM images of RAW macrophages incubated with 100 μM **C2-Lys(CF)-BPI** for 24 h. Images show cytoplasmic membranes (red channel, left), the peptide (green channel, middle) and the combination of both (overlay, right).

Murine RAW macrophages were also incubated with **C16-Lys(CF)-BPI** at a concentration of 25 μM for 12 h. Under these conditions the peptide interacted with the cell membranes and also was uptaken by cells (Figure 7).

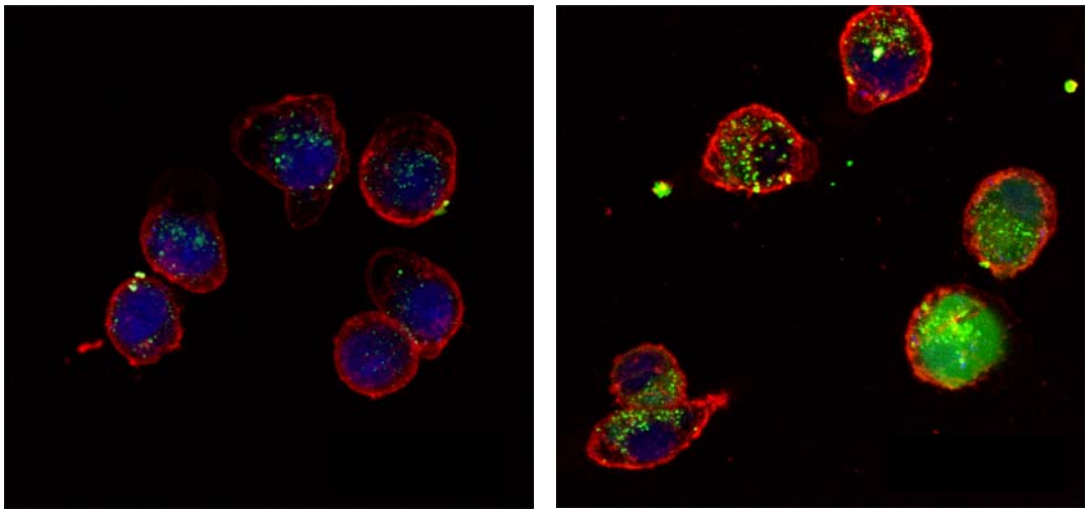


Figure 7. CLSM images of RAW macrophages incubated with 25 μM **C16-Lys(CF)-BPI** for 12 h.

These observations indicated that both peptides interacted with cell systems. However, their modes of action differed greatly. The acetylated analogue might be able to interact with cell membranes because of its amphipathicity, though with much less affinity than the palmitoylated derivative. This membrane-peptide interaction may be followed by an internalization process, which takes place only under high concentrations of peptide (100 μM) and prolonged incubation times (12-24 h). The presence of the C2-peptide

inside the cells did not compromise cell viability, probably because of the facile degradation/elimination of this peptide in/from the cytoplasmic medium. In contrast, the C16-peptide displayed a much higher degree of affinity with cell membranes at much lower concentrations (5-10 μM) and times of incubation (1.5 h). The binding to cell membranes was favored at higher concentrations (25 μM) and periods of incubation (12 h). However, this increased affinity for cell membranes resulted in cell damage. This peptide was also internalized by cells. In this case, its capacity to aggregate might be also harmful for the cell.

CONCLUDING REMARKS

Here we have described the synthesis of two new carboxyfluoresceinated derivatives of the LPS-neutralizing peptides C2- and C16-BPI. The presence of a CF molecule did not represent an increase in the toxicity of the peptides and allowed their study with cells using CLSM. The studies were conducted in HeLa cells and RAW macrophages. In both cell lines, at high concentrations and prolonged incubation times, the acetylated derivative was internalized by cells. The cell uptake of this peptide did not compromise cell viability. In contrast, the palmitoylated analogue presented an increased affinity for cell membranes and entered cells at lower concentrations. However, in this case peptide-cell interactions resulted in cell death. To quantify the extent of these internalization processes, uptake measurements by plate fluorimetry and flow cytometry will be undertaken.

EXPERIMENTAL SECTION

General. Fmoc-Rink amide MBHA resin and protected Fmoc-L-amino acids were purchased from Iris Biotech GmbH (Marktredwitz, Germany). Coupling reagents, solvents for peptide synthesis, and other reagents were purchased from commercial suppliers at the highest purity available and used without further purification. Analytical HPLC was performed using a Waters Alliance 2695 chromatography system (Waters, MA, USA) with a PDA 995 detector, reversed-phase Symmetry C_{18} (4.6 x 150 mm, 5-μm) and Sunfire C_{18} (4.6 x 100 mm, 3.5-μm) columns and linear gradients of MeCN with 0.036% TFA into H_2O with 0.045% TFA. The system was run at a flow rate of 1.0 mL/min over either 8 (C_{18} Sunfire) or 15 min (C_{18} Symmetry). Semi-preparative HPLC

was carried out on a Waters chromatography system with a dual absorbance detector 2487, a reverse-phase Symmetry C18 column (30 x 150 mm, 5-μm) and linear gradients of MeCN with 0.05% TFA into H_2O with 0.1% TFA. The system was run at a flow rate of 20.0 mL/min over 30 min. HPLC–MS was performed using a Waters Alliance 2796 instrument with a dual absorbance detector 2487 and ESI-MS Micromass ZQ chromatography system (Waters), a reverse-phase Symmetry 300 C_{18} (3.9 x 150 mm, 5-μm) column, and H_2O with 0.1% formic acid and MeCN with 0.07% formic acid as mobile phases. Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (Applied Biosystems, Foster City, CA, USA) using ACH matrix.

Peptide Synthesis. 1. Automatic Synthesis. The peptide chains were elongated by means of an ABI 433A peptide synthesizer (Applied Biosystems, Foster City) employing standard Fmoc chemistry and a FastMoc protocol. Syntheses were conducted on a 0.1-mmol scale with a 10-fold excess of Fmoc-protected L-amino acids and 0.45 M HBTU or HATU in the presence of HOBt or HOAt in DMF as coupling reagents. Syntheses were carried out with a 15-min deprotection step with piperidine-DMF (1:4) and a 35-min or 50-min coupling, as indicated in the text. When peptide assembly was completed, the peptide-resin was washed with CH_2Cl_2.

Peptide Synthesis. 2. Manual synthesis. Manual solid-phase manipulations were carried out in polypropylene syringes fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Fmoc removal was carried out with piperidine–DMF (1:4) (1 x 1 min, 2 x 10 min) and acetylation steps with Ac_2O–DIEA–DMF (1:2:7) (1 x 1 min, 2 x 5 min). Washings between deprotection, coupling, and subsequent deprotection steps were carried out with DMF (5 x 0.5 min) and CH_2Cl_2 (5 x 0.5 min) using 10 mL of solvent/g of resin each time. Couplings and washes were performed at 25 ºC. Couplings were monitored using Kaiser, de Clercq or chloranil methods.[20]

Cleavage. For the deprotection of side chain groups and concomitant cleavage of the peptide from the support, the resin was washed with CH_2Cl_2 (3 x 1 min), dried, and treated with TFA-H_2O-TIS (95:2.5:2.5), unless otherwise indicated, for 1.5 h. TFA was then removed by evaporation with nitrogen, and the peptides were precipitated with

cold anhydrous TBME, the solution was decanted, and the solid was triturated with cold TBME, which was again decanted. This process was repeated twice. Peptides were then dissolved in H_2O-MeCN (distinct mixtures used) and lyophilized.

Coupling of CF. Solid CF (5 equiv) was smashed until a powder is obtained. It was then dissolved with PyAOP (5 equiv) and HOAt in DMF-CH_2Cl_2 (9:1). This solution was then sonicated for 5 min. Next, DIEA (10 equiv) was added and the mixture was pre-activated for 10 min. After this time, the mixture was added to the resin, and left to react for a time varying from 2 to 5 h. The peptide-resin was then washed with piperidine-DMF (1:4) (2 x 1 min), and treated with a new solution of CF (5 equiv), DIC (5 equiv) and HOAt (5 equiv) in DMF-CH_2Cl_2 (9:1) for 12 h. After coupling completion, the resin was washed with consecutive 5-min treatments with piperidine-DMF (1:4) until CF-aggregates were no further observed.[21]

Synthesis of BPI analogues. First attempt. The automatic synthesis was conducted on Fmoc Rink amide MBHA resin (0.1 mmol, 0.65 mmol/g) TBTU and HOBt as coupling reagents. When the peptide assembly was completed, the peptide-resin was washed with CH_2Cl_2 and an aliquot was treated with TFA-H_2O-TIS (95:2.5:2.5) for 1.5 h. The analysis by HPLC showed a complex profile. Characterization by MALDI-TOF of **H-BPI** revealed a great number of deletions (*m/z* calcd. for $C_{92}H_{157}N_{27}O_{18}S$, 1960.19; found, 1961.08 $[M + H]^+$, 1804.99 $[(M - aa) + H]^+$, 1676.90 $[(M - 2aa) + H]^+$), 1563.82 $[(M - 3aa) + H]^+$, 1435.74 $[(M - 4aa) + H]^+$, etc.). The synthesis was thus abandoned.

Synthesis of BPI analogues. Second attempt. The synthesis was manually performed on Fmoc Rink amide MBHA resin (0.33 mmol, 0.63 mmol/g). The coupling reactions were performed with Fmoc-amino acids (4 equiv), DIC (4 equiv) and HOAt (4 equiv) in DMF for 2 h. When necessary, recouplings were done either with HATU (4 equiv) and DIEA (8 equiv) for 30 min, or with PyBOP (4 equiv), HOAt (4 equiv), and DIEA (12 equiv) for 2 h. Samples were taken every three or four amino acids and analyzed by HPLC and HPLC-MS or MALDI. The synthesis did not present any challenge until Trp^7 (10^{th} coupling). From this residue on, several recouplings were required to achieve a proper incorporation, especially for the last residues, amino acids Ile^3, Lys^2 and Ile^1. When the peptide assembly was completed an aliquot was cleaved, under the conditions explained above, and analyzed. HPLC analysis showed a major peptide (71 % peptide)

but the analysis by MALDI revealed an incorrect mass for **H-BPI** (*m*/*z* calcd. for $C_{92}H_{157}N_{27}O_{18}S$, 1960.19; found, 2089.14 and 2311.29). This synthesis was hence discarded.

Synthesis of BPI analogues. Third attempt. The process was identical as before but using aminomethyl ChemMatrix (CM) resin (Matrix Innovation, Canada). For this solid support, previous washings were required: MeOH (2 x 1 min), DMF (2 x 1 min), CH_2Cl_2 (3 x 1 min), TFA-CH_2Cl_2 (1:99) (3 x 1 min), DIEA-CH_2Cl_2 (1:19) (3 x 1 min), and CH_2Cl_2 (3 x 1 min). Next, Fmoc-Rink amide linker (4 equiv) was incorporated with HOAt (4 equiv) and DIC (4 equiv) in DMF overnight. Similarly to the previous synthesis, the purity and mass identity of the peptide was analyzed after three or four couplings. Although this solid support showed a better performance compared to the polystyrene (PS) resin, the coupling of the last three residues was again challenging. Acidic treatment to cleave an aliquot of the resin with TFA-H_2O-TIS (95:2.5:2.5) for 1.5 h rendered a peptide with a major peak observed by HPLC (78 % purity). However MALDI analysis showed that the mass was different from the target peptide (*m*/*z* calcd. for $C_{92}H_{157}N_{27}O_{18}S$, 1960.19; found, 2117.13).

Synthesis of Ac-Lys(CF)-BPI. The synthesis was performed automatically on Fmoc-Rink amide MBHA resin (0.1 mmol, 0.62 mmol/g). HATU and HOAt were selected as coupling system, coupling cycles were extended and several recouplings were programmed at positions: Lys^{11}, Trp^{7}, Lys^{6}, Ile^{3}, Lys^{2}, Ile^{1}. Cleavage of a sample taken after synthesis completion showed a major peak with the expected mass for **H-BPI** (*m*/*z* calcd. for $C_{92}H_{157}N_{27}O_{18}S$, 1960.19; found, 1961.15 $[M + H]^+$). A small amount of Met oxidation and one Ile deletion were also detected. To pursue further manipulations, one portion of the resin was transferred to a new syringe. To this portion, Fmoc-Lys(Alloc)-OH (4 equiv) was inserted with two consecutive treatments of DIC (4 equiv) and HOAt (4 equiv) (1 x 2 h, 1 x 12 h). Then, the Fmoc group was removed and the free α-amino group was acetylated (1 x 1 min, 2 x 15 min). At this point the peptide was still detectable by HPLC and MALDI but the extent of Met oxidation increased. Next, Alloc removal was accomplished after 3 x 15 min treatments of $PhSiH_3$ (10 equiv) and $Pd(PPh_3)_4$ (0.1 equiv) in CH_2Cl_2. The resin was then washed with sodium dithiodiethylcarbamate (0.02 M in DMF, 3 x 15 min) to remove possible palladium traces of the resin. CF (5 equiv) was subsequently added with a first 5-h treatment with

PyAOP (5 equiv), HOAt (5 equiv) and DIEA (10 equiv), followed by a 12-h treatment with DIC (5 equiv) and HOAt (5 equiv) in DMF-CH_2Cl_2 (9:1) as described in the general procedure. The peptide-resin portion was finally cleaved as previously explained. The final product had a 52 % purity but **C2-Lys(CF)-BPI** was totally oxidized as shown by MALDI analysis (*m*/*z* calcd. for $C_{121}H_{181}N_{29}O_{26}S$, 2488.35; found, 2393.12 $[(M - Ile + 16) + H]^+$, 2506.21 $[(M + 16) + H]^+$). Keeping these considerations in mind, the original resin containing the **H-BPI** peptide (see above) was treated in a similar manner, but using inert conditions to avoid air oxidation. This was accomplished purging the resin between different synthetic steps and performing the reactions under N_2 atmosphere. The final cleavage yielded **C2-Lys(CF)-BPI** with a 52 % purity by HPLC. Met-oxidation was not observed by MALDI. A portion of the crude peptide (27.3 mg) was finally purified by semi-preparative HPLC (linear gradient from 10 to 30 % MeCN over 30 min, flow rate 20 mL/min). Obtained: 2.3 mg (purification yield 8 %) Characterization of **C2-Lys(CF)-BPI**: HPLC (t_R = 3.8 min, from 5 to 100 % CH_3CN over 8 min, purity 95%; t_R = 6.0 min, from 10 to 40 % CH_3CN over 8 min, purity 88%), MALDI-TOF (*m*/*z* calcd. for $C_{121}H_{181}N_{29}O_{26}S$, 2488.35; found, 2489.45 $[M + H]^+$, 2512.41 $[M + Na]^+$, 2528.41 $[M + K]^+$).

Synthesis of C16-Lys(CF)-BPI. Initial peptide elongation was conducted on an automatic peptide synthesizer as explained for Ac-Lys(CF)-BPI. The MALDI analysis of the resulting **H-BPI** peptide showed the desired mass (*m*/*z* calcd. for $C_{92}H_{157}N_{27}O_{18}S$, 1960.19; found, 1961.15 $[M + H]^+$). The MALDI spectrum also showed an Ile deletion and a partially oxidized Met residue. Next, Fmoc-Lys(Alloc)-OH was coupled as described above, the Fmoc group was removed and palmitic acid (5 equiv) was inserted with PyBOP (5 equiv), HOAt (5 equiv) and DIEA (15 equiv) in DMF for 12 h. The total insertion of the aliphatic acid was checked by ninhydrin and confirmed by HPLC. Then, the Alloc group was eliminated and CF was coupled using protocols described for the synthesis of the acetylated analogue. All these manipulations were performed under N_2 atmosphere; however, Met-oxidation could not be avoided. For this reason, a small aliquot was taken and cleaved using a reductive cleavage cocktail based on reagent H.[22] HPLC and MALDI analysis after this treatment showed that this treatment was useful to reduce oxidized-Met. Therefore, the whole peptide-resin was treated with TFA-H_2O-TIS-Me_2S-NH_4I (95:2:1:1:1) for 1.5 h. Standard work-up afforded **C16-Lys(CF)-BPI** with a 33 % purity free of Met-oxidation. A portion of this crude (36.1

mg) was purified by semi-preparative HPLC (linear gradient from 30 to 45 % MeCN over 30 min, flow rate 20 mL/min). Obtained: 1.4 mg (purification yield 4 %). Characterization of **C16-Lys(CF)-BPI**: HPLC (t_R = 5.1 min, from 5 to 100 % CH_3CN over 8 min, purity 99%; t_R = 6.7 min, from 30 to 45 % CH_3CN over 8 min, purity 80%), MALDI-TOF (*m*/*z* calcd. for $C_{135}H_{209}N_{29}O_{26}S$, 2684.56; found, 2685.41 $[M + H]^+$).

Cell culture and incubation with CF peptides. HeLa cells and mouse macrophages (RAW 264.7) were obtained from ATCC (American Type Culture Collection, USA). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRI) supplemented with 10% fetal bovine serum (FBS - Gibco BRI) and 1% L-glutamine. HeLa cells were detached every 2-3 days using a trypsin-0.25% EDTA solution and subcultured into new flasks with fresh medium. Alternatively, subcultures of macrophages were prepared every 2-3 days by scraping cells into fresh medium. In both cases, the cultures were incubated at 37ºC in a humidified atmosphere of 5% CO_2. To perform the CSLM experiments, cells were seeded at a concentration of 9.3 x 10^4 cells/mL onto plastic dishes (Nalge Nunc International, Rochester, NY). Cells were then allowed to settle for 24 h. After this time, the medium was discarded and cells were washed 3 times with PBS. Next, CF-peptides and CF stock solutions, were dissolved in DMEM, passed through 0.22-μm filters (Millex-GV, PVDF, Durapore, Millipore, Billerica, MA) and added to the cells at different ranges of concentrations. Confocal studies were performed after 1.5 h to 24 h of incubation with the peptides at 37ºC under 5% CO_2. Before the observations were carried out, cells were washed with PBS.

Confocal laser scanning microscopy (CLSM). CLSM was performed using a Leica SPII microscope with a 63x objective. The experiments were carried out with live cells. CF fluorescence was excited with the 488 nm line of an argon laser, and its emission was detected over the range of 515 to 530 nm. Nuclei were stained with the dye Draq5 (Biostatus Limited, Leicestershire, UK) after a 1:1000 dilution from a 5 mM stock solution (excitation λ = 633 nm; emission λ = 681 to 697 nm) and cytoplasmic membranes with WGA-(TRITC) (Molecular probes, Paisley, UK) after a 1:1000 dilution from an initial 1 mg/mL stock (excitation λ = 543 nm; emission λ = over 580 nm).

MTT cell viability assays. Cell viability was evaluated by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. HeLa and RAW 264.7 cells were seeded in sterile 96-well microtiter plates at a density of 4 x 10^4 cells/mL and 6 x 10^4 cells/mL respectively in DMEM supplemented with 10% FBS and allowed to settle for 24 h. Peptides at concentrations of 10 μM, 5 μM and 1 μM were added to the plates and the cells were incubated for 20 h. After this time, MTT was added to a final concentration of 0.5 mg/mL. The cells with peptide and MTT were incubated for a further 4 h. After removal of the medium, the precipitated formazan crystals were dissolved in optical grade DMSO (100 μL), and the plates were read at 570 nm using a Wallac 1420 Workstation. Cell viability percentages were calculated by dividing the absorbance value of cells treated with a given compound by the absorbance value of untreated cells.

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2.3 CONCLUSIONS

The conclusions of the present chapter are the following:

1. The study of the active sequence of an LPS-neutralizing protein by means of Ala scanning and hexapeptide mapping is a useful strategy to identify the most relevant residues for biological activity. These findings can in turn be applied in the design of new minimized peptide sequences with high capacity to neutralize endotoxins. The proposed minimal LPS-binding domain of the protein LALF (the peptide LALF-14c) is susceptible to further minimization.

2. The insertion of structural motifs that induce a certain degree of secondary structure (i.e. Pro insertion) or the replacement of a disulfide bridge by an amide bond into well-known LPS-binding sequences, also serve as viable options to increase the LPS-neutralizing activity of peptides. In this regard, peptides derived from LALF-14c with these modifications displayed enhanced biological activities.

3. *N*-Acylation of LPS-neutralizing peptides is a successful strategy to obtain LPS-neutralizing peptides with improved activities. This enhancement of the biological activity can be explained by increased hydrophobic interactions of the peptide with the acyl chains of the lipid A, and the peptide's capacity to form micellar or fibril-like nanostructures. This phenomenon has been observed for peptides derived from the proteins LALF, BPI and SAP.

4. *N*-Acylated peptides bearing long acyl chains (i.e. a palmitoyl group) display cytotoxicity in cells. The study of carboxyfluorescein (CF)-labeled acylated-peptides in cell systems using CSLM is a useful strategy to elucidate this behavior. Palmitoylated derivatives of BPI present a higher affinity for cell membranes over acetylated analogues, and capacity to be internalized by cells, which might result in cytotoxicity for the cells studied.

3. PEPTIDED-BASED LPS-INHIBITORS

3.1 INTRODUCTION

Improving the biological profile of LPS-neutralizing peptides

In the previous chapter we presented a series of peptides derived from known LPS-binding proteins. We demonstrated how the study of these peptide sequences and their chemical modification (i.e. *N*-acylation) are promising strategies to obtain peptides with enhanced LPS-neutralizing activities.[1,2] However, in this field, peptides have constantly failed to reproduce the *in vitro* biological activities in septic animal models. The possible reasons for this failure have been described earlier (please see section 2.1.3 in the previous chapter). However, according to several authors, the low proteolytic stability of peptides in biological fluids together with their rapid clearance from the bloodstream are major factors.[3]

In this chapter therefore, as a first step to address these limitations, we will explore three main strategies:

1. The design of new peptide-based LPS-inhibitors based on the chemical features of the lipid A. In order to confer more proteolytic stability to the molecules, they will have a certain number of amino acids replaced by other chemical groups.
2. The conjugation of selected LPS-neutralizers to dendrimers based on poly(ethylene glycol) (PEG), in order to improve their pharmacokinetic properties.
3. The identification of small organic molecules with LPS-neutralizing activity, and their further conjugation to anti-LPS peptides. This strategy aims to exploit the synergy of two LPS-neutralizing molecules that differ in their nature and modes of action.

At the same time, these strategies seek to increase the LPS-neutralizing activity of the original peptides.

3.1.1 LPS-INHIBITORS BASED ON THE CHEMICAL FEATURES OF LIPID A

We have previously introduced with great detail LPS-neutralizing peptides with the capacity to interact with the toxic part of LPS, lipid A. Thus, the design of LPS-inhibitors should consider the chemical features of lipid A in order to exert effective neutralization. These chemical features are listed below and are depicted in Figure 3.1.

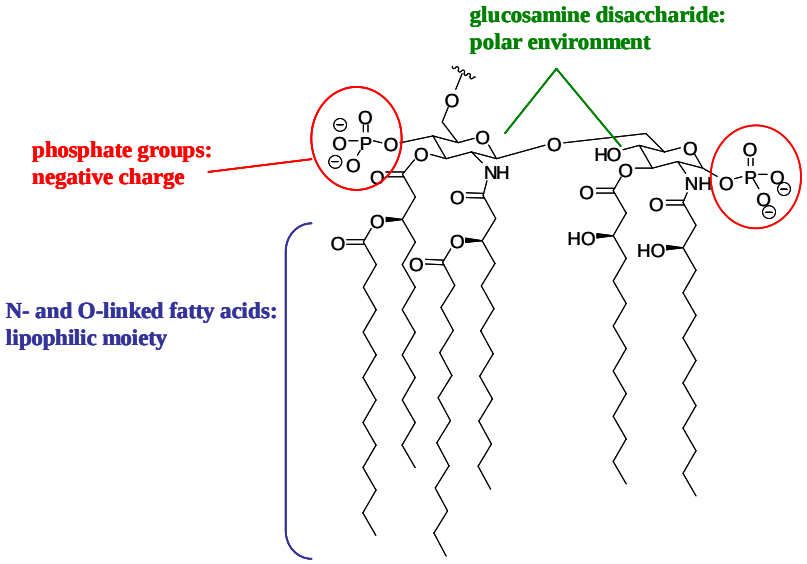

Figure 3.1: Chemical structure of lipid A. Its main chemical features are highlighted.

Lipid A contains:

i) two phosphate groups (negatively charged at a physiological pH)

ii) a 2-amino-2-deoxy-D-glucose (glucosamine) disaccharide

iii) a hydrophobic moiety that comprises several amide- and ester-linked fatty acids

The presence of cationic residues that interact with the phosphate groups of lipid A is a prerequisite for any LPS-inhibitor targeting lipid A.[4,5,6,7] In addition, the binding of peptides to lipid A appears to be relatively independent of their amino acid sequence. Therefore, the two basic amino acids do not necessarily need to be inserted in a peptide sequence and could be connected by linkers of distinct chemical natures. The introduction of these linkers provides a great range of possibilities. On the one hand

they can modulate the distance of the positive centers to optimize the interaction with the phosphates; on the other hand they can also interact with the disaccharide core. Furthermore, depending on their chemical structure they can improve the proteolytic stability and the pharmacokinetic profile of the inhibitors. David and coworkers have thoroughly studied this issue and have shown that the pharmacophore required to neutralize lipid A requires two positive groups separated by a distance of ~14 Å.[8,9] Finally, a hydrophobic component might be necessary to interact with the lipidic part of lipid A. We have previously described how hydrophobic interactions are believed to convert LPS-binders into true LPS-neutralizers (please refer to section 2.1.3 in the previous chapter). In fact, several studies showed how the insertion of long acyl chains into LPS-neutralizing peptides and organic molecules, involves an enhancement in their biological activity.[1,10,11,12,13] Therefore, the structure of LPS-neutralizers should also hold a hydrophobic acyl chain. The final design proposed is shown in Figure 3.2. This will be the subject of our research in the first part of this chapter.

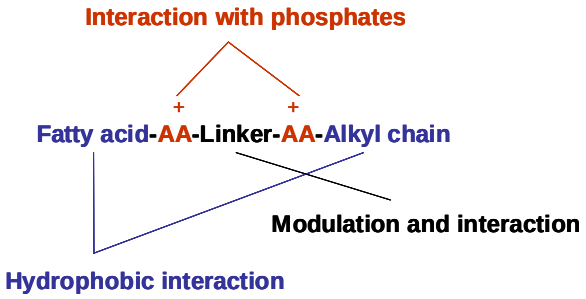

Figure 3.2: Schematic representation of the chemical features required in the design of LPS-neutralizers.

3.1.2 CONJUGATION OF LPS-INIHBITORS TO PEGYLATED DENDRIMERS AND ORGANIC MOLECULES

PEGylated dendrimers

Dendrimers have emerged as a new class of biopolymers with structural and biological properties of interest.[14,15,16,17] These compounds are highly branched polymers with a well-defined chemical composition and structure. They have a compact

globular shape, monodisperse size and controllable surface functionalities. One of the most relevant properties of dendrimers in biological systems is the concept of multivalency. Multivalency has shown to lead to strongly increased activity compared to the corresponding monomeric interaction.[14] This synergistic enhancement of a certain activity is explained by two effects: a simple additive effect (higher molar number of binding entities per mole of dendrimer) and what is know as the "cluster" or "dendritic" effect, a co-operative effect in a multivalence system that leads to a larger increase in activity than expected from the valency of the system.[18]

Dendrimers have been extensively used in biology and medicine for a wide range of activities. They have been studied for drug and gene delivery since by attaching a drug to a suitable dendrimer it is possible to enhance its aqueous solubility, increase its circulation half-life, target the drug to certain tissues, improve drug transit across biological barriers and slow drug metabolism.[19,20,21,22] Among many other applications, dendrimers have also been used as contrast agents for imaging,[23,24] and as scaffolds for tissue repair.[25]

PEGylated dendrimers, in which a multifunctional dendritic core is conjugated to polyethylene glycol (PEG), are an interesting subclass of dendrimers for these applications.[26,27] Various PEGylated dendrimers show lower toxicity and hemolytic properties, long blood circulation times, low organ accumulation, and high accumulation in tumor tissue.[28,29,30] Some of these unique advantages are in part due to the presence of PEG chains. PEG is non-toxic and it has no immunogenicity or antigenicity. It is highly flexible and has the capacity to solubilize very insoluble small molecule compounds.[31] Additionally, the presence of PEG reduces kidney ultrafiltration, improves the bioavailability and solubility of the drug and, in general, facilitates its administration.[32,33]

However, despite the great number of biological applications described for dendrimers, to the best of our knowledge only a few examples with LPS-neutralizing activity have been reported in the literature.[34,35] In these studies, Cromer *et al*. examined a variety of amine-terminated poly(amidoamine) (PAMAM) dendrimers.[36] The authors derivatized the surface amines of these dendrimers with lipopolyamines and obtained a multi-

branched dendritic structure that neutralized LPS-induced inflammatory responses *in vitro* and afforded protection in a murine model of endotoxic shock.[35] No reports are found in the literature concerning other types of dendrimers. Therefore, the second part of this chapter will address the conjugation of LPS-neutralizing compounds to PEG-based dendrimers. This study will explore the effect of multivalency on the biological activity of the selected inhibitors as well as the modulation of their cytotoxicity.

Identification of small organic molecules with LPS-neutralizing activity

Finally, we were also interested in identifying small organic molecules with LPS-neutralizing activity to further conjugate them to known LPS-neutralizing peptides. Peptide hybrids containing active sequences from two or more LPS-binding proteins have shown synergistic effects in terms of enhanced LPS-neutralizing activities. This is the case for example of peptides MBI-27 and MBI-28, both of which are derived from the active sequences of cecropin and melittin.[37] However, constructs or hybrid molecules based on small organic molecules and peptides with LPS-neutralizing capacity have yet to be reported in the literature.

In the search for new lead compounds, there are four classical strategies:

i) The systematic screening of sets of compounds arbitrarily chosen for their diversity, by selected biological assays. Methodologies of high-throughput screening (HTS) currently available have been successfully applied in numerous cases to identify new hits. However, HTS presents some limitations such as low hit rates or the frequent obtaining of compounds with poor bioavailability or toxicity.[38,39]
ii) The modification and improvement of existing active molecules in order to achieve higher activity profiles, better pharmacokinetics, improved safety and easier formulations. These molecules are sometimes also called “me-too compounds”.
iii) The serendipitous finding of unexpected biological information. The antibiotic activity of *Penecillium notatum* or the effect of a 5 cGMP phosphodiesterase on male erectile dysfunction[40] are good examples. The latter represented a clinical observation of a side-effect and research programs based on such information are of great interest, since they are based on activities observed directly in man.

iv) The fourth route is the rational drug design based on the knowledge of the molecular structure responsible for a given disease. This approach strongly depends on the progress made in basic research, especially in the structural characterization of proteins.

However, other strategies are available for the discovery of new hits of relevance. Of great interest is the SOSA approach (selective optimization of side activities).[41,42] This approach consists of testing "old" drugs, which are known to be safe and bioavailable in humans, on new pharmacological targets. The SOSA approach proceeds in two steps. In the first place, a smart library of about 1000 compounds is screened. Since bioavailability and toxicity studies have already been performed for these drugs and their usefulness in humans has been proved, they are expected to be "drug-like". Next, hits are optimized in order to transform the observed side-activity into the main effect as well as to reduce or abolish the initial pharmacological activity. This approach aims to reduce the distance between basic research and clinical applications.

One of these "new types" of chemical libraries is the *Prestwick Chemical Library*.[43] This library contains 880 biologically active compounds[44] with high chemical and pharmacological diversity as well as known bioavailability and safety in humans. Over 85% of the compounds are well-established drugs and 15% are bioactive alkaloids. This library has recently been successfully screened to obtain specific inhibitors to human tissue transglutaminase (TGM2) with potencies at the nanomolar range.[45]

In the present chapter we will screen this library in order to identify new hits with LPS-neutralizing activity Hits will be optimized and finally conjugated to the previously described LPS-neutralizing peptide RLKWc.[1]

3.1.3 OBJECTIVES

The objectives of the present chapter are:

1. To develop a solid-phase methodology for the facile synthesis of potent LPS-neutralizers. These peptide-based compounds will be designed on the basis of the chemical features of the toxic moiety of LPS, lipid A and will have several amino acid residues substituted by different chemical groups.

2. To conjugate the most interesting peptide-based LPS-inhibitor to PEGylated dendrimers, and to study the effect of the conjugation over the original molecule on the biological activity and the toxicity profile.

3. To identify new hits for LPS-neutralization using the *Prestwick Chemical Library*, and to conjugate the most potent compound to the LPS-neutralizing peptide RLKWc. The biological activity of this new hybrid molecule will be evaluated.

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3.2 RESULTS AND DISCUSSION

"Design and facile solid-phase synthesis of peptide-based LPS-inhibitors containing PEG-like functionalities"

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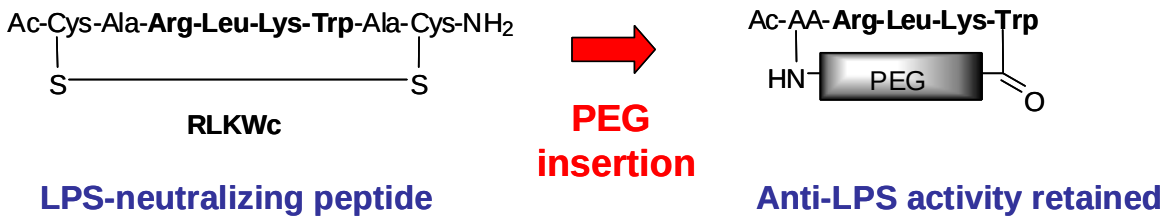
ChemMedChem, *submitted (December 2008)*

RESUM

La següent publicació presenta la síntesi senzilla d'inhibidors del LPS mitjançant metodologies en fase sòlida. Els compostos varen ser dissenyats basant-se en les característiques químiques de la part tòxica del LPS, el lípid A. En aquest disseny es va assajar la substitució de diversos aminoàcids per altres grups químics. La presencia de cadenes de polietilenglicol (PEG) va donar lloc als compostos més actius. Aquesta estratègia també es va aplicar amb èxit en el pèptid cíclic RLKWc, tot retenint la seva capacitat per neutralitzar el LPS.



Contribucions a aquest treball:

- En Carlos Mas va realitzar el disseny, la síntesi i la caracterització de tots els compostos descrits, els estudis de toxicitat en cèl·lules i els estudis d'estabilitat proteolítica. Va portar tot els pes de la preparació del manuscrit.

- La Laura Cascales va realitzar els assaigs d'activitat biològica de tots els compostos.

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Design and facile solid-phase synthesis of peptide-based LPS-inhibitors containing PEG-like functionalities

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LPS release from Gram-negative bacteria can result in sepsis, a serious systemic inflammatory response to infection that can lead to septic shock and multiple organ failure. Thus, easy-to-synthesize, effective and safe LPS-inhibitors are required in order to develop new agents for the treatment of sepsis. On the basis of the chemical features of the toxic part of LPS, lipid A, here we present peptide-based LPS-neutralizers that can be readily obtained using solid-phase methodologies. The presence of PEG-like moieties yielded the most active compounds, thereby indicating that these functionalities may be of great value in the design of new inhibitors. In this regard, the substitution of several amino acids by PEG-like chains in a previously reported cyclic anti-LPS peptide (the peptide RLKWc) rendered a new derivative that retained the activity of the original peptide. We foresee that this strategy could be successfully applied to other LPS-neutralizing peptides.

Introduction

Sepsis, defined as a systemic response to infection, and severe sepsis-associated organ failure are the first cause of mortality in Intensive Care Units (UCIs).[1] In 2006, sepsis was the 10th leading cause of death in the U.S.[2] and with approximately 750,000 cases per year, sepsis accounts for 2% of all hospitalizations.[3,4] The lipopolysaccharide (LPS), a bacterial endotoxin present in the outer leaflet of Gram-negative bacteria, plays a major role in Gram-negative sepsis.[5] LPS is a pathogen-associated molecular pattern (PAMP) that is recognized by the innate immune system. Continuous exposure to LPS in the mammalian bloodstream induces the deregulation of inflammatory cytokine release, which eventually leads to the pathological condition of sepsis.[6] LPS triggers the innate immune response by several mechanisms;[5] however, the main mechanism described involves the binding of LPS to LPS-binding protein (LBP) to form the complex (LBP)-LPS. LPS is then transferred to the CD14 receptor, which in turn signals through the Toll-like receptor 4 (TLR4)-MD-2 complex, an event that initiates the transcription of cytokine genes.[7-9] Over the last two decades intensive research has been devoted to target pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and other inflammatory mediators. However, although some promising results have been obtained in animal models, the results in humans have been disappointingly modest.[10] Only Xigris® (activated drotecogin alfa), a recombinant form of human activated protein C, has been approved by the FDA.[11] This drug decreases microvascular dysfunction by reducing inflammation and coagulation, and increases fibrinolysis. Drotecogin alfa improves survival in critically ill adult patients.[12,13] Nevertheless, there is some debate concerning the side-effects of this drug and hence complementary agents would improve the therapeutic outcome.[14,15] In this regard, the design of therapeutic agents that extracellularly neutralize LPS or its toxic part, namely lipid A, before the immune response is triggered, is considered a promising approach to provide useful lead compounds of pharmacological relevance. In this regard, small organic molecules[16] and peptides derived from LPS-binding proteins[17] have been extensively studied. The LPS-neutralizing activity of these compounds derives from their capacity to recognize and bind to lipid A, the moiety that confers LPS its endotoxicity[18,19] (Figure 1A). Therefore, effective LPS-binders should interact with the chemical features of LPS: two phosphate groups (negatively charged at physiological pH), a glucosamine disaccharide and a lipophilic moiety (Figure 1B). A clear paradigm for optimal LPS-neutralization has been described for the antibiotic polymyxin B (PMB),[20] a cyclic peptide that contains five positive charges and an *N*-linked acyl chain. However, the pronounced nephrotoxicity of PMB precludes its systemic use.[21] On the basis of the chemical structure of lipid A and previous results obtained in our laboratory, here we present the design and solid-phase synthesis of peptide-based molecules that interact with high affinity with LPS. Moreover, the use of poly(ethylene glycol) (PEG)-like chains in our design can be used to modify anti-LPS cyclic peptides.

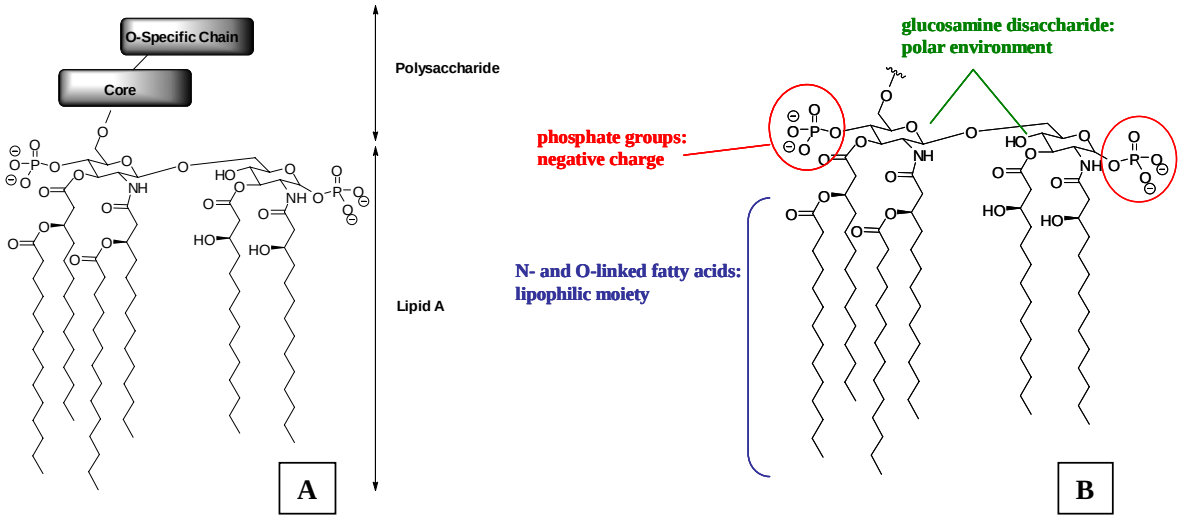


Figure 1. A. General structure of LPS showing the chemical detail of the lipid A moiety. LPS comprises three covalently linked domains: i) an O-specific chain, an immunogenic, highly variable, repeating polysaccharide that extends into the external medium; ii) an inner core oligosaccharide; and iii) a glycolipid, the so-called lipid A, formed by a 1,4'-bis-phosphorylated glucosamine disaccharide that carries several amide- and ester-linked fatty acids.[22,23] The lipid A structure is conserved in all species of Gram-negative bacteria. **B.** The main chemical features of lipid A are highlighted.

Results and Discussion

Design of LPS-neutralizers

Our design was focused on three main chemical features to exert effective binding to lipid A: i) two basic (cationic) groups with the capacity to interact tightly with the phosphate groups; ii) a suitable linker that easily modulates the distance of the positive centers, and also interacts with the sugar units; and iii) a lipophilic moiety to establish hydrophobic interactions with the fatty acyl chains. Several authors have studied the optimal amino acid sequences that result in efficient LPS binding,[17] and an interesting related study described that LPS-neutralizing peptides share a common motif for neutralization, the BHPHB (where B=basic, H=hydrophobic and P=polar).[24] This amphipathic cationic pattern is present in several peptides, such as LALF[25] or BPI,[26] which neutralize bacterial endotoxins. In this regard, the basic amino acid Arg is a candidate of interest, since this residue might promote ionic hydrogen bonding between its guanidyl group and a phosphate group. In fact, Pristovsek et al. demonstrated that arginine plays a pivotal role in LPS-LALF complexes.[27] Furthermore, we have also described how this residue is critical for LPS-neutralizing activity.[28] In the aforementioned LPS-binding motif, the two basic residues are separated by three amino acids. We believe that these amino acids can be substituted by different linkers that can control and modulate the length of this distance. David and coworkers have thoroughly studied this issue and have shown that the pharmacophore necessary to neutralize lipid A requires two positive groups separated by a distance of ~14 Å.[29,30] In our design, we considered aliphatic amino acids and poly(ethylene glycol) (PEG)-like chains. The introduction of PEG functionalities is of interest in terms of improved water solubility and pharmacokinetic profiles.[31] Moreover, the presence of PEG chains in our design may provide the required flexibility to the molecule, thereby allowing a good interaction with the polar disaccharide unit. Finally, the hydrophobic moiety can be incorporated as an acyl chain at the *N*-terminus of an Arg residue. A number of studies have proposed that the optimal acyl chain to facilitate binding to LPS varies from 12C to 18C.[32-34] In fact, recent studies by our laboratories showed that a C16 acyl chain modification on LPS-binding peptides improved LPS-neutralization.[35] The final design strategy is summarized in Figure 2.

Facile solid-phase synthesis of LPS-neutralizers

The synthesis of LPS-neutralizers was performed step-wise on solid-phase using Rink MBHA resin as solid support (see Scheme 1). Fmoc-6-aminohexanoic acid (Fmoc-Ahx-OH) was inserted into the resin using *N,N'*-diisopropylcarbodiimide (DIC) and 1-hydroxy-7-azabenzotriazole (HOAt) as coupling method. In contrast, the bulky Fmoc-Arg(Pbf)-OH required two consecutive treatments with the more reactive aminium salt O-(7-azabenzotriazol-1-yl)tetramethyluronium hexafluorophosphate (HATU) to achieve completion.[36] The next step involved the introduction of the diverse linkers. These linkers require both a free carboxylic acid and a protected amino group to be suitable for an Fmoc strategy on solid-phase. To introduce aliphatic linkers, commercially available Fmoc-amino acids were used: Fmoc-beta-alanine (Fmoc-βAla-OH), Fmoc-gamma-aminobutiric acid (Fmoc-GABA) and Fmoc-6-aminohexanoic acid (Fmoc-Ahx-OH). In the case of linkers containing PEG-like functionalities, there are also several Fmoc-protected amino acids containing these chemical groups; however, they are expensive and the diversity of structures commercially available is limited. We therefore adapted a previously described method[37] to easily convert PEG-diamines, a cheap starting material, into conveniently functionalized Fmoc-amino acids. Hence, diamines 1,4-bis(3-aminopropoxy)butane and 4,7,10-trioxa-1,13-tridecanediamine were respectively converted to Fmoc-protected 1,4-bis(3-aminopropoxy)butane succinimic acid (**linker 1**) and Fmoc-protected 4,7,10-trioxa-1,13-tridecanediamine succinimic acid (**linker 3**). The former contains poly(butylene glycol) in its structure, whereas the latter is PEG-based. Finally, Fmoc-8-amino-3,6-dioxaocatonic acid (**linker 2**) was purchased from commercial sources and included in the design (Scheme 2). Linkers were coupled overnight to ensure high yields; in this case HATU was replaced by the phosphonium salt benzotriazol-1-yloxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP) to prevent *N*-terminus guanidinylation.[38] After the second Arg coupling, palmitic acid was incorporated overnight and the compounds were finally cleaved from the resin using standard trifluoroacetic acid (TFA)-cleavage mixtures. All compounds were purified and characterized as described in the Experimental Section.

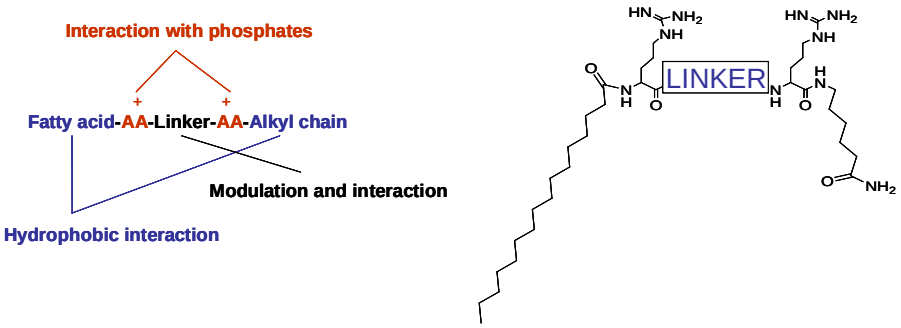
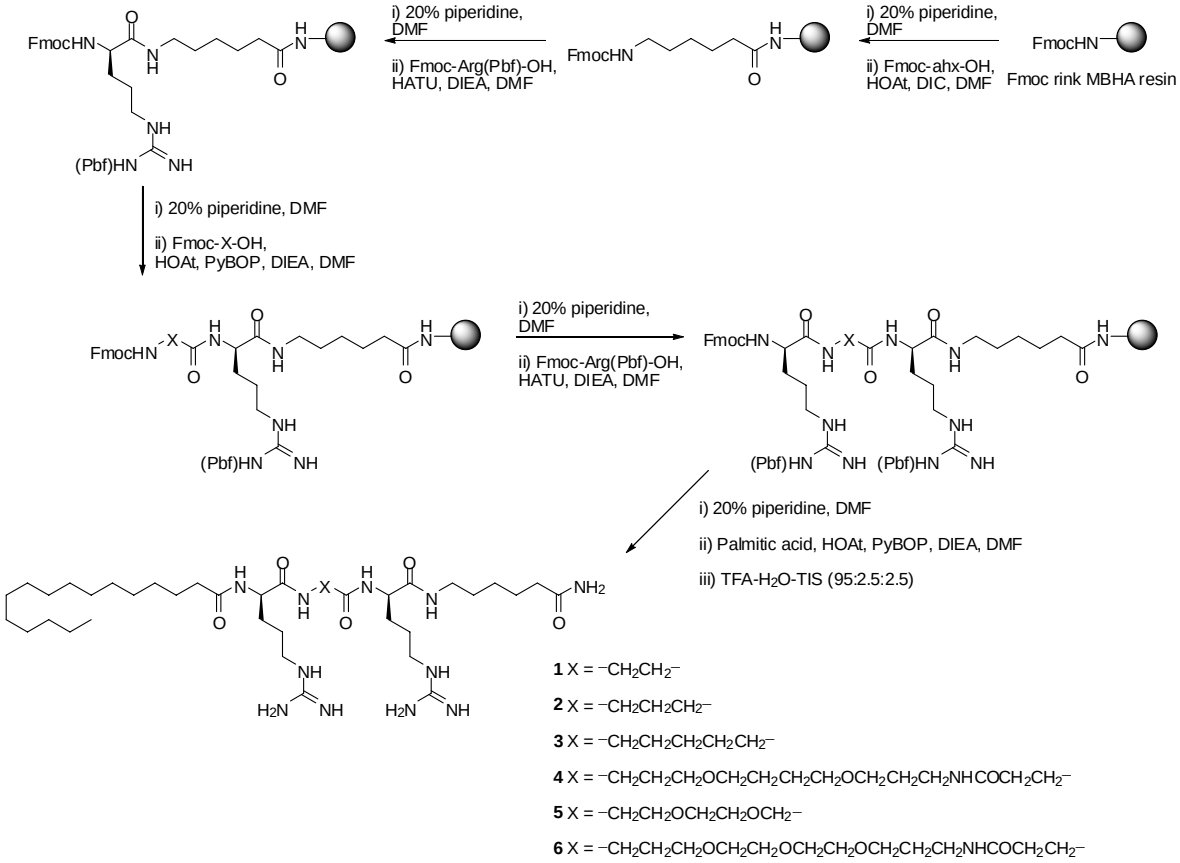
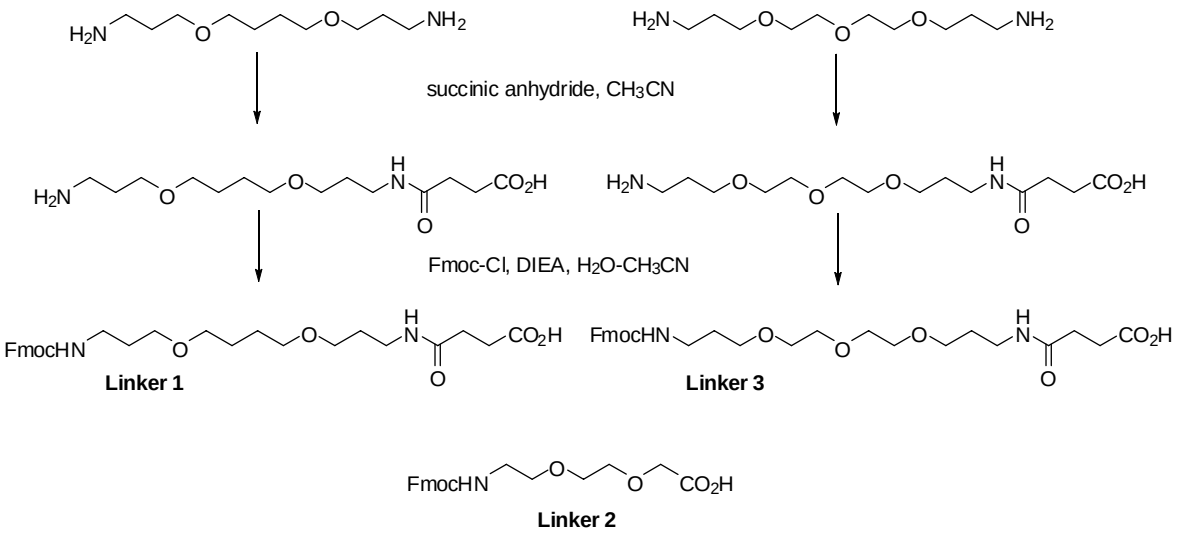


Figure 2. Schematic view of the molecules designed (AA = amino acid). These compounds contain a variable linker, two Arg and two aliphatic tails, consisting of palmitic and aminohexanoic acids.



Scheme 1. Synthesis of compounds **1** to **6** on solid-phase. See the Experimental Section for details.



Scheme 2. Chemical structure of the PEG-like linkers used in the synthesis of LPS-neutralizers. The synthesis of **linker 1** and **2** is described. **Linker 2** was purchased from commercial sources.

LPS-neutralizing activity of compounds 1 to 6

Compounds **1** to **6** were assayed for anti-LPS activity using the chromogenic LAL assay.[39] LALF-14c was used in the assay as a reference of well-known LPS-neutralizing peptide[25] (Table 1).

Table 1. LPS-neutralization activity (IC_{50}) of compounds **1** to **6**

Compound	IC_{50} (μM) ± SD
LALF-14c	37 ± 1
1	34 ± 1
2	34 ± 1
3	36 ± 1
4	18 ± 1
5	36 ± 1
6	17 ± 1

Compounds **1** to **3** showed an LPS-neutralizing activity comparable to that of the reference compound LALF-14c. These results are in agreement with previous data on a structurally related class of bis(Args) *gemini* surfactants[40] which showed a good inhibition of the LPS-induced production of TNF-α and NO release from LPS-challenged macrophages.[41] In our study, no significant difference was observed between compounds **1** to **3**, suggesting that the small changes in the linker length are too slight to modify their activity. The PEG-containing **5** had a similar behavior. In contrast, compounds **4** and **6** showed a 2-fold increase in LPS-neutralizing activity. Compound **5** contained **linker 2** (see scheme 2), which has a similar length to the aminohexanoic spacer of **3**, and the extra oxa groups may not represent any improved interaction with the lipid A. However, the length of **linker 1** and **linker 3** (see scheme 2) is close to the optimal 16 Å distance reported in the literature[29] and resulted in an enhancement of the biological activity of **4** and **6**. Moreover, the presence of an extra carboxamide group and the higher flexibility of these chains might contribute to the interaction with the disaccharide unit. As a first step to further analyze the biological profile of these compounds in biological systems, cytotoxicity assays in RAW macrophages were pursued (Figure 3).

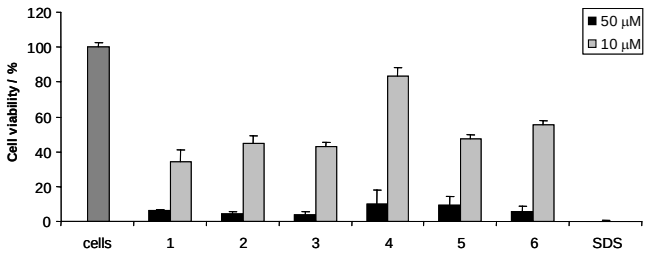


Figure 3. Cell viability is shown at two compound concentrations: 50 and 10 μM. Cell viability was evaluated by MTT assay in RAW 264.7 cells after 24 h of incubation with compounds **1** to **6**. The assay is described in the Experimental Section.

LPS-inhibitors were assayed at 10 and 50 μM, concentrations below and above their IC_{50} values, respectively. At these concentrations, the compounds displayed toxicity. These results were not unexpected given the pronounced membrane activity reported for this class of compounds.[41] The incorporation of PEG-chains involved a slight decrease in toxicity, as is the case of **4,** which is tolerable for this cell system at 10 μM.

Overall, this methodology provides a powerful tool for the preparation of a large number of new LPS inhibitor candidates. We believe that further modifications of these compounds aimed to modulate their toxicity could result in highly active agents of promising therapeutic value.

Substitution of amino acids by PEG-like chains

Having demonstrated that PEG or PEG-derived chains may be helpful in the design of LPS inhibitors, we considered that the same idea could be applied to cyclic LPS-neutralizing peptides, in which several "non-essential" residues could be substituted by PEG moieties. For this purpose, we chose the peptide **RLKWc**.[28] This peptide contains the minimal LPS-binding region of LALF-14c: Arg^{41}-Leu^{42}-Lys^{43}-Trp^{44}. However, this small sequence required extra residues in order to achieve proper cyclization, which was obtained by the addition of Cys-Ala pairs at the *C*- and *N*-termini.[28] Now we reasoned that extra Ala and Cys residues could be replaced by any of the linkers described above, which are suitable building-blocks for solid-phase peptide synthesis (SPPS) (Figure 4).

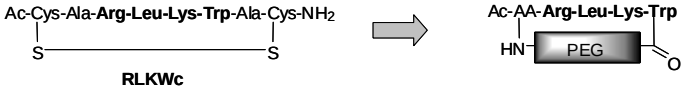
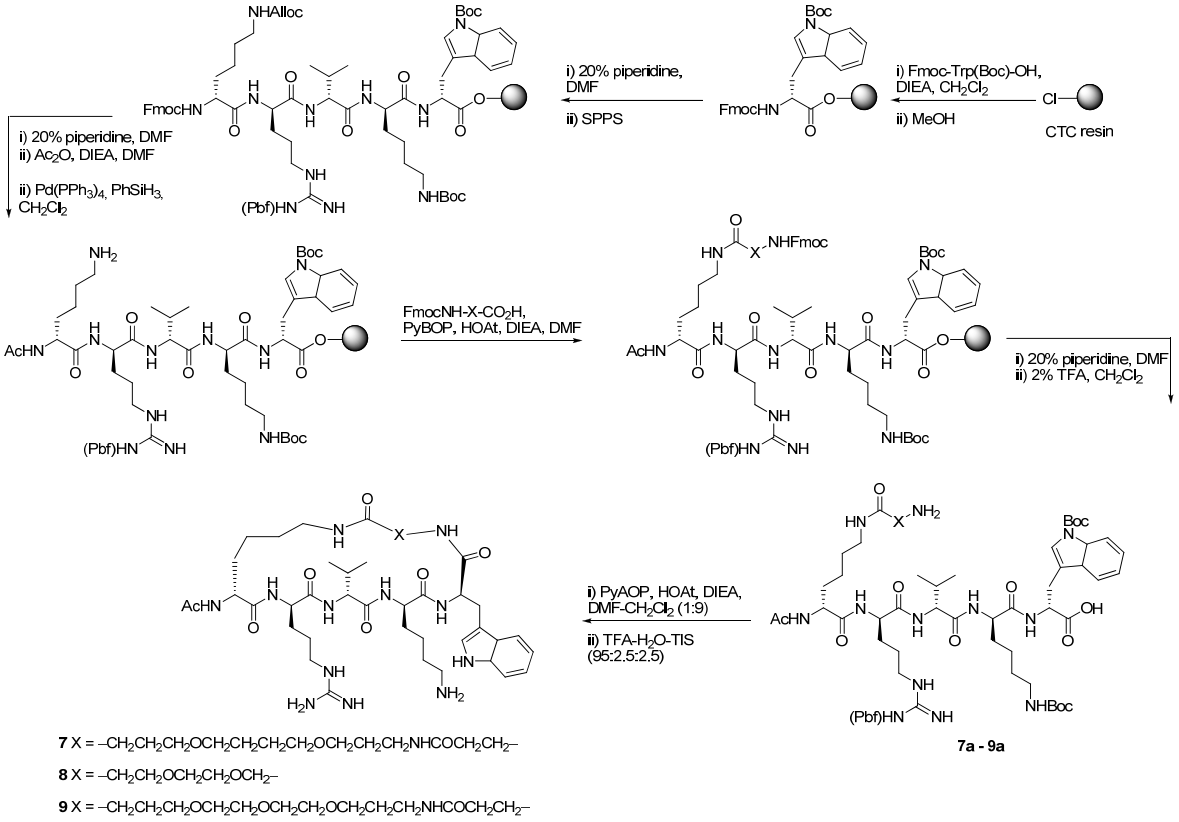


Figure 4. Chemical structure of the peptide **RLKWc** (amino acids are written using the three-letter code). Replacement of Cys and Ala residues by a PEG-linker is shown.

Thus, PEG-modified RLKWc peptides were synthesized on solid-phase to obtain compounds **7** to **9** (see Scheme 3). In order to have a free carboxylic acid at the *C*-terminus, Fmoc-Trp(Boc)-OH was inserted into 2-chlorotrytil chloride resin (CTC). The peptide was then elongated using standard SPPS (see the Experimental Section for details) with the final introduction of an extra residue, the orthogonally protected Fmoc-Lys(Alloc)-OH, which allowed the specific functionalization of the ε-amino group of the Lys side chain with the PEG-linkers. Hence, after selective Fmoc removal, the α-amino of Lys was acetylated. Pd-catalyzed elimination of the Alloc group rendered the Lys free ε-amino group. It is important to acetylate the peptide at this point, since prior elimination of the *N*-ε-Alloc group may result in undesired *N*-α-Fmoc removal.[42] The resin was divided in three portions and **linkers 1** to **3** were introduced overnight using PyBOP and HOAt as coupling reagents. The peptide was then cleaved from the solid support with mild acidic treatments. The side-chain protecting groups were not altered by this cleavage. Cyclization was carefully carried out in solution with 7-azabenzotriazol-1-yloxy-tris-(pyrrolidino)phosphonium hexafluorophosphate (PyAOP) and followed by HPLC (see the Experimental Section for details).[43] Cyclization was achieved after 2 h. After an adequate work-up to remove solvents and by-products, compounds were finally treated with TFA mixtures to deprotect amino acid side-chains. Compounds **7** to **9** were purified by HPLC to obtain optimal purities.



Scheme 3. Synthesis of compounds **7** to **9**. See the Experimental Section for details.

LPS-neutralizing activity of compounds 7 to 9

The LPS-neutralizing activity of compounds **7** to **9** was evaluated using the LAL test. In this case, the **RLKWc** peptide was used as a positive control. Neutralization values are expressed by means of IC_{50} and are shown in Table 2.

Table 2. LPS-neutralization activity (IC_{50}) of compounds **7** to **9**

Compound	IC_{50} (μM) ± SD
RLKWc	30 ± 1
7	35 ± 1
8	n.c.[a]
9	n.c.
n.c. not calculated	

The LPS-neutralizing activity of **7** was similar to that of the **RLKWc** control peptide, thereby suggesting that the substitution of several amino acids by the **linker 1** is not detrimental to activity. In fact, this 5-amino acids cyclic peptide contains the LPS-binding domain described for LPS neutralization and retains its biological activity. In contrast, surprisingly, compounds **8** and **9** showed poor neutralizing activity at 100 μM, and therefore their IC_{50} values were not calculated. While linear compounds **1** to **6** were active *per se*, peptides derived from LALF required a cyclic conformation to be active.[25,28] For these peptides, the introduction of a PEG-linker that disturbs the "active" cyclic conformation may imply a loss in activity, as might be the case of **linker 2** and **3**, which are respectively shorter in length and have a distinct chemical structure to that of **linker 1**. In this case, small changes may be crucial for effective LPS-binding.

Finally, the cytotoxicity and the enzymatic stability of the most promising candidate, **7**, were studied and compared to **RLKWc**. In this case, **7** did not show toxicity at the concentrations previously tested (Figure 5), thereby indicating that PEG insertion does not imply any kind of unspecific toxicity. Finally, the stability of both compounds to human serum was studied (see the Experimental Section). **RLKWc** showed a slightly better half-live of 14.1 ± 0.3 h in human serum whereas **7** had a half-life of 9.9 ± 0.3 h.

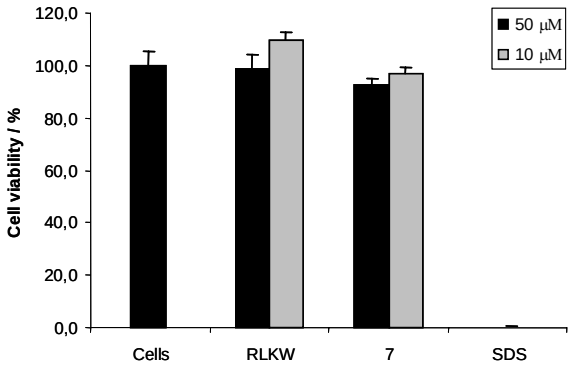


Figure 5. Cell viability is shown at two compound concentrations: 50 and 10 μM. Cell viability was evaluated by MTT assay in RAW 264.7 cells, after 24 h of incubation with compounds **RLKWc** and **7**. The assay is described in the Experimental Section.

Conclusion

Here we present a new solid-phase methodology for the facile synthesis of potent LPS-neutralizers. On the basis of the chemical features of the toxic moiety of LPS, lipid A, we synthesized a series of compounds and assayed them for anti-LPS activity. All compounds showed activity comparable to that of the highly active LPS-neutralizing peptide LALF-14c. In particular, the introduction of PEG-like chains resulted in enhanced activity. These functionalities were used to replace several non-essential amino acids of the anti-LPS peptide RLKWc. In this regard, we report the synthesis, biological activity and toxicity profile of **7,** a PEG-like-containing peptide that displays promising LPS-neutralizing activity and that is non-toxic to cells. This approach will also be useful for the modification of other cyclic bioactive peptides.

Experimental Section

General

Resins, amino acids, coupling reagents, solvents and other reagents were purchased from commercial suppliers at the highest purity available and used without further purification. Analytical HPLC was performed using a Waters Alliance 2695 (Waters, MA, USA) chromatography system with a PDA 995 detector, reverse-phase Symmetry C_{18} (4.6 x 150 mm, 5-μm) and Sunfire C_{18} (4.6 x 100 mm, 3.5-μm) columns and linear gradients of MeCN with 0.036% TFA into H_2O with 0.045% TFA. The system was run at a flow rate of 1.0 mL/min over either 8 (C_{18} Sunfire) or 15 min (C_{18} Symmetry). Semi-preparative HPLC was carried out on a Waters chromatography system with a dual absorbance detector 2487, reverse-phase Symmetry C_{18} columns (7.8 x 100 mm 5-μm or 30 x 150 mm, 5-μm) and linear gradients of MeCN with 0.05% TFA into H_2O with 0.1% TFA. The system was run at a flow rate of either 3.0 or 20.0 mL/min over 30 min, depending on the column used. HPLC-MS was performed using a Waters Alliance 2796 with a dual absorbance detector 2487 and ESI-MS Micromass ZQ (Waters) chromatography system, a reversed-phase Symmetry 300 C_{18} (3.9 x 150 mm, 5-μm column) and H_2O with 0.1% formic acid and MeCN with 0.07% formic acid as mobile phases. Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (Applied Biosystems, Foster City, CA, USA) using ACH matrix.

Solid-phase synthesis

Compounds **1**-**6** and peptides **RLKWc** and **7**-**9** were synthesized manually using the Fmoc strategy in polypropylene syringes, each fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Washings between deprotection, couplings and subsequent deprotection steps were carried out with DMF and CH_2Cl_2, using 10 mL of solvent/g of resin each time. When syntheses were performed on 2-chlorotrityl chloride resin (CTC), the CH_2Cl_2 used was treated with alumina. The Fmoc group was removed by treatment with piperidine–DMF (1:4, v/v) and acetylation steps were performed with Ac_2O–DIEA–DMF (1:2:7). Couplings and washes were performed at 25 ºC. Couplings were monitored using Kaiser, de Clercq or chloranil methods.[44]

General procedure for the synthesis of compounds 1-6

Fmoc-Rink Amide MBHA resin (1.0 g, 0.7 mmol/g) was placed in a 20 mL-polypropylene syringe fitted with a polyethylene filter disk. After Fmoc removal, Fmoc-6-aminohexanoic acid (4 equiv) was coupled with DIC (4 equiv) and HOAt (4 equiv) in DMF for 6 h. Fmoc-Arg(Pbf)-OH (4 equiv) was inserted with 2 x 45 min consecutive treatments using HATU (4 equiv) and DIEA (8 equiv) as coupling reagents. At this point, the resin was split into six portions. Aliphatic amino acids Fmoc-β-Ala-OH (5 equiv), Fmoc-4-aminobutiric acid (5 equiv) and Fmoc-6-aminohexanoic (5 equiv); and Fmoc-protected PEG-like amino acids Fmoc-8-amino-3,6-dioxaocatonic acid (5 equiv), Fmoc-protected 4,7,10-trioxa-1,13-tridecanediamine succinimic acid (5 equiv) and Fmoc-protected 1,4-Bis(3-aminopropoxy)butane succinimic acid (5 equiv) were each coupled overnight with PyBOP (5 equiv), HOAt (5 equiv), and DIEA (15 equiv). The following step involved a second coupling of Fmoc-Arg(Pbf)-OH under the conditions explained above. Finally, palmitic acid (5 equiv) was inserted overnight with PyBOP (5 equiv), HOAt (5 equiv) and DIEA (15 equiv). For the cleavage of the compounds from the support and concomitant deprotection of side-chain groups, the resin was washed with CH_2Cl_2 (3 x 1 min), dried, and treated with TFA–H_2O–triisopropylsilane (TIS) (95:2.5:2.5) for 2 h. TFA was then removed by evaporation with nitrogen, and peptides were precipitated with cold anhydrous *t*-butyl methyl ether (TBME), dissolved in H_2O–MeCN (1:1) and then lyophilized. Compounds were purified by semi-preparative HPLC (linear gradient from 0 to 100 % MeCN over 30 min, flow rate 20 mL/min.) up to purities higher than 95 %. Compounds were characterized by HPLC and mass spectrometry. **1** HPLC (t_R = 10.19 min, from 0 to 100 % CH_3CN over 15 min, purity 96%), ESMS (*m/z* calcd. for $C_{37}H_{73}N_{11}O_5$ 751.6, found 752.2 $[M+H]^+$, 376.4 $[M+H]^+/2$). **2** HPLC (t_R = 10.24 min, from 0 to 100 % CH_3CN over 15 min, purity >99%), ESMS (*m/z* calcd. for $C_{38}H_{75}N_{11}O_5$ 765.6, found 766.1 $[M+H]^+$, 383.3 $[M+H]^+/2$). **3** HPLC (t_R = 10.37 min, from 0 to 100 % CH_3CN over 15 min, purity 98%), ESMS (*m/z* calcd. for $C_{40}H_{79}N_{11}O_5$ 793.6, found 794.0 $[M+H]^+$, 397.5 $[M+H]^+/2$). **4** HPLC (t_R = 10.42 min, from 0 to 100 % CH_3CN over 15 min, purity 98%), MALDI-TOF (*m/z* calcd. for $C_{48}H_{94}N_{12}O_8$ 966.73, found 967.81 $[M+H]^+$). **5** HPLC (t_R = 10.19 min, from 0 to 100 % CH_3CN over 15 min, purity >99%), MALDI-TOF (*m/z* calcd. for $C_{40}H_{79}N_{11}O_7$ 825.62, found 825.66 $[M+H]^+$). **6** HPLC (t_R = 10.27 min, from 0 to 100 % CH_3CN over 15 min, purity >99%), MALDI-TOF (*m/z* calcd. for $C_{48}H_{94}N_{12}O_9$ 982.73, found 983.73 $[M+H]^+$).

Synthesis of Linkers 1 and 3

Fmoc-protected 1,4-Bis(3-aminopropoxy)butane succinimic acid (**linker 1**). 1,4-Bis(3-aminopropoxy)butane (2.04 g, 10 mmol) was dissolved in 50 mL of CH_3CN and placed in a 250-mL flask with magnetic stirring. Succinic anhydride (1.00 g, 10 mmol) dissolved in 25 mL of CH_3CN was added dropwise for 1 h. The mixture was then left to react for 3 more hours at room temperature. After this time, a white waxy product appeared in the flask. The product was allowed to settle and the organic solvent was decanted and discarded. The solid was then redissolved in 100 mL of H_2O-CH_3CN (1:1) and cooled in an ice bath for 30 min. 9-fluorenylmethyl chloroformate (Fmoc-Cl) (3.36 g, 13 mmol) dissolved in 25 mL of CH_3CN was then added. The pH was adjusted to 9 with DIEA and the reaction was stirred overnight. The solvents were removed *in vacuo* and the resultant product was dissolved in 100 mL of 5% $NaHCO_3$ and washed with AcOEt (3 x 50 mL). To minimize the formation of emulsions at this point, brine was also added. The aqueous phase was then acidified with HCl 2N to pH 2 and extracted with AcOEt (3 x 50 mL). The organic phase was finally washed with distilled H_2O (3 x 25 mL) and dried over anhydrous Na_2SO_4. The evaporation of the organic solvent yielded the expected compound as a white-cream solid (3.56 g, 68%). HPLC (t_R = 11.43 min, from 0 to 100 % CH_3CN over 15 min), ESMS (m/z calcd. for $C_{29}H_{38}N_2O_7$ 526.3, found 527.9 $[M+H]^+$).

Fmoc-protected 4,7,10-trioxa-1,13-tridecanediamine succinimic acid (**linker 3**). 4,7,10-trioxa-1,13-tridecanediamine (2.22 g, 10 mmol) was treated as explained above for **linker 1**. This treatment gave the desired product as a yellow oil (2.58 g, 48%). HPLC (t_R = 10.84 min, from 0 to 100 % CH_3CN over 15 min), ESMS (m/z calcd. for $C_{29}H_{38}N_2O_8$ 542.3, found 543.8 $[M+H]^+$).

General procedure for the synthesis of compounds 7-9

Fmoc-Trp(Boc)-OH (1 equiv) and DIEA (10 equiv) were sequentially added to CTC resin (500 mg, 1.00 mmol/g) and the resin was stirred for 1 h. The incorporation was followed by a capping step with MeOH (411 μL). Fmoc-amino acids (4 equiv) were coupled using HOAt (4 equiv) and DIC (4 equiv) in DMF for 2 h, with the exception of Fmoc-Arg(Pbf)-OH, which was coupled with HATU (4 equiv.) and DIEA (8 equiv.), 2 x 45 min. Acetylation was performed as previously described and Alloc removal was accomplished after 3 x 15 min treatments of $PhSiH_3$ (10 equiv) and $Pd(PPh_3)_4$ (0.1 equiv) in CH_2Cl_2. The resin was then washed with sodium dithiodiethylcarbamate (0.02 M in DMF, 3 x 15 min) to remove any remaining palladium traces of the resin. The resin was divided in three, and **linkers 1** to **3** were inserted overnight with HOAt (5 equiv), PyBOP (5 equiv) and DIEA (15 equiv). After Fmoc removal, peptides were cleaved using TFA–CH_2Cl_2 (1:99) (5 x 0.5 min). The solvents were evaporated and the crude products lyophilized to obtain **7a** to **9a** as white solids. These linear precursors were then cyclized without any intermediate purification.

General procedure for cyclization and purification of compounds 7-9

The linear precursors **7a-9a** were placed in a round-bottom flask to which HOAt (2 equiv) dissolved in 2 mL of DMF was added. The mixture was stirred and CH_2Cl_2 was added to obtain a CH_2Cl_2-DMF (9:1) solution at a final concentration of 1mg/mL. Solid PyAOP (2 equiv) was then added, followed by the addition of DIEA (3 equiv). The reaction was then stirred for 2 h and checked by HPLC. The organic phase was then washed with aqueous $NaHCO_3$ (3 x 10 mL) and a saturated solution of NH_4Cl (3 x 10 mL). The organic phase was finally dried over anhydrous Na_2SO_4. After evaporation of the organic solvent, the protected compounds were treated with TFA–H_2O–TIS (95:2.5:2.5) for 2 h. After this time, TFA was evaporated with nitrogen and peptides were precipitated with cold anhydrous TBME. After centrifugation-decantation, peptides were dissolved in H_2O–MeCN (1:1) and lyophilized to afford **7-9**. Peptides were then purified by HPLC (linear gradient from 10 to 30 % MeCN over 30 min, flow rate 3 mL/min) to achieve optimal purities. Peptides were characterized by HPLC and MALDI-TOF.

Cyclization and purification of compound 7

The linear precursor **7a** (20 mg, 0.013 mmol) was treated as described in the general procedure with HOAt (3.54 mg, 0.026 mmol), PyAOP (13.55 mg, 0.026 mmol) and DIEA (9 μL, 0.052 mmol) to afford **7** as a white powder (8.0 mg, 58%). Compound **7** was then purified by HPLC to obtain 4.70 mg (purification yield 59 %). Characterization of **7**: HPLC (t_R = 14.02 min, from 10 to 30 % CH_3CN over 15 min, purity 93%), MALDI-TOF (*m/z* calcd. for $C_{51}H_{85}N_{13}O_{10}$ 1039.65, found 1040.72 $[M+H]^+$).

Cyclization and purification of compound 8

8a (20 mg, 0.015 mmol) was treated with HOAt (4.08 mg, 0.030 mmol), PyAOP (15.63 mg, 0.030 mmol) and DIEA (10.45 μL, 0.060 mmol) as described above. Compound **8** was obtained as a white powder (7.5 mg, 56%). Purification of **8** was accomplished by HPLC to yield 1.11 mg (purification yield 15 %). Characterization of **8**: HPLC (t_R = 11.10 min, from 10 to 30 % CH_3CN over 15 min, purity >99%), MALDI-TOF (*m/z* calcd. for $C_{43}H_{70}N_{12}O_9$ 898.54, found 899.61 $[M+H]^+$, 921.60 $[M+Na]^+$, 937.58 $[M+K]^+$).

Cyclization and purification of compound 9

9a (11.5 mg, 0.008 mmol) was cyclized with HOAt (2.04 mg, 0.016 mmol), PyAOP (7.80 mg, 0.016 mmol) and DIEA (5.23 μL, 0.032 mmol), as previously explained, to afford **9** as a white powder (3.6 mg, 37%). HPLC-purification gave 3.6 mg of **9** (purification yield, quantitative). Characterization of **9**: HPLC (t_R = 12.55 min, from 10 to 30 % CH_3CN over 15 min, purity 88%), MALDI-TOF (*m/z* calcd. for $C_{51}H_{85}N_{13}O_{11}$ 1055.65, found 1056.71 $[M+H]^+$).

Synthesis of RLKWc

Fmoc-Rink Amide MBHA resin (100 mg, 0.45 mmol/g) was placed in a 10 mL-polypropylene syringe fitted with a polyethylene filter disk. After Fmoc removal, Fmoc-amino acids were coupled with DIC (4 equiv) and HOAt (4 equiv) in DMF for 2 h. Side chains of Fmoc-amino acids were protected as follows: Lys and Trp were protected with the *tert*-butyloxycarbonyl group (Boc), Cys with the trityl group (Trt) and Arg with the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl group (Pbf). For the deprotection of side-chain groups and concomitant cleavage of the peptide from the solid support, the resin was washed with CH_2Cl_2 (3 × 1 min), dried and treated with a TFA–H_2O–TIS (95:2.5:2.5) mixture for 1.5 h. TFA was then removed by evaporation with nitrogen, and the peptide was precipitated with cold anhydrous TBME, dissolved in H_2O–MeCN (1:1) and then lyophilized to afford the linear precursor (29 mg, 68%). Next, the linear peptide (8.9 mg, 0.009 mmol) was dissolved with H_2O–MeCN (1:1) at a concentration of 0.5 mg/ml in a round-bottom flask. The pH was then adjusted to 8-9 with a 20% solution of NH_3. The solution was stirred at room temperature for 24 h to allow air oxidation. Cyclization was easily monitored either by Ellman's test[45] and/or by RP-HPLC. The solution was then acidified with a 4 % AcOH solution and lyophilized to yield the cyclic peptide **RLKWc** (5.2 mg, 58 %). **RLKWc** was finally purified by HPLC (linear gradient from 0 to 50 % MeCN over 30 min, flow rate 3 mL/min) to obtain 1.6 mg (purification yield 31 %). Characterization of **RLKWc**: HPLC (t_R = 5.42 min, from 0 to 50 % CH_3CN over 8 min, purity >99 %), MALDI-TOF (*m/z* calcd. for $C_{41}H_{66}N_{14}O_8S_2$ 946.46, found 947.26 $[M+H]^+$, 969.23 $[M+Na]^+$).

LPS-neutralizing activity

All solutions used in the LPS-neutralizing activity assay were tested to ensure they were endotoxin-free and material was sterilized by heating for 3 h at 180 ºC. LPS from *E. Coli* 055:B5 and Polymyxin B were purchased from Sigma. LPS-neutralizing activity was measured using the chromogenic *Limulus* Amebocyte Lysate (LAL) test,[39] following the manufacturer's instructions (Cambrex). LAL reagent contains a clottable protein that is activated in the presence of non-neutralized LPS and is an extremely sensitive indicator of the presence of endotoxin. When activated, this enzyme catalyses the release of p-nitroaniline (pNA) from the colorless chromogenic substrate Ac-Ile-Glu-Ala-Arg-pNA. The pNA released can be measured photometrically at 405 nm. Compounds were initially incubated at 100 μM with LPS (100 pg/mL) in a 96-well microtiter for 60 min at 37 ºC. Polymyxin B (10 μg/mL) was used as positive control. LAL (12.5 μL) was added to start the reaction at 37 ºC. After 10 min, non-neutralized LPS was detected after a 5-8 min incubation with the chromogenic substrate (25 μL). Acetic acid (25 % v/v final concentration) was added to stop the reaction and the absorbance was monitored at 405 nm in a Multiskan Ascent microtiter plate reader (ThermoLabsystems). Compounds that showed a LPS-neutralization above 75 % at this concentration were tested to determine their IC_{50} (the concentration necessary to *in vitro* neutralize 50% of LPS). IC_{50} values were determined by a serial dilution assay using 100 pg/mL of LPS and a range of compound concentrations (50 to 0.001 μM). All assays were run in triplicate, and the curves were automatically adjusted by non-linear regression using "Prism 4" (GraphPad) software.

Cell culture

Mouse macrophages (RAW 264.7) were obtained from ATCC (American Type Culture Collection, USA). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRI) supplemented with 10% fetal bovine serum (FBS - Gibco BRI) and 1% L-glutamine. The cultures were incubated at 37ºC in a humidified

atmosphere of 5% CO_2- 95% air. Subcultures of macrophages were prepared every 2-3 days by scraping cells into fresh medium.

MTT cell viability assays

Cell viability was evaluated by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. RAW 264.7 cells were seeded in sterile 96-well microtiter plates at a density of 6 x 10^4 cells/mL in DMEM supplemented with 10% FBS and allowed to settle for 24 h. Various concentrations of the compounds were added to the plates and the cells were further incubated for 24 h. After removal of the medium, the precipitated formazan crystals were dissolved in optical grade DMSO (100 μL), and the plates were read at 570 nm using a Wallac 1420 Workstation.

Proteolytic stability

Compound **7** and **RLKWc** were initially dissolved in H_2O–CH_3CN (1:1) at a concentration of 500 μM and further diluted to 250 μM with 90% human serum (from human male AB plasma, sterile-filtered, Sigma) in Hanks' Balanced Salt Solution (HBSS, Gibco). Compounds were incubated at 37 ºC and 50-μL aliquots were periodically taken at 1 h to 120 h. Aliquots were poured into 200 μL of MeOH to precipitate serum proteins and cooled at 4 ºC for 30 min. Samples were then centrifuged at 10,000 rpm for 15 min at 4 ºC and the supernatants were analyzed by HPLC (linear gradient from 0 to 50 % MeCN over 8 min, flow rate 1 mL/min). The kinetics analysis was performed by plotting the log % A from the HPLC peak versus time using the least-squares method. The slope of the straight-line half-life was calculated for each compound. Experiments were performed in duplicate.

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Keywords: sepsis · LPS-inhibitors · PEG · biological activity · amino acid substitution

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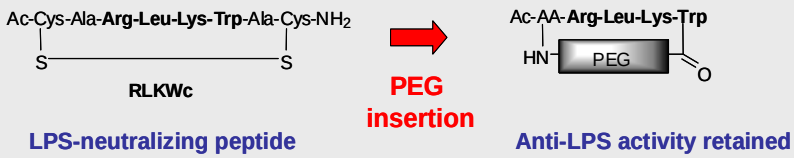

Easy-to-synthesize LPS inhibitors are presented here. These compounds were designed on the basis of the chemical features of lipid A, the endotoxic portion of LPS, and synthesized using solid-phase methodologies. The presence of PEG-like functionalities rendered the most active compounds. Moreover, we successfully replaced several amino acids from a cyclic anti-LPS peptide by PEG-like chains. The synthesis, biological activity and toxicity of a new PEG-like-containing cyclic peptide are discussed.

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Page No. – Page No.

Design and facile solid-phase synthesis of peptide-based LPS-inhibitors containing PEG-like functionalities

"Conjugation of bacterial endotoxin neutralizers to dendritic PEGylated platforms: synthesis, biological activity and toxicity"

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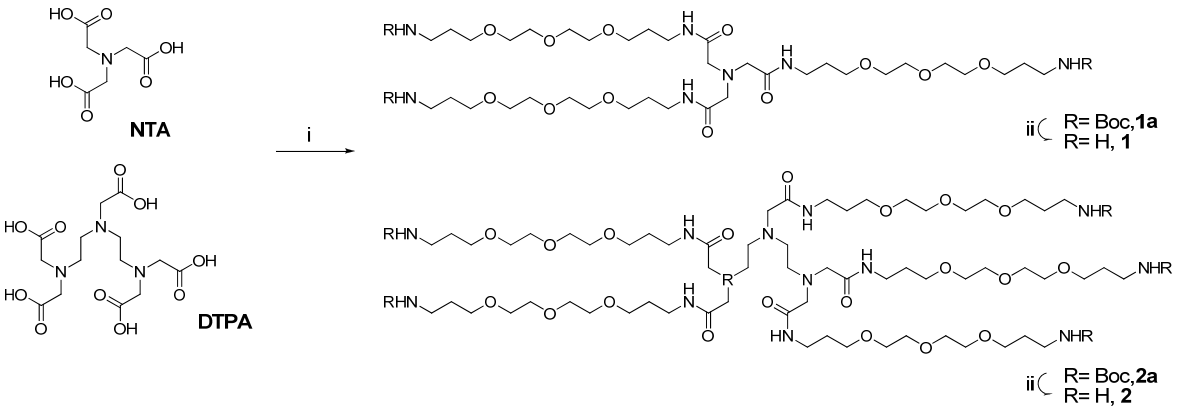
* These two authors contributed equally to the present study

RESUM

En el següent treball es presenta la síntesi d'un nou tipus d'arquitectures dendrimèriques basades en polietilenglicol (PEG) per a la conjugació de compostos amb activitat biològica. La utilitat d'aquestes plataformes va ser avaluada mitjançant la conjugació d'un compost amb gran capacitat per neutralitzar endotoxines descrit per nosaltres anteriorment. La conjugació d'aquesta molècula a les noves estructures va comportar un augment en la seva activitat neutralitzant del LPS i va reduir-ne la toxicitat. A més a més, aquestes arquitectures han demostrat ser útils per incrementar la solubilitat dels compostos conjugats.



Contribucions a aquest treball:

- L'Angela Torres va dissenyar els dendrimers basats en PEG, i va realitzar la seva síntesi. Va realitzar les conjugacions dels dendrimers amb els inhibidors del LPS i va treballar en la seva caracterització. També va col·laborar en l'elaboració del manuscrit.

- En Carlos Mas va dissenyar i sintetitzar els inhibidors del LPS en fase sòlida i va participar en les reaccions de conjugació. En Carlos va realitzar els assaigs biològics d'activitat i els estudis de toxicitat, així com va participar activament en la preparació del manuscrit.

Conjugation of bacterial endotoxin neutralizers to dendritic PEGylated platforms: synthesis, biological activity and toxicity†

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PEGylated dendrimers are an interesting subclass of dendrimers for biological applications. Here we present the synthesis of new dendritic tri- and pentavalent structures based on poly(ethylene glycol) units (PEG). To evaluate whether these PEGylated platforms could be useful for the conjugation of bioactive compounds, a well-known lipopolysaccharide (LPS) inhibitor, developed in our laboratory, was selected and conjugated. The conjugated compound displayed an increased LPS-neutralizing activity and a reduced toxicity profile.

Introduction

The conjugation of bioactive compounds to oligo- and polymeric materials constitutes a useful strategy of outstanding interest in the field of medicinal chemistry.[1,2] These materials can be used either as carrier systems for drug-delivery or as bioactive multivalent platforms for the presentation of several copies of a pharmacological agent with a specific topology. Overall, these systems should allow the administration of much lower drug doses with higher efficiency, thereby minimizing unwanted side-effects. Additionally, for substances with poor water solubility, these systems offer the possibility to increase this property and hence their effectiveness.

In this regard, dendritic or dendrimeric structures have emerged as a new class of biopolymers with structural properties[3,4,5] and biological applications of interest.[6,7,8,9,10] These compounds are highly branched polymers with a well-defined chemical composition and structure. They have a compact globular shape, monodisperse size and controllable surface functionalities. One of the most relevant properties of dendrimers in biological systems is the concept of multivalency. Multivalency leads to a strongly increased activity compared to the corresponding monomeric interaction. This synergistic enhancement of a certain activity is explained by two effects: a simple additive effect, and what is known as the "cluster" or "dendritic" effect, a co-operative effect in a multivalence system that leads to a larger increase in activity than expected from the valency of the system.[11]

PEGylated dendrimers, in which a multifunctional dendritic core is attached to polyethylene glycol (PEG), are a subclass of dendrimers that amplify the special properties of PEG.[12,13] PEG is non-toxic and has no immunogenicity or antigenicity. It is highly flexible and has the capacity to solubilize very insoluble molecules.[14] PEG maintains the original biological functions of the bioactive molecules and increases *in vivo* their stability to degradation. In addition, the presence of PEG reduces kidney ultrafiltration, improves the bioavailability and solubility of a drug, and, in general, facilitates its administration.[15,16] In fact, various PEGylated dendrimers show lower toxicity and hemolytic properties, long blood circulation times, low organ accumulation, and high accumulation in tumor tissue.[17,18,19]

Here we describe the synthesis of a very promising class of tri- and pentavalent PEG-based dendritic platforms, which consist of multiple copies of monodisperse PEG units attached to a multifunctional core (Figure 1). To evaluate whether these PEGylated platforms could be useful for the conjugation of bioactive compounds, a well-known lipopolysaccharide (LPS) inhibitor developed in our laboratory, compound **3**, was selected (Figure 1).[20] LPS is a bacterial endotoxin present in the outer leaflet of Gram-negative bacteria that has a major role in Gram-negative sepsis.[21] Sepsis is a serious systemic response to infection that represents the foremost cause of death in Intensive Care Units (UCIs),[22] and accounts annually for 750,000 hospitalizations in the U.S.[23,24] The selected LPS-inhibitor, compound **3**, was successfully conjugated to the trivalent dendritic platform, thereby rendering a new construct with improved endotoxin-neutralizing activity and toxicity profile.

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† Electronic Supplementary Information (ESI) available: 1H NMR spectra of compunds **1a** and **2a**, and HPLC chromatograms and MALDI spectra of compound **5** and **6**. See DOI: 10.1039/b000000x/

‡ These authors contributed equally to the present study

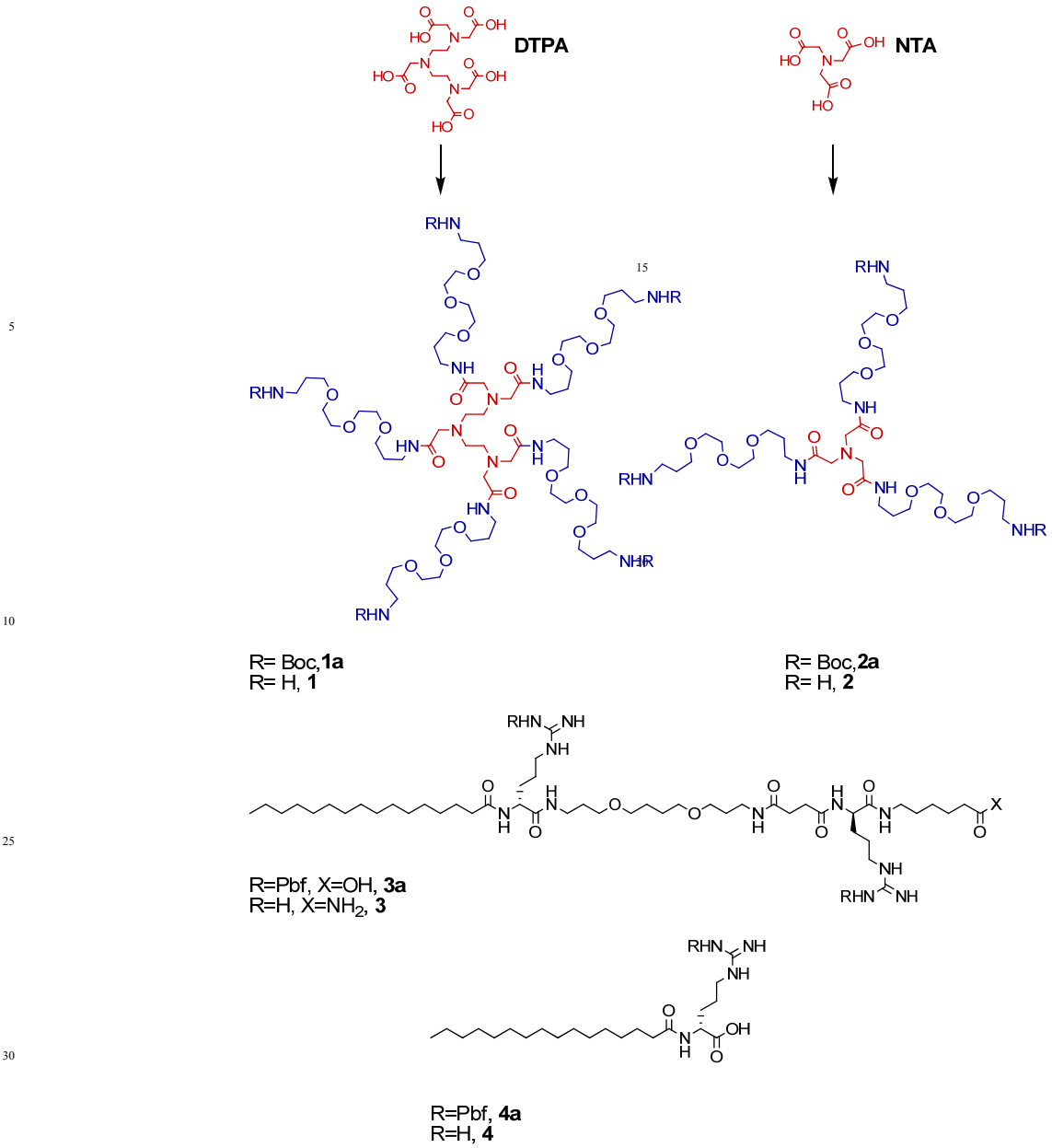


Fig. 1 The two dendritic platforms **1** and **2,** containing the multifunctional cores (**DTPA** or **NTA**), and the PEG chains are shown. The potent LPS-neutralizing agent **3** and the amphipathic monomer **4** are also shown.

Results and discussion

Design of drug-dendritic platform conjugates with LPS-neutralizing activity

Dendrimers have been assayed for a wide range of biological applications; however, to the best of our knowledge, to date only one example of dendrimers with LPS-neutralizing activity has been reported.[25,26] In these studies, David and coworkers examined a variety of amine-terminated poly(amidoamine) (PAMAM) dendrimers.[27] The authors derivatized the surface amines of these dendrimers with lipopolyamines and obtained a multi-branched dendritic structure that neutralized LPS-induced inflammatory responses *in vitro* and afforded protection in a murine model of endotoxic shock.26 No reports concerning other types of dendrimers are found in the literature.

Here we present the synthesis of two PEGylated platforms with tri- and pentavalency (Compounds **1** and **2**, Figure 1). These structures were obtained by convergent synthesis from the condensation of a commercial propylene glycol (TOTA) and a multifunctional core (**DTPA** or **NTA)**. These types of polycarboxylic acids have been used *in vivo* as complexing agents for metals and as carriers for Magnetic Resonance Imaging (MRI) contrast reagents.[28,29] Since their physiological removal has been studied,[30] they were considered excellent cores to design further biological applications.

To evaluate whether our dendritic PEGylated platforms would be useful for the conjugation of biologically active compounds, we conjugated **3** (see Figure 1) to the dendritic structure **2**. In an ongoing research program performed in our laboratory, compound **3** has recently been reported to show high LPS-neutralizing activity.[20] This compound displays unique chemical features for optimal LPS binding: two Arg residues conveniently separated by a PEG linker and a fatty

acid linked at the *N*-terminus. In fact, the cationic residues interact with the negative phosphate groups of lipid A[31] (the endotoxic moiety of the LPS)[32,33] while the palmitic acid promotes hydrophobic interactions with the lipophilic part of the endotoxin[34] and confers amphipathicity to the whole molecule, a prerequisite for LPS neutralization.[35] Moreover, the presence of a PEG spacer is of interest in terms of improved water solubility and pharmacokinetic profiles.[15] In addition, the palmitoyl Arg monomer **4** was included in the design to further evaluate the relevance of the topology of positive charges in terms of LPS interaction (Figure 1).

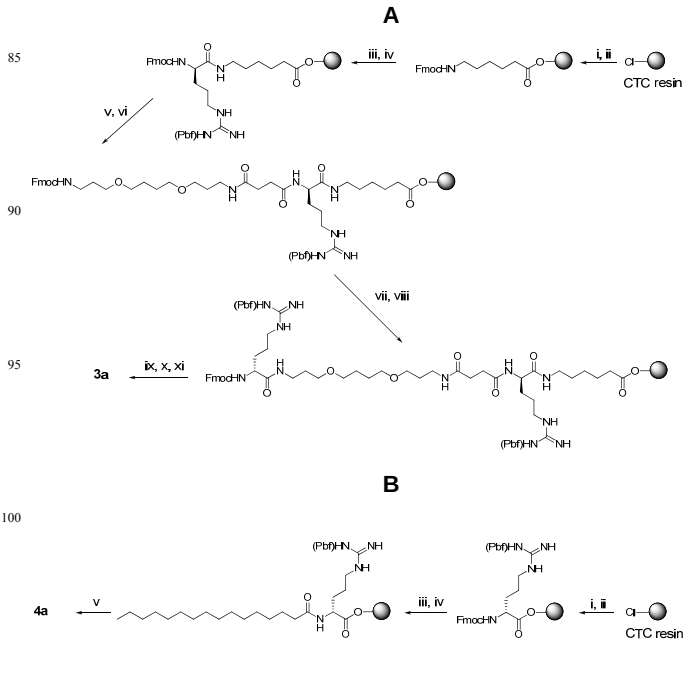
Synthesis of dendritic PEGylated platforms

The synthesis of the PEGylated platform **1** was achieved after conjugation of conveniently mono-protected TOTA into the **DTPA** core. From the reaction conditions assayed, the use of DIC in the presence of HOBt and TEA in DMF–DCM (7:3) for 48 h gave the best results. To ensure an optimal yield, an excess of base was required to prevent amine neutralization. The use of Fmoc as the protecting group of TOTA was precluded in this design because of partial Fmoc removal during the coupling reaction. Hence, Boc-TOTA was coupled to DTPA under the conditions described above to yield the Boc-protected dendrimer **1a** (92% yield, 54% purity). The protected compound was then purified using an automated flash chromatography system with a reverse-phase C_{18} column under acidic conditions (see the Experimental section for details). It is essential to purify the dendrimer while it is protected in order to separate it efficiently from other by-products. Finally, the Boc group was removed by treatment with TFA 50% in DCM for 30 min to obtain dendrimer **1**. The synthesis of the PEGylated platform **2** was performed using the optimized conditions described for the pentavalent structure. In this case, the insertion of Boc-TOTA into NTA afforded the Boc-protected dendrimer **2a** (95% yield, 67% purity). The compound was purified as explained above, and then treated with TFA to render dendrimer **2** (see the Experimental section).

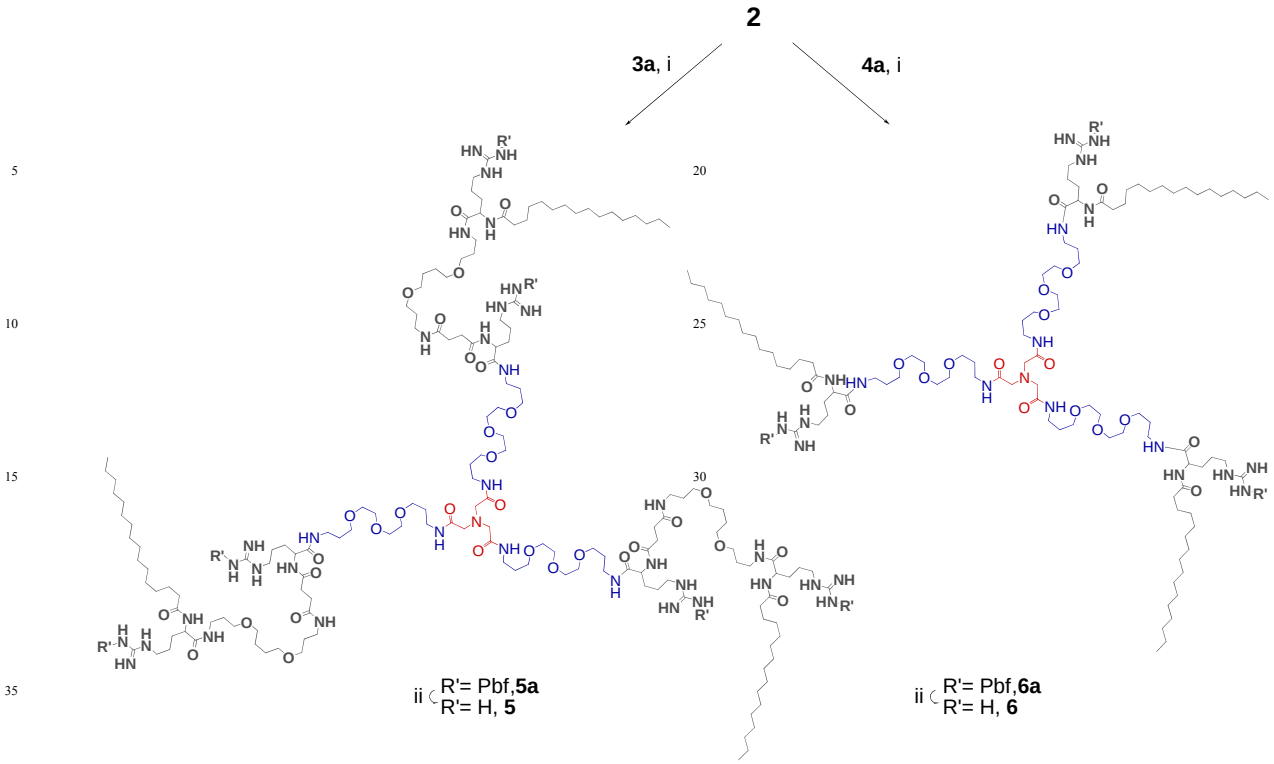
Conjugation of bioactive compounds to a dendritic PEGylated platform

To achieve proper conjugation, the carboxamide group of compound **3** was replaced by a free carboxylic acid and Arg side chains were kept protected with the Pbf group (see Figure 1, **3a**). The synthesis of **3** has been reported in detail elsewhere.[20] Here we used CTC resin as solid support in order to obtain the free carboxylic acid required for amide bond conjugation. After the insertion of Fmoc-6-aminohexanoic acid (Fmoc-Ahx-OH) using DIEA as a base, Fmoc-Arg(Pbf)-OH was coupled with two consecutive treatments with the reactive aminium salt HATU.[36] Next, Fmoc-protected 1,4-bis(3-aminopropoxy)butane succinimic acid was coupled overnight with the phosphonium salt PyBOP to prevent *N*-terminus guanidylation.[37] After the second Arg coupling, palmitic acid was inserted overnight. The compound was finally cleaved from the solid support with mild acidic treatments to yield **3a** (see the Experimental Section for details, Scheme 1A). In turn, **4a** was easily obtained on solid-phase after insertion of Fmoc-Arg(Pbf)-OH into CTC, and subsequent Fmoc removal and palmitic acid coupling (see the Experimental section for details, Scheme 1B).

Thus, **3a** and **4a** were carefully conjugated to **2** with the coupling reagent PyBOP in the presence of HOBt and DIEA under anhydrous conditions (Scheme 2). The reaction was followed by HPLC. As an example, HPLC chromatograms of the conjugation of **3a** with **2** are shown in Figure 2. It is noteworthy that the polarity of **3a** (Figure 2A) did not diminish drastically after conjugation with the dendritic structure (Figure 2B), even though the conjugated compound, **5a**, contains three palmitoyl residues and protected side-chains. The same effect was observed for the conjugate **6a** (data not shown). This behavior illustrates how PEGylated dendritic structures are useful platforms to solubilize hydrophobic compounds. After completion of the conjugation, a series of work-up methods were evaluated. We found that simple extraction and precipitation procedures were useful to obtain the protected conjugates with optimal purities (Figure 2C) and hence tedious and both compound- and time-consuming HPLC purifications were avoided (see the Experimental Section for details). Finally, the protected conjugates were treated with TFA to yield the desired conjugates **5** (Figure 2D) and **6**.



Scheme 1 Solid-phase synthesis of compounds **3a** (A) and **4a** (B). **1A:** (i) Fmoc-Ahx-OH, DIEA, DCM; (ii) MeOH; (iii) piperidine, DMF; (iv) Fmoc-Arg(Pbf)-OH, HATU, DIEA, DMF; (v) piperidine, DMF; (vi) PEG-linker, HOAt, PyBOP, DIEA, DMF; (vii) piperidine, DMF; (viii) Fmoc-Arg(Pbf)-OH, HATU, DIEA, DMF; (ix) piperidine, DMF; (x) palmitic acid, HOAt, PyBOP, DIEA, DMF; (xi) TFA-DCM. **1B: (**i) Fmoc-Arg(Pbf)-OH, DIEA, DCM; (ii) MeOH; (iii) piperidine, DMF; (iv) palmitic acid, HOAt, PyBOP, DIEA, DMF; (v) TFA-DCM



Scheme 2 Synthesis of the conjugates **5** and **6**. (i) PyBOP, HOBt, DIEA; (ii) TFA-H_2O-TIS

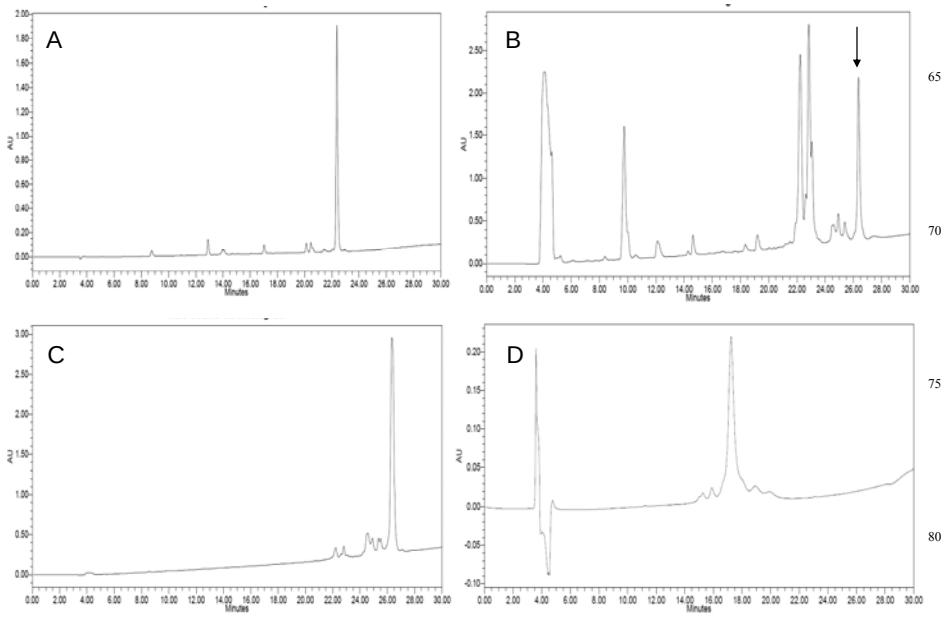


Fig. 2 Analytical HPLC chromatograms showing the formation of the conjugate **5a**. A: Compound **3a**. B: Crude product of the synthesis at 72 h. The peak corresponding to the starting material is due to the excess equivalents used. Final compound **5a** is indicated by an arrow. C: Conjugate **5a** after work-up treatment. D: Conjugate **5** (**5a** deprotected).

LPS-neutralizing activity

The LPS-neutralizing activity of the PEGylated dendritic constructs **5** and **6** was assayed using the chromogenic *Limulus* amebocyte lysate assay (LAL).[38] The activity of these compounds was compared to the unconjugated **3** and **4** to study the effect of the conjugation. Finally, the dendritic platforms **1** and **2** were also included to determine whether these structures themselves displayed anti-LPS activity. All compounds were tested at 100 μM and the assay was performed as described in the Experimental Section. The anti-LPS peptide polymyxin B (PMB)[39] was used as a positive control in this assay (Figure 3).

Dendritic structures **1** and **2** did not display LPS-binding activity. These results correlate well with other studies that describe how, although necessary, cationicity is not enough for effective LPS binding and neutralization.[35] Moreover, the incapacity of these dendritic platforms to neutralize LPS is relevant, since it ensures that such platforms will not interfere with the biological activity of bioactive conjugated molecules. At the concentration assayed, **3** showed high LPS-neutralizing activity, which is consistent with published data.[20] In contrast, the monomeric compound **4** was inactive, thereby suggesting that two positive residues are required to interact with lipid A, regardless of amphipathicity. Finally, conjugates **5** and **6** showed interesting behaviors. Construct **5** retained the neutralizing activity of the inhibitor **3** and totally neutralized LPS at 100 μM. In contrast, compound **6** had a poor activity, even though it exposes three copies of the acyl-Arg monomer to LPS. Then, as previously noticed with other class of LPS-binders, more than the number of positive charges is their geometrical distribution what is crucial to appropriate LPS neutralization.[35,40] To further analyze the effect of conjugation and multivalency, compounds **3** and **5** were subjected to serial dilutions and their IC_{50} values (i. e. the concentration required to neutralize 50% of LPS *in vitro*) were calculated (Table 1).

The experimental IC_{50} values obtained revealed that the conjugation of **3** to the PEGylated dendritic structure involved a 2-fold enhancement in its LPS-neutralizing activity. Such improvement correlates with the presence of three equal copies of the bioactive compound **3** in construct **5**, suggesting an almost full exposure of **3** in the dendritic construct (trivalency).

The dendritic structures here proposed are cationic in nature. It is known that some amphipathic cationic compounds could present basal toxicity associated to cell membrane activity.[41,42] Thus, we wanted to examine the toxicity, if any, of these conjugates in cells. In order to chose an appropriate cellular model we selected RAW 264.7 murine macrophages. These cells are a powerful model to study LPS-induced cell signaling as well as the efficacy of LPS-inhibitors, since they are critical members of the innate immune system and play a major role in the pathogenesis of sepsis.[21] In fact, the inhibition of inflammatory mediators such as TNF-α from LPS-challenged macrophages[43] is usually measured to determine the efficacy of LPS-neutralizers.[34,44] However, cationic dendrimers have recently been described to induce apoptosis in this cell model.[45] Thus, in a first attempt to determine whether these dendritic platforms are viable agents for future *in vitro* and *in vivo* studies, the cytotoxicity of compounds **3** and **5** was evaluated using MTT assays. These compounds were tested at a concentration close to their IC_{50} values, 10 μM. At this concentration, both compounds were non-toxic (Table 1). It has already been reported that **3** is tolerable for these cells at 10 μM, although it becomes moderately toxic at higher concentrations.[20] Interestingly, **5** was devoid of any toxic effect even though it contains three copies of **3**. These findings are consistent and support the notion that both the PEGylation and the conjugation of molecules to cationic dendrimers is a viable strategy to reduce or even remove their intrinsic toxicity while maintaining the bioactive properties of the drug.[46]

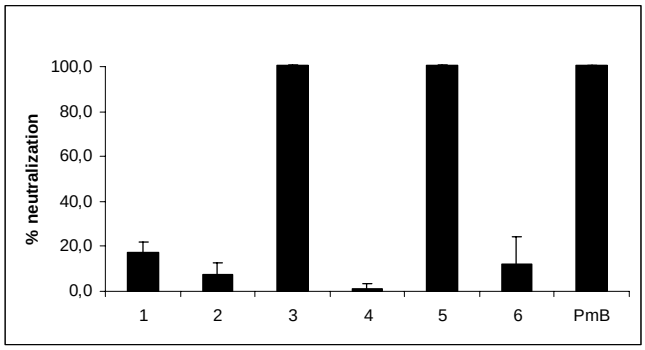


Fig. 3 The inhibitory activity of the compounds was determined using the chromogenic LAL assay. PMB was included as a positive control. Compounds were tested at a 100 μM concentration in the presence of 100 pg/mL of LPS. The LPS-binding assay was performed in three independent assays as described in the Experimental Section. Data are represented with ±SD.

Table 1 LPS-neutralization activity (IC_{50}) and cell viability of compounds **3** and **5**

Compound	IC_{50} (μM)[a]	% of Cell viability[b]
3	18 ± 1	109 ± 18
5	9 ± 1	110 ± 14

[a] The inhibition of the compounds was determined using the chromogenic LAL assay. The inhibitory activity is represented as IC_{50}. Standard deviations (SD) are also included. The assay was performed as described in the Experimental section.

[b] Cell viability was evaluated in RAW 264.7 cells, after 24 h of incubation in the presence of 10 μM concentration of compounds by MTT assay. The assay is described in the Experimental Section.

Conclusions

Here we have presented the synthesis of novel dendritic tri- and pentavalent PEGylated structures. These platforms were obtained by convergent synthesis from the condensation of a commercial propylene glycol (TOTA) and **NTA** and **DTPA** as multifunctional cores. The utility of these PEGylated platforms for the conjugation of bioactive compounds was assayed with a previously described LPS-neutralizer, compound **3**. As a proof of concept, compound **3** was conjugated to the trivalent dendritic structure **2** to render the construct **5**. The conjugated compound displayed improved

LPS-neutralizing activity and a reduced toxicity profile over the parent compound, thus proving the efficacy of these platforms for biological applications.

Experimental

Abbreviations

ACH: α-cyano-4-hydroxycinnamic acid; Boc-TOTA: *N-tert*-butiloxycarbonyl-4,7,10-trioxa-1,13-tridecanediamine; CTC: 2-chlorotrityl chloride, DCM: dichloromethane; DIPCDI (DIC): *N,N'*-diisopropylcarbodiimide; DIEA: *N,N*-diisopropylethylamine; DMF: *N,N'*-dimethylformamide; DTPA: dietilene-triaminepentaacetic acid; ESI-MS: electrospray ionization mass spectrometry; Fmoc: 9-fluorenylmethoxycarbonyl; FA: Formic acid; HATU: 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo-[4,5-b]pyridinium hexafluorophosphate 3-oxide; HPLC: high performance liquid chromatography; HOAt: 1-hydroxy-7-azabenzotriazole; HOBt: 1-hydroxybenzotriazole; LC: liquid chromatography; MALDI: Matrix-assisted laser desorption/ionization; MeCN: acetonitrile; NTA: nitriloacetic acid; Pbf: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl group; PyBOP, benzotriazol-1-yloxy-tris(pyrrolidino)phosphonium hexafluorophosphate; RP: reverse-phase; SA: sinapinic acid; TBME: *tert*-butyl methyl ether; TEA: triethylamine; TFA: trifluoroacetic acid; TIS: triisopropylsilane.

General

Resins, amino acids, coupling reagents, solvents and other reagents were purchased from commercial suppliers at the highest purity available and were used without further purification. Analytical HPLC was performed using a Waters Alliance 2695 (Waters, MA, USA) chromatography system with a solvent degasser, quaternary pump, autosampler, column compartment and a PDA 995 detector. Semi-preparative HPLC was carried out on a Waters chromatography system with a dual absorbance detector 2487 and HPLC-MS was performed using a Waters Alliance 2796 with a dual absorbance detector 2487 and ESI-MS Micromass ZQ (Waters) chromatography system. Reverse-phase columns were used for all HPLC systems (see Chromatography Conditions above for details). Flash chromatography with cartridges was carried out with a Combi Flash system from Isco Teledyne. Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (Applied Biosystems, Foster City, CA, USA) using ACH or SA matrices. ^{1}H NMR spectra were recorded using a Varian Mercury 400 apparatus (400 MHz, DO_2).

Chromatography Conditions

Analytical RP-HPLC was carried out either on a Symmetry C_4 column (3.5 x 250 mm, 5µm, linear gradients of MeCN with 0.1% TFA into H_2O with 0.1% TFA) or a Symmetry C_{18} column (4.6 x 150 mm, 5µm, linear gradients of MeCN with 0.036% TFA into H_2O with 0.045% TFA). The system was run at a flow rate of 1.0 mL/min over varying times. Several linear gradients are defined above (conditions A-D). Semi-preparative RP-HPLC was performed on a Symmetry C_{18} column (30 x 150 mm, 5µm, linear gradients of MeCN with 0.05% TFA into H_2O with 0.1% TFA). The system was run at a flow rate of 20 mL/min over 30 min (conditions E-F). Alternatively, RP-LC was performed on a flash chromatography system with a RediSep C_{18} cartridge (4g) and linear gradients of MeCN with 0.05% TFA into H_2O with 0.1% TFA). The system was run at a flow rate of 20 mL/min over 20 min (condition G). HPLC-MS was performed on a reversed-phase Symmetry 300 C_{18} column (3.9 x 150 mm, 5µm column) and H_2O with 0.1% formic acid and MeCN with 0.07% formic acid as mobile phases. The system was run at a flow rate of 1.0 mL/min over 15 min (condition H). The columns and conditions used are summarized in Table 2.

Table 2. Chromatography Conditions

Condition	Column	Gradient (% MeCN)	Time (min)
A	C4 (3.5 x 250 mm)	0-65	30
B	C4 (3.5 x 250 mm)	0-100	30
C	C18 (4.6 x 150 mm)	0-100	15
D	C18 (4.6 x 150 mm)	50-100	15
E	C18 (30 x 150 mm)	0-100	30
F	C18 (30 x 150 mm)	50-100	30
G	C18 (RediSep column 4g)	0-100	20
H	C18 (3.9 x 150 mm)	50-100	15

Solid-phase synthesis

Compounds **3a**-**3** and **4a**-**4** were synthesized manually using the Fmoc strategy in polypropylene syringes fitted with polyethylene porous disks. Solvents and soluble reagents were removed by suction. Washings between deprotection, couplings and subsequent deprotection steps were carried out with DMF and DCM using 10 mL of solvent/g of resin each time. When syntheses were performed on 2-chlorotrityl chloride resin (CTC), DCM used was treated with alumina. The Fmoc group was removed by treatment with piperidine–DMF (1:4, v/v). Couplings and washes were performed at 25 ºC. Couplings were monitored using standard colorimetric tests for solid-phase synthesis.[47]

Synthesis of *(Boc-TOTA)$_5$DTPA* (1a)

DTPA (25.0 mg, 0,063 mmol; 1 equiv), DIC (49.5 µL, 0.32 mmol; 5 equiv) and HOBt (47.7 mg, 0.32 mmol; 5 eq) were dissolved in 10 ml of DCM–DMF (7:3) and stirred for 15 min. Next, Boc-TOTA (124.90 mg, 0.39 mmol; 6 equiv) and TEA (88.0 µL, 0.63 mmol; 10 equiv) were dissolved in 10 ml of DCM–DMF (7:3) and added to the preactivated mixture. The mixture was allowed to react under N_2 with stirring at room temperature for 48 h and the reaction was checked by RP-HPLC (condition A). Finally, the reaction mixture was dried under vacuum and the residue was dissolved in DCM and washed with saturated $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$ and evaporated to dryness to obtain 1a as yellow oil (110.44 mg, 92% yield, 54% purity). Protected dendrimer **1a** was purified by means of an Isco system with a RP C18 column (condition G). From the fractions collected,

we obtained **1a** with an 85% yield over the amount loaded into the column. **1a** HPLC (t_R = 26.8 min, purity 77%, condition A), MALDI-TOF (ACH matrix + FA 0.1% in H_2O–ACN (1:1); *m/z* calcd. for $C_{89}H_{173}N_{13}O_{30}$ 1905.4, found 1906.2 $[M+H]^+$), RMN 1H (400 MHz, D_2O): δ ppm 1.38 (s, 45H), 1.64-1.79 (m, 20H), 2.48-2.65 (m, 10H), 3.03 (bs, 2H), 3.13-3.19 (m, 8H), 3.28 (dd, J= 6.58, 12.5 Hz, 10H), 3.40 (s, 8H), 3.47 (t, J = 6.00, 6.00 Hz, 20H), 3.51-3.61(m, 40H), 5.09 (bs, 5H), 7.68 (bs, 5H).

Synthesis of *(TOTA)$_5$DTPA* (1)

After purification, dendrimer **1a** was treated with 15 mL of TFA–DCM (1:1) for 30 min. TFA was then removed by evaporation to dryness in order to obtain **2** (30.7 mg, 77% yield). **1** HPLC (t_R = 12.9 min, purity 94%, condition A), MALDI-TOF (ACH matrix + FA 0.1% in H_2O–ACN 1:1; *m/z* calcd. for $C_{64}H_{133}N_{13}O_{20}$ 1403.98, found 1405.0 $[M+H]^+$.

Synthesis of *(Boc-TOTA)$_3$NTA* (2a)

NTA (12.0 mg, 0,063 mmol; 1 equiv), DIC (29.4 µL, 0.19 mmol; 3 equiv) and HOBt (28.3 mg, 0.19 mmol; 3 equiv) were dissolved in 10 ml of DCM–DMF (7:3) and stirred for 15 min. Boc-TOTA (73.6 mg, 0.23 mmol; 3.6 equiv) and TEA (52.74 µL, 0.38 mmol; 6 equiv) were then dissolved in 10 ml of DCM–DMF (7:3), and added to the preactivated nitrilotriacetic acid. Amide formation was then carried out under the conditions explained above and checked by RP-HPLC (condition A). After completion of the reaction, the mixture was dried under vacuum and the residue was dissolved in DCM and washed with saturated $NaHCO_3$ and brine. The organic layer was dried over MgSO4 and evaporated to dryness to obtain **2a** as a yellow oil (76.2 mg, 95% yield, 67% purity). Protected dendrimer **2a** was purified by means of a flash chromatography system with a RP C18 column (condition G). From the fractions collected, we obtained **2a** with an 83% yield over the amount loaded into the column. 2a HPLC (t_R = 23.1 min, purity 97%, condition A), MALDI-TOF (ACH matrix + FA 0.1% in H_2O–ACN 1:1; m/z calcd. for $C_{51}H_{99}N_7O_{18}$ 1097.74, found 1098.6 [M+H]+), RMN 1H (400 MHz, D_2O): δ ppm 1.43 (s, 27H), 1.71-1.84 (m, 12H), 3.21 (m, 6H), 3.26 (bs, 6H), 3.36 (dd, J= 6.8, 12.4 Hz, 6H), 3.53 (t, J = 6.02, 6.02 Hz, 12H), 3.56-3.66 (m, 24H), 5.12 (bs, 3H), 7.73 (bs, 3H).

Synthesis of *(TOTA)$_3$NTA* (2)

After purification, dendrimer **2a** was treated with 15 mL of TFA–DCM (1:1) for 30 min. Next, TFA was removed by evaporation to dryness in order to obtain **2** (41.1 mg, 96% yield). **1** HPLC (t_R = 8.1 min, purity 88%, condition A), MALDI-TOF (ACH matrix + FA 0.1% in H_2O-ACN (1:1); *m/z* calcd. for $C_{36}H_{75}N_7O_{12}$ 797.55, found 798.6 $[M+H]^+$).

Synthesis of 3a

Fmoc-6-aminohexanoic acid (1 equiv) and DIEA (10 equiv) were sequentially added to CTC resin (300 mg, 1.00 mmol/g) and the resin was stirred for 1 h. The incorporation was followed by a 10-min capping step with MeOH (240 µL). After Fmoc removal, Fmoc-Arg(Pbf)-OH (4 equiv) was inserted with 2 x 45-min consecutive treatments with HATU (4 equiv) in DMF as coupling reagent in the presence of DIEA (8 equiv) to the resin. Next, the Fmoc group was removed and Fmoc-protected 1,4-Bis(3-aminopropoxy)butane succinimic acid (4 equiv) was coupled overnight using HOAt (4 equiv), PyBOP (4 equiv) and DIEA (12 equiv) as coupling system. The following step involved the removal of the Fmoc group and a second coupling of Fmoc-Arg(Pbf)-OH using the conditions explained above. Finally, palmitic acid (5 equiv) was inserted overnight with HOAt (5 equiv), PyBOP (5 equiv) and DIEA (15 equiv). The compound was then cleaved using TFA–DCM (1:99) (5 x 0.5 min). The solvents were evaporated, the compound was dissolved in H_2O–MeCN (1:1) and then lyophilized to afford **3a** (139.1 mg, 32% yield). The crude product was purified by semi-preparative HPLC (condition F) to yield **3a** with optimal purity. **3a** HPLC (t_R = 14.9 min, purity 97%, condition D), ESI-MS (m/z calcd. for $C_{74}H_{125}N_{11}O_{15}S_2$ 1471.9, found 738 [M+H]+/2).

Synthesis of 3

The synthesis of **3** has been described elsewhere.[20] Briefly, Fmoc-6-aminohexanoic acid (4 equiv) was coupled to Rink Amide MBHA resin with DIC (4 equiv) and HOAt (4 equiv) in DMF for 6 h. Subsequent steps in solid-phase were carried out as explained for the synthesis of **3a**. Finally, the cleavage of the compound from the solid support and concomitant deprotection of side-chain groups was done with TFA–H_2O–TIS (95:2.5:2.5) for 2 h. TFA was then removed by evaporation with nitrogen, the compound was precipitated with cold anhydrous TBME, dissolved in H_2O–MeCN (1:1) and then lyophilized. The crude compound was purified by semi-preparative HPLC (condition E) to yield the desired **3** with excellent purity. **3** HPLC (t_R = 10.4 min, purity 98%, condition C), MALDI-TOF (*m/z* calcd. for $C_{48}H_{94}N_{12}O_8$ 966.73, found 967.81 $[M+H]^+$).

Synthesis of 4a

Fmoc-Arg(Pbf)-OH (1 equiv) in DCM and DIEA (10 equiv) were sequentially added to CTC resin (218 mg, 1.00 mmol/g) and the resin was stirred for 1 h. Next, a 10 min treatment with MeOH (175 µL) was carried out to cap the resin. Then, the Fmoc group was removed and palmitic acid (5 equiv) was introduced overnight with HOAt (5 equiv), PyBOP (5 equiv) and DIEA (15 equiv) as coupling reagents. Mild acidic treatments with TFA–DCM (1:99) (5 x 0.5 min) afforded the cleavage of the compound from the resin. The solvents were evaporated, the compound dissolved in H_2O–MeCN (1:1) and then lyophilized to obtain **4a** (102.7 mg, 71% yield) which was used without purification. **4a** HPLC (t_R = 16.8 min, purity 95%, condition D).

Synthesis of 4

Compound **4a** was treated with TFA–H_2O–TIS (95:2.5:2.5) for 1 h. TFA was then removed by evaporation and the product was dissolved in MeOH and precipitated with cold TBME. It was then dissolved in H_2O–MeCN (1:1) and then lyophilized to obtain 4.2 mg of **4** with 86% of yield. **4** HPLC (t_R = 19.5 min, purity 89%, condition B), MALDI-TOF (*m/z* calcd. for $C_{22}H_{44}N_4O_3$ 412.34, found 413.01 $[M+H]^+$).

Synthesis of compound *(3a-TOTA)$_3$NTA* (5a)

A solution of compound **3a** (20.0 mg, 0.0136 mmol; 1 equiv) in 10 ml of DCM–DMF (7:3) was preactived for 15 min by addition of PyBOP (7.1 mg, 0.0136 mmol; 1 equiv) and HOBt (2.0 mg, 0.0136; 1 equiv). After the preactivation, deprotected compound **2** (3.30 mg, 0.0041; 0.3 equiv) and DIEA (4.6 µL, 0.0272 mmol; 2 equiv) dissolved in 10 ml of DCM–DMF (7:3) were added. The mixture was allowed to react under N_2 with stirring at room temperature for 72 h and it was checked by RP-HPLC (condition B). When the reaction was completed, the mixture was dried under vacuum and the residue was dissolved in DCM and washed with saturated $NaHCO_3$, aqueous 10% HCl and brine. The organic layer was dried over $MgSO_4$ and evaporated to dryness. The crude product was dissolved in MeOH and **5a** was obtained by precipitation with TBME (18.8 mg, 70% yield). **5a** HPLC (t_R = 26.3 min, purity 80%, condition B), MALDI-TOF (*m/z* calcd. for $C_{258}H_{444}N_{40}O_{54}S_6$ 5162.91, found 5180.02 $[M+18]^+$).

Synthesis of compound *(3-TOTA)$_3$NTA* (5)

5a (18.8 mg, 0.005 mmol) was then treated with TFA–H_2O–TIS (95:2.5:2.5) for 3 h. After this time TFA was evaporated with nitrogen and the peptide was precipitated with cold anhydrous TBME. After centrifugation-decantation, the compound was dissolved in H_2O–MeCN (1:1) and lyophilized to afford **5** as a white solid (6.1 mg, 86% yield). **5** HPLC (t_R = 17.2 min, purity 83%, condition B), MALDI-TOF (*m/z* calcd. for $C_{180}H_{348}N_{40}O_{36}$ 3648.94, found 3649.93 $[M+H]^+$).

Synthesis of *(4a-TOTA)$_3$NTA* (6a)

A mixture of compound **4a** (20.0 mg, 0.030 mmol; 1 equiv), PyBOP (15.7 mg, 0.030 mmol; 1 equiv) and HOBt (4.5 mg, 0.030 mmol; 1 equiv) was dissolved in 10 mL of DCM–DMF (7:3) and stirred for 15 min. Compound **2** (7.26 mg, 0.0091 mmol; 0.3 equiv) and DIEA (10.2 µL, 0.060 mmol; 2 equiv) were dissolved in 10 mL of DCM–DMF (7:3) and after stirring 10 min this mixture was added to the preactivated compound **4a** and was allowed to react under N_2 with stirring at room temperature for 72 h. The reaction was checked by RP-HPLC (condition B). After this time, the mixture was dried under vacuum and the residue was dissolved in DCM and washed with saturated $NaHCO_3$sat, aqueous 10% HCl and brine. The organic layer was dried over $MgSO_4$ and evaporated to dryness to obtain **6a**. This compound was dissolved in MeOH and precipitated by the slow addition of MeCN (20.2 mg, 81% yield). **6a** HPLC (t_R = 26.6 min, purity 84%, condition B), MALDI-TOF (*m/z* calcd. for $C_{141}H_{249}N_{19}O_{27}S_3$ 2738.79, found 2762.01 $[M+Na]^+$).

Synthesis of *(4-TOTA)$_3$NTA* (6)

The protected precursor (20.2 mg, 0.0073 mmol) was finally treated with TFA, as explained for compound **5**. Work-up yielded **6** as a white solid (8.6 mg, 88% yield). **6** HPLC (t_R = 14.9 min, purity 92%, condition B), MALDI-TOF (*m/z* calcd. for $C_{102}H_{201}N_{19}O_{18}$ 1981.8, found 1981.78 $[M+H]^+$).

LPS-neutralizing activity

All solutions used in the LPS-neutralizing activity assay were tested to ensure they were endotoxin-free and the material was sterilized by heating 3 h at 180 ºC. LPS from *E. Coli* 055:B5 and Polymyxin B were purchased from Sigma. LPS-neutralizing activity was measured using the chromogenic *Limulus* Amebocyte Lysate (LAL) test, following the manufacturer's instructions (Cambrex). LAL reagent contains a clottable protein that is activated in the presence of non-neutralized LPS and it is an extremely sensitive indicator of the presence of endotoxin. When activated, this enzyme catalyses the release of p-nitroaniline (pNA) from the colorless chromogenic substrate Ac-Ile-Glu-Ala-Arg-pNA. The pNA released can be measured photometrically at 405 nm. Compounds were initially incubated at 100 µM with LPS (100 pg/mL) in a 96-well microtiter for 60 min at 37 ºC. Polymyxin B (10 µg/mL) was used as positive control. LAL (12.5 µL) was added to start the reaction at 37 ºC. After 10 min, non-neutralized LPS was detected after a 5-8 min incubation with the chromogenic substrate (25 µL). Acetic acid (25 % v/v final concentration) was added to stop the reaction and the absorbance was monitored at 405 nm in a Multiskan Ascent microtiter plate reader (ThermoLabsystems). At this concentration, compounds that showed a LPS-neutralization above 75 % were tested to determine their IC_{50} (the concentration required to neutralize 50% of LPS *in vitro*). IC_{50} values were determined by a serial dilution assay using 100 pg/mL of LPS and a range of compound concentrations (50 to 0.001 µM). All assays were run in triplicate, and the curves were automatically adjusted by non-linear regression using "Prism 4" (GraphPad) software.

Cell culture

Mouse macrophages (RAW 264.7) were obtained from ATCC (American Type Culture Collection, USA). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco BRI) supplemented with 10% fetal bovine serum (FBS - Gibco BRI) and 1% L-glutamine. The cultures were incubated at 37ºC in a humidified atmosphere of 5% CO_2-95% air. Subcultures of macrophages were prepared every 2-3 days by scraping cells into fresh medium.

MTT cell viability assays

Cell viability was evaluated by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. RAW 264.7 cells were seeded in sterile 96-well microtiter plates at a seeding density of 6 x 10^4 cells/mL in DMEM supplemented with 10% FBS and allowed to settle for 24 h. Compounds were added at a 10 µM concentration to the plates and the cells were further incubated for 24 h. After removal of the medium, the precipitated formazan crystals were dissolved in optical grade DMSO (100 µL), and the plates were read at 570 nm using a Wallac 1420 Workstation.

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Supplementary Material (ESI) for Organic & Biomolecular Chemistry

Conjugation of bacterial endotoxin neutralizers to dendritic PEGylated platforms: synthesis, biological activity and toxicity

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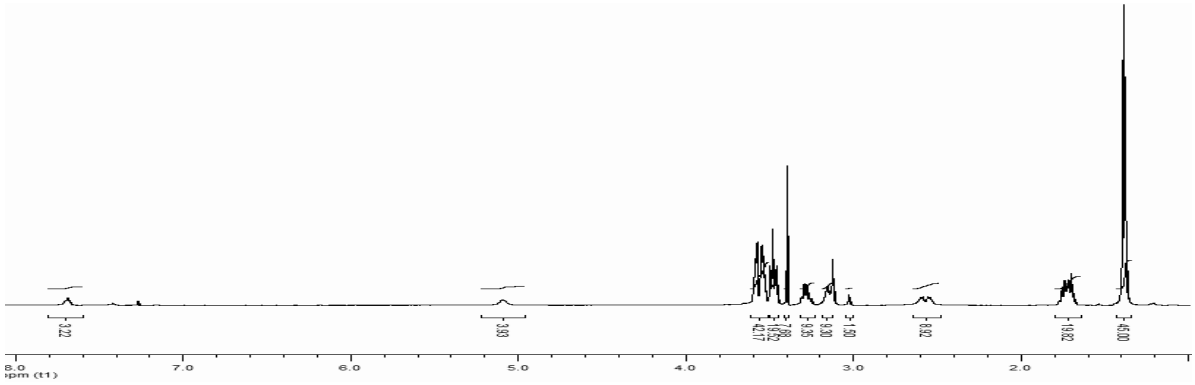
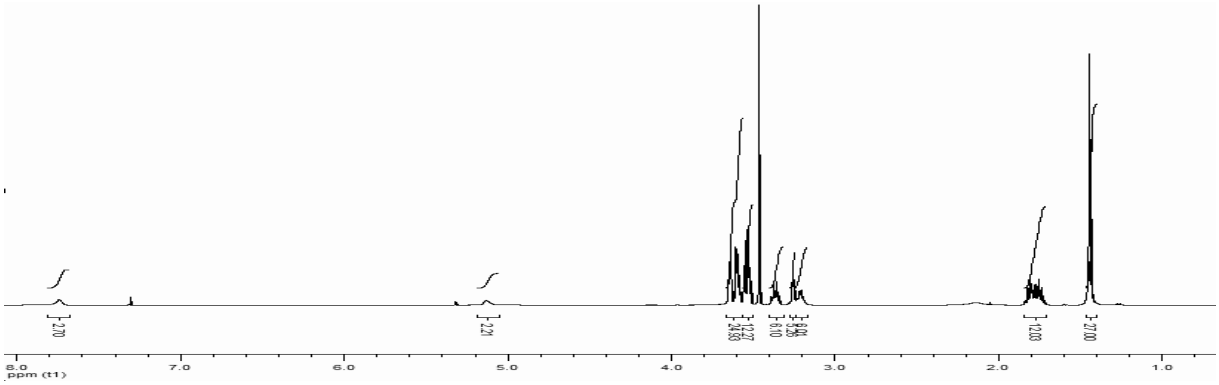
Electronic Supplementary Information (ESI)

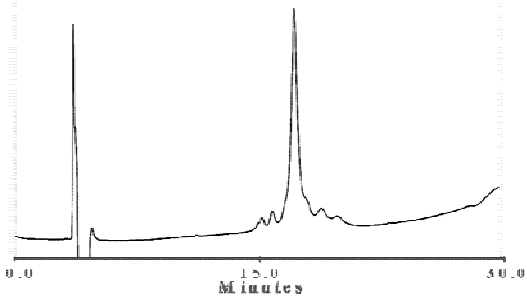
***(Boc-TOTA)$_5$DTPA* (1a)**

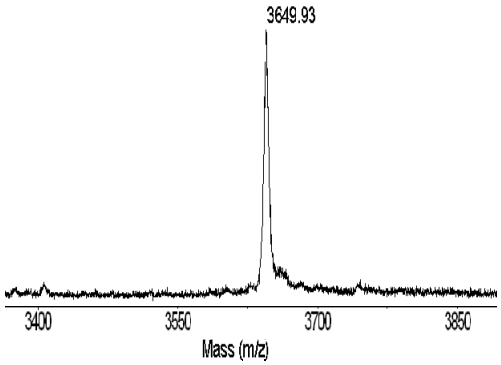


(Boc-TOTA)$_3$NTA (2a)



Compound (3-TOTA)$_3$NTA (5)





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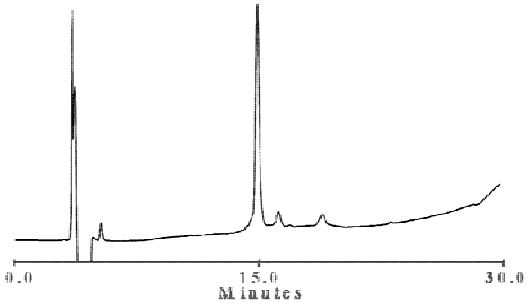

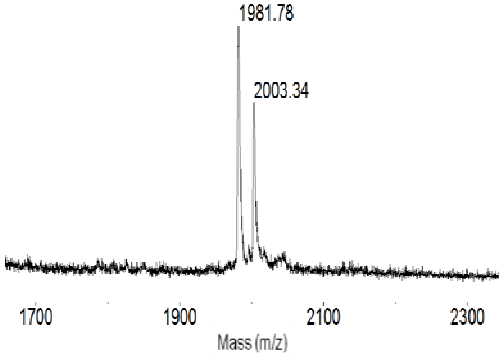

"Tiratricol neutralizes bacterial endotoxins and reduces lipopolysaccharide-induced TNF-α production in the cell"

Laura Cascales, Carlos Mas-Moruno, Silvia Tamborero, José Luis Aceña, Juan F. Sanz-Cervera, Santos Fustero, Luis J. Cruz, Puig Mora, Fernando Albericio, Enrique Pérez-Payá

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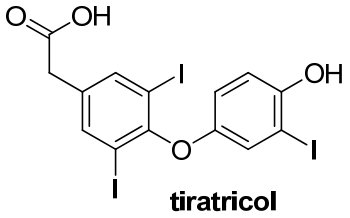
RESUM

En aquest treball s'ha explorat una nova estratègia per al descobriment de nous fàrmacs: el "screening" d'una quimioteca comercial de 880 compostos amb gran diversitat estructural i activitat farmacològica coneguda, la *Prestwick Chemical Library*. Aquests tipus de quimioteques són de gran utilitat per al descobriment de nous fàrmacs ja que els compostos que contenen tenen una bona disponibilitat oral i no presenten toxicitat. L'anàlisi d'aquesta quimioteca va permetre identificar una nova molècula amb capacitat neutralitzant del LPS, el tiratricol. Aquesta molècula no presenta toxicitat, ni activitat antibacteriana, i és capaç d'inhibir la producció de TNF-α induïda pel LPS en macròfags. En aquesta publicació també es presenta el disseny i la síntesi de diversos anàlegs del tiratricol. Aquests anàlegs van permetre estudiar relacions d'estructura-activitat i obtenir compostos més actius.



tiratricol

Contribucions a aquest treball:

- La Laura Cascales i la Silvia Tamborero van realitzar el "screening" de la quimioteca. La Laura Cascales, a més, va realitzar l'estudi d'inhibició del TNF-α en macròfags i la major part d'assaigs biològics.

- En Carlos Mas va realitzar la síntesi dels anàlegs del tiratricol fets en fase sòlida. També va col·laborar en els assaigs d'activitat biològica dels anàlegs i va participar activament en el procés d'elaboració del manuscrit.

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Tiratricol Neutralizes Bacterial Endotoxins and Reduces Lipopolysaccharide-Induced TNF-α Production in the Cell

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The screening of a commercially available library of compounds has proved a successful strategy for the identification of a lead compound in a drug discovery programme. Here, we analysed 880 off-patent drugs, which initially comprised the Prestwick Chemical library, as sources of bacterial endotoxin neutralizers. We identified 3,3′,5-triiodo-thyroacetic acid (tiratricol) as a non-antibacterial compound that neutralizes the toxic lipopolysaccharide.

Key words: endotoxin, lipid A, lipopolysaccharide, lipopolysaccharide-antagonists, sepsis, septic shock, tumour necrosis factor-α

Abbreviations: CMC, critical micellar concentration; CTC, 2-chlorotrityl chloride resin; DMEM, Dulbecco's Modified Eagle's Medium; DPH, 1,3-diphenyl-1,3,5-hexatriene; FBS, fetal bovine serum; LBP, lipopolysaccharide-binding protein; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PMB, polymyxin B; TNF-α, tumour necrosis factor-α.

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Sepsis is a systemic inflammatory response to infection caused by a bacterial endotoxin and can lead to multiple organ failure known as 'septic shock'. Gram-negative sepsis is the 10th leading cause of overall mortality and the main cause of death in intensive care units (1). Sepsis and related disorders are caused by lipopolysaccharide (LPS) present in the outer leaflet of Gram-negative bacteria. The basic LPS molecular structure consists of two distinct regions: the hydrophilic carbohydrate portion and outer core region, and the hydrophobic toxic lipid A component. The latter is highly conserved among Gram-negative bacteria and contributes to the toxicity of LPS (2).

Research efforts have addressed the characterization of all components involved in cascade recognition events and the full elucidation of the LPS-signalling pathway, to define pharmacological targets (2). After the lysis of bacteria, LPS is released from the leaflet; the circulating LPS-binding protein (LBP) binds to LPS, and transfers it to inositol phosphate anchored protein (CD14) receptor (3). The complex LPB/LPS/CD14 then activates Toll-like receptors (TLR4), which participate in the transduction of the LPS signal to the cell nucleus, thereby initiating the transcription of cytokine genes (4,5). Continuous presence of LPS in mammalian bloodstream induces a deregulation of the expression of inflammatory cytokines thereby leading to the pathological condition that provokes sepsis or septic shock (6). However, despite the incidence of sepsis and the efforts made towards the identification of molecules that could ameliorate LPS-induced diseases, currently, only drotrecogin alfa (Xigris®) (Eli Lilly and Company, Indianapolis, IN, USA) has been approved as a treatment. This drug reduced mortality in a large phase III study, although the results of this and other studies raised questions about the use of this pharmacological agent (7).

Hits/leads as LPS-neutralizers in basic research programmes should: (i) bind to and neutralize LPS, (ii) show no or weak antibacterial activity (because we consider that current antibacterial treatments are effective and interference with them could have a negative outcome), (iii) increase the critical micellar concentration of LPS, to enhance the natural lipoprotein-dependent LPS clearance mechanism. Lipopolysaccharide is released from bacterial cell wall in an inactive micellar form that dissociates to the active monomeric form at slow rate (8). It has been proposed that the dissociation is catalysed by LBP (3,4,9,10). Then, the complex LPS(monomer)–LBP could define an alternative detoxification mechanism that involves the participation of additional lipid-binding proteins (11).

In a previous study, we have successfully identified a family of LPS-neutralizing *N*-alkylglycine trimers able to neutralize the LPS toxic activity (12). Now, we are improving the pharmacological features of this class of compounds (to be published elsewhere) mainly through the synthesis of nano-sized hybrid constructs that covalently combine the bioactive agent with a polymer (13). Alternatively, the

screening of compound collections with known pharmacology properties (14) may reduce the distance between basic research and clinical applications. In this regard, here we screened the Prestwick Chemical Library® as a source of LPS-neutralizing molecules and characterized initial hits under the premises described above. The initial library contained 880 biologically active compounds with high chemical and pharmacological diversity as well as known bioavailability and safety in humans. We identified 3,3′,5-triiodo-thyroacetic acid (tiratricol) as a non-antibacterial compound that neutralizes LPS activity. Here, we also demonstrate that tiratricol induces a shift in the micellar to monomeric form equilibrium of LPS and decreases TNF production in macrophages challenged with LPS.

Methods and Materials

General

The resins, coupling reagents, solvents and other reagents were of the highest purity available and were purchased from commercial suppliers and used without further purification. Analytical HPLC was performed using a Waters Alliance 2695 (Waters Milford, MA, USA) chromatography system with a PDA 995 detector, a reversed-phase Symmetry C_{18} column (4.6 × 150 mm, 5 μm) and linear gradients of MeCN with 0.036% TFA into H_2O with 0.045% TFA. The system was run at a flow rate of 1.0 mL/min over 15 min. Semi-preparative HPLC was performed on a Waters chromatography system with a dual absorbance detector 2487, reverse-phase Symmetry C_{18} columns (7.8 × 100 mm, 5.0 μm or 30 × 150 mm, 5 μm) and linear gradients of MeCN with 0.05% TFA into H_2O with 0.1% TFA. The system was run at a flow rate of either 3.0 or 20.0 mL/min over 30 min depending on the column used. HPLC-MS was performed using a Waters Alliance 2796 with a dual absorbance detector 2487 and ESI-MS Micromass ZQ (Waters) chromatography system, a reversed-phase Symmetry 300 C_{18} (3.9 × 150 mm, 5-μm column) and H_2O with 0.1% formic acid and MeCN with 0.07% formic acid as mobile phases. Flash column chromatography was performed with the indicated solvents on silica gel 60 (particle size 0.040–0.063 mm). Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (Applied Biosystems, Foster City, CA, USA) using ACH matrix. High-resolution mass spectra were obtained by the Mass Spectrometry Service at the *Universidad de Valencia*. The ^1H NMR spectra of compounds were recorded using a Varian Mercury 400 apparatus (Varian, Palo Alto, CA, USA) (400 MHz, CD_3OD) or a Bruker AC300 apparatus (300 MHz) (Bruker Biospin, Rheinstetten, Germany). Chemical shifts (δ) are expressed in parts per million downfield from tetramethylsilyl chloride. Coupling constants are expressed in Hertz. The letters m, s, d and t stand for multiplet, singlet, doublet and triplet, respectively. The letters br indicate that the signal is broad.

LPS assay materials

All solutions used in the LPS-neutralizing activity assay were endotoxin-free and material was sterilized by heating for 3 h at 180 °C. Endotoxin-free water and LPS from *Escherichia Coli* 0111:B4 were from BioWhittaker (Malkersville, MD, USA); LPS from *E. Coli* 055:B5 and polymyxin B (PMB) were from Sigma (St Louis, MO, USA). The Prestwick Chemical Library® and also replacements for biological assays were purchased from Prestwick Chemical Inc. (Illkirch, France).

In vitro LPS-neutralizing activity was measured using the chromogenic *Limulus* Amebocyte Lysate (LAL) test, following the manufacturer's instructions (BioWhittaker). *Limulus* Amebocyte Lysate reagent contains a clottable protein that becomes activated in the presence of non-neutralized LPS, then the enzyme catalyses the splitting of *p*-nitroaniline (*p*NA) from the colourless chromogenic substrate Ac-Ile-Glu-Ala-Arg-*p*NA. The *p*NA released was measured photometrically at 405 nm. The library compounds (tested at a final concentration of 100 μM – in PBS containing 1.25% DMSO) were incubated with LPS (100 pg/mL) or lipid A (100 pg/mL) in a 96-well microtitre plate for 45 min at 37 °C. PMB (50 μg/mL) was used as positive control (12). *Limulus* Amebocyte Lysate (50 μL) was added to start the reaction at 37 °C. After 16 min, non-neutralized LPS was detected by a 10-min incubation with the chromogenic substrate (100 μL). Acetic acid (25% v/v final concentration) was added to stop the reaction and the absorbance was monitored at 405 nm in a Rosys Anthos 2010 microtitre plate reader (Tecnomara AG) (Zurich, Switzerland). The intrinsic absorbance at 405 nm of the library compounds, if any, was subtracted using the appropriate controls in the plate. IC_{50} values (the concentration necessary to *in vitro* neutralize 50% of LPS) were determined by a serial dilution assay using 100 pg/mL of LPS and a range of compound concentrations (50–0.001 μM]. All assays were run in triplicate, and the curves were automatically adjusted by non-linear regression using 'Prism 4' (GraphPad) software (GraphPad Software, Inc, La Jolla, CA, USA).

Fluorescence spectroscopy studies

The critical micellar concentration (CMC) of lipid A was determined in the presence and absence of LPS-neutralizing compounds by measuring the fluorescence emission spectra (Perkin-Elmer LS-50 spectrofluorimeter) (Norwalk, CT, USA) of the fluorescent probe 1,3-diphenyl-1,3,5-hexatriene (DPH) (Sigma) (15). To determine the CMC of lipid A, the fluoresescent probe DPH at 5 μM was excited at 340 nm and the emission spectra was recorded between 400 and 500 nm. Increasing concentrations from 0 to 20 μM of lipid A were used. At the CMC, the lipid A forms micelles and the fluorescent probe is hidden inside the micelles leading an increase in the fluorescence intensity. The test compounds were added to the cuvette at 10 μm together with DPH whilst increasing the concentration of lipid A. The results are expressed as relative intensity of fluorescence.

Antibacterial activity

Antibacterial activity was assayed in a 96-well format in a growth-inhibition assay against *E. coli* DH5α. The exponential phase from an overnight culture grown at 37 °C in LB broth at a final OD_{600} (dilution 1/1000) = 0.001 was used in all assays. Fifty micrometres of each compound tested was added to 50 μL of the 1/1000 diluted bacterial suspension in 100 μL of LB broth. The plates were incubated overnight at 37 °C in a termomixer. The relative percentage growth of the bacteria found for each mixture was determined by measuring absorbance at 600 nm every 30 min during 8 h using a microtitre plate reader.

Cell culture

Mouse macrophages (RAW 264.7) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown

in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Rockville, MD, USA) supplemented with 15% fetal bovine serum (FBS; Gibco BRL) and 1% L-glutamine. The cultures were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Subcultures of macrophages were prepared every 2–3 days by scraping cells into fresh medium.

MTT cell viability assays

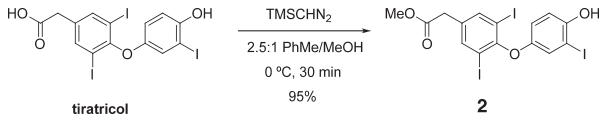
Cell viability was evaluated by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. RAW 264.7 cells were seeded in sterile 96-well microtitre plates at a seeding density of 25×10^3 cells/mL in DMEM supplemented with 1% FBS and allowed to settle for 24 h. Various concentrations of the compounds were added to the plates and the cells were further incubated for 24 h. After removal of the medium, the precipitated formazan crystals were dissolved in optical grade DMSO (100 μL) and the plates were read at 570 nm using a Wallac 1420 Workstation (Perkin Elmer, Inc, Wellesley, MA, USA).

Evaluation of TNF-α expression

RAW 264.7 cells (100 000 cells/well in a 6-well microplate) were seeded in 6-well plates at density of 100 000 cells/mL in DMEM supplemented with 1% FBS and allowed to settle for 24 h. Lipopolysaccharide (1 μg/mL) was then added in the absence or the presence of tiratricol (a range of concentrations from 1 to 25 μM). After 24 h, supernatants were collected and centrifuged for 10 min at 400 × *g* and stored at −20 °C until determination of cytokine content. Tumour necrosis factor-α in the cell supernatant was determined using a commercial ELISA kit (BD Bioscience, San Diego, CA, USA) following the manufacturer's protocol. Samples were 20-fold diluted with buffer. Colour changes at 450 nm were measured using a microtitre plate reader. Levels of cytokines were expressed as picograms per millilitre. Detection range of the ELISA kit was 0–1000 pg/mL. The TNF-α content of each sample was determined thrice.

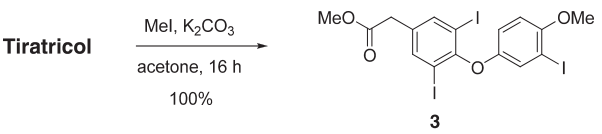
Solution-phase synthesis of tiratricol analogues

Tiratricol methyl ester (2)



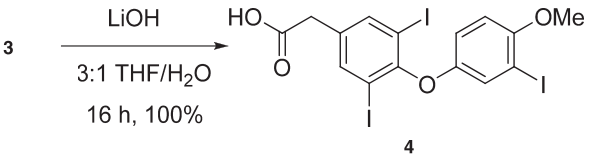
$TMSCHN_2$ (24 μL, 0.048 mmol) was added drop-wise to a solution of tiratricol (20 mg, 0.032 mmol) in MeOH (0.30 mL) and toluene (0.75 mL) cooled to 0 °C. After stirring at 0 °C for 30 min, several drops of HOAc were added and the solution was concentrated under vacuum. The product was purified by column chromatography on silica gel (hexane/EtOAc, 1:2) to give **2** (19.5 mg, 95%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.51 (s, 2H), 3.68 (s, 3H), 4.99 (br, 1H), 6.62 (dd, J = 9.0, 2.9 Hz, 1H), 6.84 (d, J = 9.0 Hz, 1H), 7.03 (d, J = 2.8 Hz, 1H), 7.71 (s, 2H). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 39.1, 52.4, 85.3, 90.9, 115.1, 117.3, 124.6, 134.7, 141.0, 150.1, 150.4, 153.1, 170.8. HRMS (FAB) calcd. for $C_{15}H_{11}I_3O_4^{3+}$: 635.7791, found: 635.7809.

Tiratricol methyl ester, methyl ether (3)



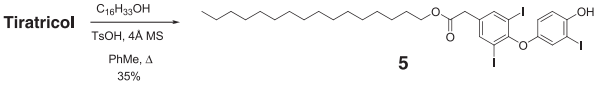
K_2CO_3 (49 mg, 0.35 mmol) and MeI (11 μL, 0.18 mmol) were added to a solution of tiratricol (22 mg, 0.035 mmol) in acetone (0.5 mL). After stirring at room temperature for 16 h, the product was treated with saturated NH_4Cl, extracted with EtOAc, dried over Na_2SO_4 and concentrated under vacuum. The product was purified by column chromatography on silica gel (hexane/EtOAc, 2:1) to give **3** (23 mg, 100%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.50 (s, 2H), 3.68 (s, 3H), 3.77 (s, 3H), 6.60–6.68 (m, 2H), 7.19–7.21 (m, 1H), 7.71 (s, 2H). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 39.1, 52.4, 56.8, 86.1, 90.9, 111.1, 115.8, 126.6, 134.7, 141.0, 150.3, 153.1, 153.7, 170.8. HRMS (FAB) calcd. for $C_{16}H_{13}I_3O_4^{3+}$: 649.7948, found: 649.7950.

Tiratricol methyl ether (4)



LiOH (6 mg, 0.128 mmol) was added to **3** dissolved in THF (0.3 mL) and H_2O (0.1 mL). After stirring at room temperature for 16 h, the product was treated with HCl 1M, extracted with EtOAc, dried over Na_2SO_4 and concentrated under vacuum. The product was purified by column chromatography on silica gel (hexane-EtOAc, 1:5) to render **4** (22 mg, 100%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.55 (s, 2H), 3.77 (s, 3H), 6.60–6.68 (m, 2H), 7.18–7.21 (m, 1H), 7.72 (s, 2H). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 39.0, 56.8, 86.2, 91.0, 111.1, 115.8, 126.6, 133.9, 141.1, 150.3, 153.3, 153.8, 175.8. HRMS (FAB) calcd. for $C_{15}H_{11}I_3O_4^{3+}$: 635.7791, found: 635.7781.

Tiratricol palmitic ester (5)



$C_{16}H_{33}OH$ (10.5 mg, 0.045 mmol), $TsOH{\cdot}H_2O$ (4 mg, 0.020 mmol) and molecular sieve of 4 Å were added to a solution of tiratricol (24.5 mg, 0.039 mmol) in toluene (0.5 mL). After stirring at reflux for 2 days, the solution was diluted with CH_2Cl_2, filtered, washed with saturated $NaHCO_3$, dried over Na_2SO_4 and concentrated under vacuum. The product was purified by column chromatography on silica gel (CH_2Cl_2-EtOAc, 15:1) to give **5** (21.5 mg, 35%). ^{1}H NMR

(300 MHz, $CDCl_3$) δ 0.81 (t, J = 6.7 Hz, 3H), 1.15–1.30 (m, 26H), 1.52–1.62 (m, 2H), 3.49 (s, 2H), 4.06 (t, J = 6.6 Hz, 2H), 5.05 (br, 1H), 6.61 (dd, J = 8.9, 2.9 Hz, 1H), 6.83 (d, J = 8.9 Hz, 1H), 7.03 (d, J = 2.9 Hz, 1H), 7.72 (s, 2H). ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 14.1, 22.7, 25.9, 28.5, 29.2, 29.3, 29.5, 29.6, 29.7, 31.9, 39.4, 65.5, 85.3, 90.8, 115.1, 117.2, 124.6, 134.9, 141.0, 150.1, 150.4, 153.0, 170.5. HRMS (FAB) calcd. for $C_{30}H_{41}I_3O_4^{\ 3+}$: 846.0139, found: 846.0136.

Solid-phase synthesis of tiratricol analogues

The synthesis of analogues on solid-phase was performed manually in polypropylene syringes, each fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Washings between reaction steps were done with DMF and CH_2Cl_2 using 10 mL of solvent/g of resin each time. The synthesis of analogue **6** was performed on 2-chlorotrityl chloride resin (CTC) (1.55 mmol/g). For this resin, CH_2Cl_2 treated with alumina was used and cleavage was achieved using TFA–CH_2Cl_2 (1:99) (5 × 0.5 min). Alternatively, analogues **7** and **8** were synthesized on Fmoc-Rink Amide MBHA resin (0.65 mmol/g). In this case, the Fmoc group was removed by treatment with piperidine-DMF (1:4) for 20 min. Compounds were cleaved from the solid support by treatment with a TFA-H_2O-TIS (95:2.5:2.5) mixture for a time ranging from 1 to 1.5 h.

Tiratricol-phenolic-aminohexanoic ester (6)

Tiratricol (1 eq.) and DIEA (10 eq.) were sequentially added to CTC resin (160 mg, 1.55 mmol/g) and the resin was stirred for 1 h. The incorporation was followed by a capping step with MeOH (128 μL). Then, 6-Boc-aminohexanoic acid (10 eq.) and DIC (5 eq.) were dissolved in CH_2Cl_2 and added to the resin. Next, 4-dimethylaminopyridine (0.1 eq.) was added to the resin. After 3 h of reaction, the compound was cleaved from the resin as described above. After TFA evaporation, a stronger acidic treatment with TFA–CH_2Cl_2 (40:60) for 30 min was required to remove the Boc group. Compound **6** was purified by semi-preparative HPLC. ^{1}H-NMR, CD_3OD: 7.88 (s, 1H), 7.21 (d, J = 2.9 Hz, 1H), 7.06 (d, J = 8.9 Hz, 1H), 6.80 (dd, J = 2.9 and 8.9 Hz, 1H), 3.64 (s, 2H), 2.95 (m, 2H), 2.68 (t, J = 7.17 Hz, 2H), 1.82 (m, 2H), 1.72 (m, 2H), 1.57 (m, 2H). Purity of 99% (from 30 to 100% MeCN over 15 min, t_R = 5.85 min). MALDI-TOF (m/z calcd. for $C_{20}H_{20}I_3NO_5$ 734.85; found, 735.76 $[M + H]^+$).

Tiratricol-Glu (7)

After Fmoc removal, the coupling of Fmoc-Glu(^{t}Bu)-OH (33.2 mg, 0.08 mmol) into Rink Amide resin (30 mg, 0.65 mmol/g) was done using HOAt (10.6 mg, 0.08 mmol) and DIC (12.1 μL, 0.08 mmol) in DMF for 3 h. Tiratricol (24.2 mg, 0.04 mmol) was added to the resin with two consecutive treatments (2 × 24 h) with PyAOP (20.3 mg, 0.04 mmol), HOAt (5.3 mg, 0.04 mmol) and DIEA (20.4 μL, 0.12 mmol) in DMF. The cleavage step was performed with TFA–H_2O–TIS (95:2.5:2.5) for 1 h. TFA was evaporated with nitrogen and the compound was dissolved in H_2O–ACN (50:50). The lyophilized compound **7** (7.2 mg, 48%) was obtained with good purity. ^{1}H-NMR, CD_3OD: 7.87 (s, 1H), 7.03 (d, J = 2.9 Hz, 1H), 6.74 (d, J = 8.9 Hz, 1H), 6.60 (dd, J = 2.9 and 8.9 Hz, 1H), 4.06 (m, 1H), 3.56 (s, 2H), 2.37–2.27 (m, 4H). Purity of 99% (from 30 to 100% MeCN over 15 min, t_R = 6.40 min). MALDI-TOF (m/z calcd. for $C_{19}H_{17}I_3N_2O_6$ 749.82; found, 749.85 $^{3+}$, 772.83 $[M + Na]^+$, 788.80 $[M + K]^+$).

Tiratricol-Arg (8)

After Fmoc removal, Fmoc-Arg(Pbf)-OH (51.4 mg, 0.08 mmol) was coupled to Rink Amide resin (30 mg, 0.65 mmol/g) with PyAOP (41.2 mg, 0.08 mmol), HOAt (10.8 mg, 0.08 mmol) and DIEA (41.4 μL, 0.24 mmol) in DMF overnight. The resin was washed, the Fmoc group removed and tiratricol (24.2 mg, 0.04 mmol) was cou-

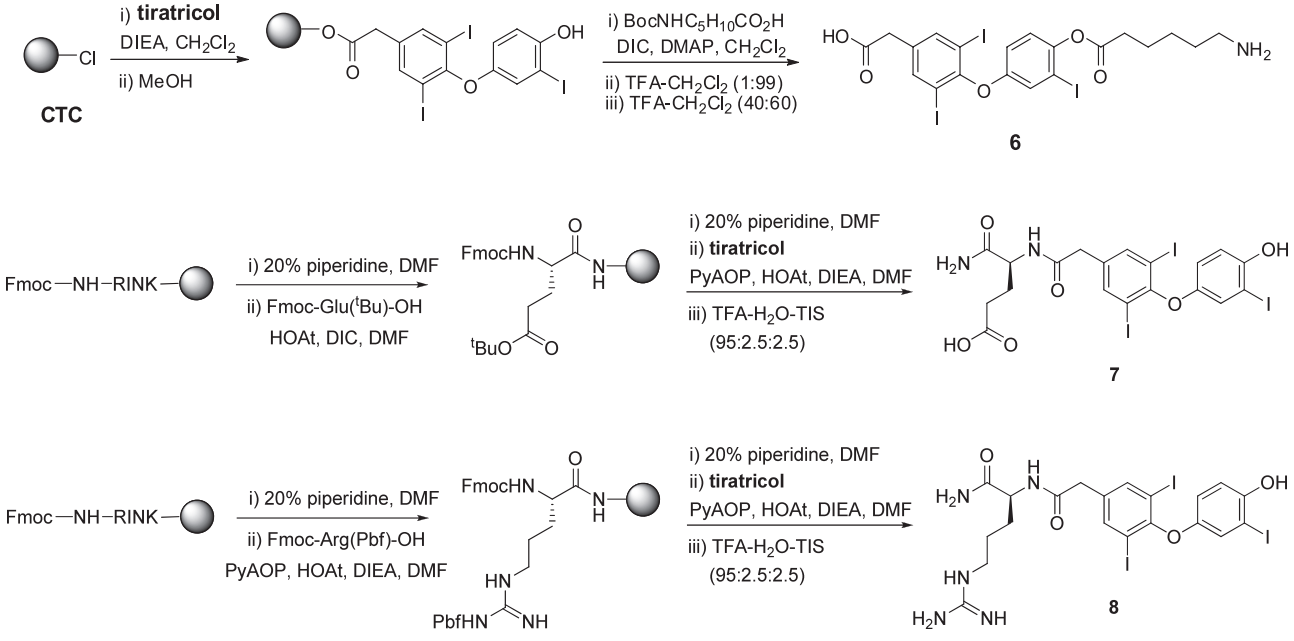
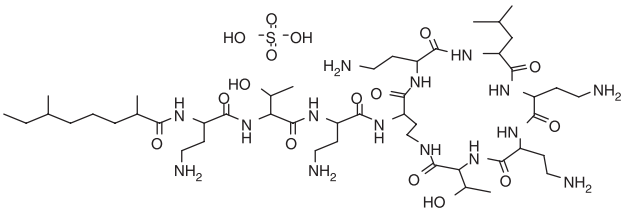
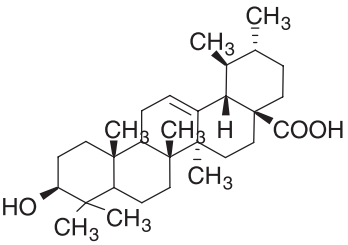
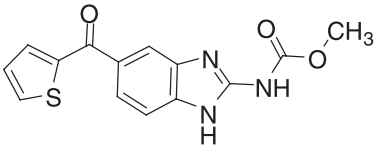
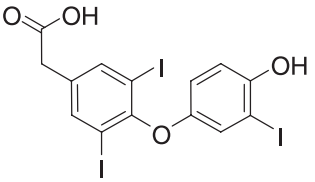
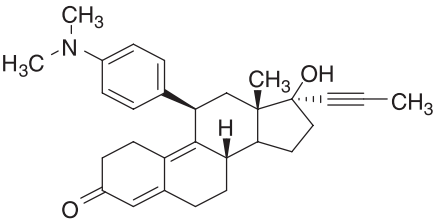
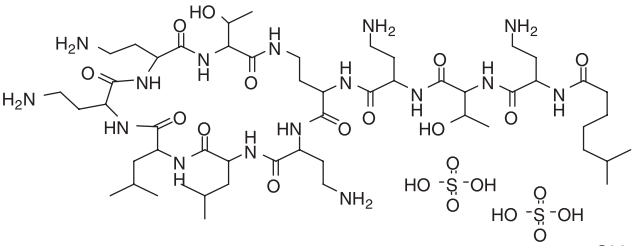
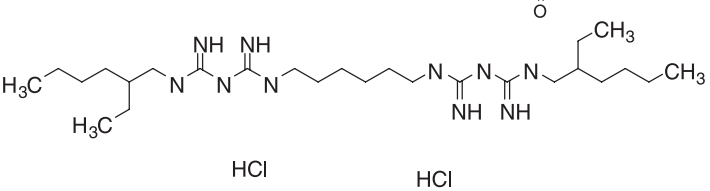


Table 1: Biological activity of the library compounds identified as LPS neutralizers

Compound			
Name	Chemical structure	IC_{50} for LPS (μM)	IC_{50} for lipid A (μM)
Polymyxin B (ctrl)		<1	–
Ursolic acid		21	86
Nocodazole		39	>100
Tiratricol		20	32
Mifepristone		18	70
Colistin sulfate		48	56
Alexidine		10	8

Data expressed as mean ($n > 3$). In all cases, SE < 10%.

pled after 2 × 24 h treatments with PyAOP (20.3 mg, 0.04 mmol), HOAt (5.3 mg, 0.04 mmol) and DIEA (20.4 μL, 0.12 mmol) in DMF. The cleavage step was performed with TFA–H_2O–TIS (95:2.5:2.5) for 1.5 h. TFA was evaporated with nitrogen and the compound was dissolved in H_2O–ACN (50:50) and lyophilized to yield **8** (6.0 mg, 39%) with optimal purity. ^{1}H-NMR, CD_3OD: 7.88 (s, 1H), 6.98 (d, *J* = 2.9 Hz, 1H), 6.75 (d, *J* = 8.9 Hz, 1H), 6.64 (dd, *J* = 2.93 and 8.9 Hz, 1H), 4.39 (dd, *J* = 5.6 and 8.2 Hz, 1H) 3.58 (s, 2H), 3.21 (m, 2H), 1.89–1.68 (m, 4H). Purity of 88% (from 30 to 100% MeCN over 15 min, t_R = 4.54 min). MALDI-TOF (*m/z* calcd. for $C_{20}H_{22}I_3N_5O_4$ 776.88; found, 777.89 $[M + H]^+$, 799.85 $[M + Na]^+$).

Results and Discussion

The LPS-neutralizing activity of the Prestwick Chemical Library® was evaluated by the chromogenic LAL assay (16). Controls of the LPS neutralizer PMB were included in each plate. As a result of the screening, six compounds, all with LPS-neutralizing activity above the initial threshold (100 μM), were selected and their IC_{50} values were determined (Table 1). Four of these compounds, namely alexidine, colistin sulphate, nocodazole and mifepristone, were not pursued further. Alexidine and colistin sulphate are antibiotic agents (17,18) and in this study our focus was on non-antibacterial molecules. Nocodazole is an antineoplastic agent that shows some neurotoxic side-effects (19). Mifepristone is an antagonist of glucocorticoid receptors and may cause secondary effects (20). In contrast, ursolic acid is anti-inflammatory and antihistaminic and has analgesic properties (21). Furthermore, tiratricol (triiodothyroacetic acid) is a natural thyroid hormone analogue. Among other properties, tiratricol binds to low density lipid-binding proteins and protects them against oxidation (22). Here, we describe the mechanisms of action of tiratricol and ursolic acid as LPS neutralizers. Initially, we evaluated their antibiotic properties and cell toxicity. Both compounds were safe for eukaryotic cells, as deduced from MTT experiments in RAW 264.7 macrophages and were devoid of any antibacterial activity.

Tiratricol and ursolic acid inhibited the activity of the lipid A moiety, with IC_{50} values of 32 and 86 μM, respectively (Table 1). As stated above, compounds that could decrease the ability of LPS to form micelles would favour its monomeric state and hence their binding to LPB and other lipoproteins of the detoxification way. We analysed the effect of the selected compounds on the micellar organization of lipid A using the fluorescent probe DPH. Incorporation of DPH into the core of micelles is accompanied by strong enhancement of its fluorescence (15). The presence of ursolic acid did not modify the CMC of lipid A (data not shown). However, tiratricol increased the CMC of lipid A from 2 to 10 μM (Figure 1), thereby suggesting a significant effect of this molecule on the monomer to micelle equilibrium of lipid A.

We sought to improve the LPS-neutralizing activity of tiratricol, including potency and batch-to-batch solubility. Several tiratricol derivatives with modifications at the phenol and/or carboxylic acid were synthesized in solution and in solid-phase modes and evaluated (Table 2). Esterification of the carboxylic acid (**2**) or etherification of the phenol **(3)** resulted in a loss of activity. This

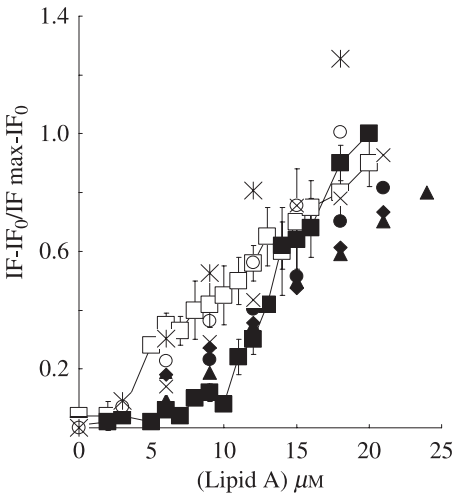
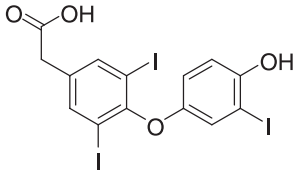
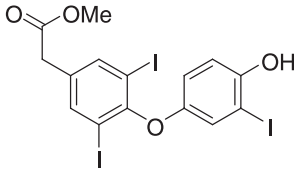
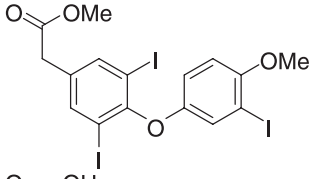
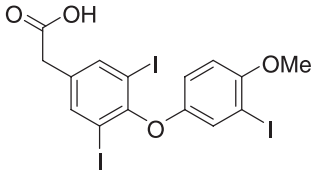
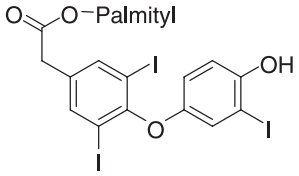
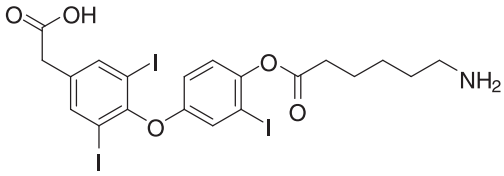
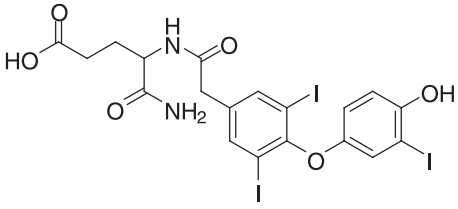
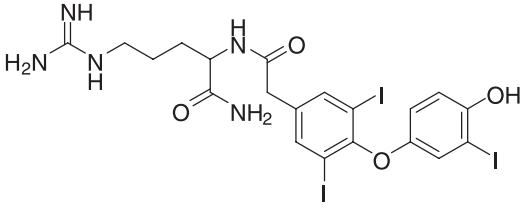


Figure 1: Fluorescence spectroscopy analysis of the effect of tiratricol and tiratricol-derivatives on the CMC of lipid A. The fluorescence intensity of the DPH probe (5 μM) was obtained at 430 nm upon excitation at 380 nm with (IF) or without lipid A (IF_0). Spectra were acquired in the absence (white squares) or in the presence of 10 μM concentration of tiratricol (black squares); and tiratricol derivatives **2** (black diamond), **3** (asterisk), **4** (white circle), **6** (black cross), **7** (black triangle) and **8** (black circle). Data expressed as mean ($n > 3$). Error bars are only shown in those data represented by the white and black squares for clarity. In all cases, SE < 10%.

was particularly detrimental for analogue **4** which contained both modifications. Only the palmityl ester derivative (**5**) retained activity. This result is consistent with previous studies that highlight the importance of acyl chains for interaction with the hydrophobic moiety of the lipid A. The most promising candidates for future investigation were the aminohexanoic ester (**6**) derivative together with the glutamic acid (**7**) and arginine (**8**) derivatives. These compounds were synthesized on solid-phase. For the synthesis of analogue **6**, tiratricol was inserted into CTC and treated with the *N*-protected aminohexanoic acid. Alternatively, derivatives **7** and **8** were obtained through coupling of tiratricol, via amide formation, with the corresponding amino acids loaded into Rink Amide resin. The modification of the carboxylic acid or the phenol moieties in this case did not decrease LPS-neutralizing activity, probably because these modifications did not imply a suppression of the partial or total polarity of the molecule: a phenol group was replaced by an amino group (**6**) or the carboxylic acid was replaced by another acid (**7**) or a guanidyl group (**8**). Tiratricol derivatives were evaluated on their effect on the micellar organization of lipid A using the DPH-based fluorescence assay (Figure 1). As expected from the LPS-neutralization experiments, derivatives **6**, **7** and **8** increased the CMC of lipid A while derivatives **3** and **4** had minor impact on such CMC value. The behavior of derivative **2** was a bit aside provided that showed lower activity than tiratricol in the LPS-neutralizing assay, while was still able to decrease the CMC of the lipid A. Derivative **5** precipitated out from the solution in the experimental conditions required for this assay.

Table 2: Biological activity of tiratricol derivatives as LPS neutralizers

Compound (tiratricol)	Chemical structure	IC_{50} for LPS (μM)
1		20
2		60
3		47
4		>100
5		20
6		5
7		3
8		4

Data expressed as mean ($n > 3$). In all cases SE < 10%.

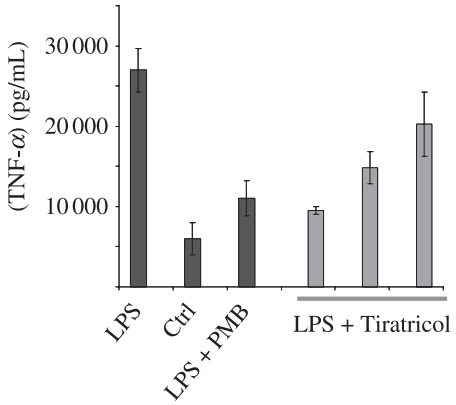


Figure 2: Inhibition of LPS-induced TNF-α release in macrophages by tiratricol. Cells were treated with 1 μg/mL of LPS or with LPS in the presence of 50 μg/mL of PMB (LPS + PMB), or in the presence of tiratricol (grey bars – 20, 10 and 5 μM from the left). Data expressed as mean ± SD ($n > 5$).

Finally, in a large number of septic shock patients elevated levels of TNF-α correlate with prolonged fatal multiple organ failure. Using RAW264.7 cells, we assessed the effects of tiratricol on LPS activity in a cellular model. Administration of this compound markedly decreased the levels of LPS-induced TNF-α (Figure 2).

In conclusion, here we screened a library of highly diverse drug molecules for which bioavailability has been proven and we identified tiratricol as a hit molecule for the neutralization of LPS (14). In the light of our results, we propose that tiratricol binds to the lipid A endotoxic moiety of LPS and induces the formation of mixed (tiratricol–lipid A) micelles. This, together with the reported capacity of tiratricol to bind to lipoproteins and protect them against oxidative processes (22), would probably favour lipoprotein-dependent LPS detoxification. Furthermore, tiratricol decreased LPS-dependent TNF-α production in macrophages.

Tiratricol is an orphan drug for use in combination with levothyroxine to treat euthyroid goiter and to suppress thyroid-stimulating hormone in patients with well-differentiated thyroid cancer (23). However, the Food and Drug Administration warned against consuming products containing tiratricol. In fact, there are questions that still need to be addressed; however, safe and pharmacologically acceptable derivatives of tiratricol, devoid of secondary effects, could advance as LPS-neutralizing molecules for the treatment of endotoxic shock. We have early reported how an *N*-alquilglycine inhibitor of the apoptosome, a key holoenzyme in the signalling pathway of apoptosis (24), was converted, from a compound with modest efficiency in cellular models to the first antiapoptotic polymeric nano-medicine upon conjugation to poly-L-glutamic acid (13). Similarly, we are developing polymer-based derivatives of our previously reported LPS-neutralizer named peptoid-7 (12), that improve the biological activity of peptoid-7 and increase the survival curves in an animal model of septicaemia (to be published elsewhere). We will apply similar procedures to tiratricol. Although still speculative, intravenous delivery of polymer-based derivatives of tiratricol will increase the blood time circulation and would decrease unspecific interactions that could decrease adverse effects.

Acknowledgments

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COMPLEMENTARY EXPERIMENTAL DATA FOR:

Tiratricol neutralizes bacterial endotoxins and reduces lipopolysaccharide-induced TNF-α production in the cell

Laura Cascales, Carlos Mas-Moruno, Silvia Tamborero, José Luis Aceña, Juan F. Sanz-Cervera, Santos Fustero, Luis J. Cruz, Puig Mora, Fernando Albericio, Enrique Pérez-Payá

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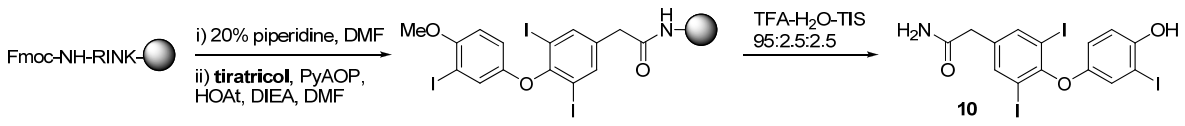
Solid-phase synthesis of tiratricol analogues

Tiratricol-amide (10)

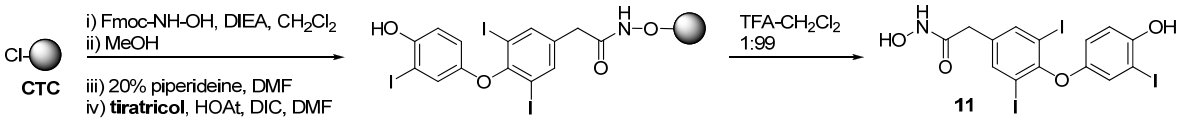
After Fmoc removal **tiratricol** (14.5 mg, 0.02 mmol) was coupled overnight to Fmoc-Rink Amide resin (30 mg, 0.65 mmol/g) with PyAOP (12.2 mg, 0.02 mmol), HOAt (3.2 mg, 0.02 mmol) and DIEA (12.2 μL, 0.07 mmol) in DMF. The compound was then cleaved from the solid support upon treatment with TFA as described in the Methods and Materials section for 1 h. TFA was evaporated with nitrogen and the compound was dissolved in H_2O–ACN (50:50) and lyophilized to yield **10** (4.8 mg, 47%). ^{1}H-NMR, CD_3OD: 7.87 (s, 1H), 7.02 (d, J = 3.0 Hz, 1H), 6.74 (d, J = 8.9 Hz, 1H), 6.62 (dd, J = 3.0 and 8.9 Hz, 1H), 3.5 (s, 2H). Purity of 88 % (from 30 to 100 % MeCN over 15 min, t_R = 7.72 min). MALDI-TOF (*m/z* calcd. for $C_{14}H_{10}I_3NO_3$ 620.78; found, 620.80 $[M]^+$).



Tiratricol-hydroxamic acid (11)

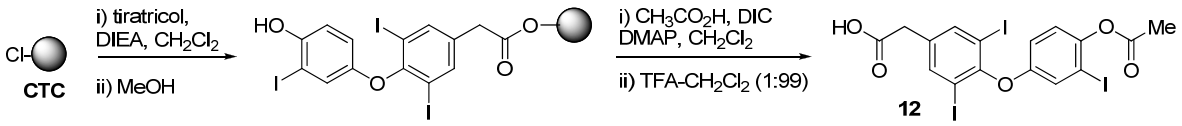
N-Fmoc-hydroxylamine (25.5 mg, 0.1 mmol) and DIEA (17.4 μL, 0.1 mmol) were added to CTC resin (21.6 mg, 1.55 mmol/g), and the mixture was allowed to react overnight. Next, a capping step with MeOH (50 μL) was carried out and the Fmoc

group was removed by treatment with piperidine–DMF (50:50) for 30 min. Then, **tiratricol** (27.4 mg, 0.04 mmol) was coupled to the new aminooxy-2-chlorotrityl resin (21.6 mg, 1 mmol/g) with HOAt (6.0 mg, 0.04 mmol) and DIC (6.8 μL, 0.04 mmol) in DMF for 3 h. The resin was washed and the compound cleaved under mild acidic conditions as described in the Methods and Materials section. After lyophilization, **6** (3.3 mg, 26 %) was obtained. ^{1}H-NMR, CD_3OD: 7.87 (s, 1H), 7.00 (d, J = 2.9 Hz, 1H), 6.75 (d, J = 8.9 Hz, 1H), 6.61 (dd, J = 2.9 and 8.9 Hz, 1H), 3.4 (s, 2H). Purity of 99 % (from 30 to 100 % MeCN over 15 min, t_R = 6.97 min). MALDI-TOF (*m/z* calcd. for $C_{14}H_{10}I_3NO_4$ 636.77; found, 636.76 $[M]^+$).



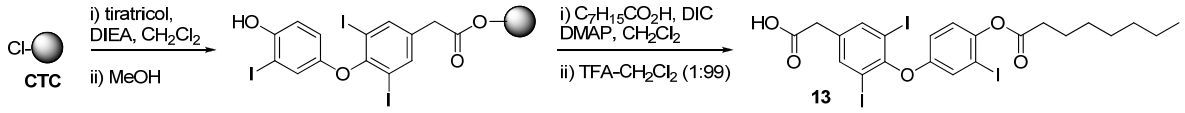
Tiratricol-phenolic-acetic ester (12)

Tiratricol (1 eq.) and DIEA (10 eq.) were sequentially added to CTC resin (160 mg, 1.55 mmol/g) and the resin was stirred for 1 h. The incorporation was followed by a capping step with MeOH (128 μL). Then, acetic acid (10 eq.) and DIC (5 eq.) were dissolved in CH_2Cl_2 and added to the resin. Next, 4-dimethylaminopyridine (DMAP) (0.1 eq.) was added to the resin. After 3 h of reaction, the compound was cleaved as previously described. Analogue **12** was purified by semi-preparative HPLC. ^{1}H-NMR, CD_3OD: 7.88 (s, 1H), 7.20 (d, J = 2.8 Hz, 1H), 7.06 (d, J = 8.9 Hz, 1H), 6.79 (dd, J = 2.8 and 8.9 Hz, 1H), 3.64 (s, 2H), 2.31 (s, 3H). Purity of 91 % (from 30 to 100 % MeCN over 15 min, t_R = 10.28 min).



Tiratricol-phenolic-octanoic ester (13)

The synthesis of compound **13** was performed as described for the analogue **12** but using octanoic acid as the carboxylic acid in the esterification reaction. Compound **13** was purified by semi-preparative HPLC. ^{1}H-NMR, CD_3OD: 7.88 (s, 1H), 7.20 (d, J = 2.9 Hz, 1H), 7.04 (d, J = 8.9 Hz, 1H), 6.80 (dd, J = 2.9 and 8.9 Hz, 1H), 3.62 (s, 2H), 2.62 (t, J = 7.38 Hz, 2H), 1.77-1.37 (m, 10H), 0.92 (t, J = 6.73 Hz, 3H). Purity of 97 % (from 50 to 100 % MeCN over 15 min, t_R = 13.08 min).



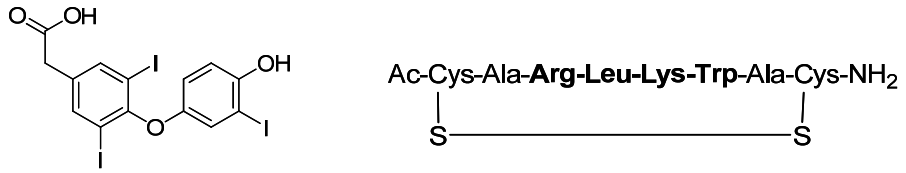
Note: The LPS-neutralizing activity of these analogues (**10** to **13**) was poor (IC_{50} > 100 μM) and thus were not included in the final version of the paper.

“RLKWc-ttcol: a new hybrid molecule that neutralizes LPS”

Unpublished results

INTRODUCTION

In this chapter we have identified 3,3',5-triiodo-thyroacetic acid (tiratricol) as a non-antibacterial compound that neutralizes the toxic lipopolysaccharide.[1] We have also demonstrated that tiratricol binds LPS via its toxic moiety, lipid A. This result is somewhat surprising given the chemical structure of tiratricol (Figure 1). Lipid A binders usually promote electrostatic interactions between their positively charged groups and the phosphate groups of lipid A. However, tiratricol does not contain functional groups with this capacity. Quite the opposite, it has a carboxylic group that might be negatively charged at a physiological pH. What is more, chemical modification of this functional group (i.e. esterification) resulted in analogues with a significantly reduced LPS-neutralizing activity. A distinct molecule with LPS-neutralizing activity is the cyclic peptide RLKWc (Figure 1).[2] This peptide was proposed as a minimized LPS-binding domain of the protein LALF in the previous chapter. It contains basic and hydrophobic residues and consequently binds to lipid A.



tiratricol **RLKWc**

Figure 1: Chemical structure of tiratricol and the peptide RLKWc. For this peptide amino acids are represented using the three-letter code.

Though speculative, we can assume that these two compounds bind to lipid A via distinct mechanisms. This different behavior would open the possibility to use these molecules together in order to study whether a synergistic effect is observed in terms of biological activity. In the present work, we will study the conjugation of tiratricol to RLKWc to obtain a new hybrid molecule, compound **RLKWc-ttcol**, and evaluate its LPS-neutralizing activity.

RESULTS AND DISCUSSION

Design and synthesis of the hybrid molecule RLKWc-ttcol

The initial question addressed in our design was how to conjugate the two molecules. An easy approach would be to bind the two molecules via an amide bond between the carboxylic acid of tiratricol and the *N*-terminal amino group of RLKWc. However, as we have previously explained, the modification of the carboxylic group of tiratricol may be deleterious for its biological activity. We therefore chose the analogue tiratricol-Glu (see Figure 2)[1]. This compound showed a superior LPS-neutralizing activity over tiratricol and included an extra carboxylic group in its structure. This compound would therefore allow the formation of an amide bond with RLKWc, thereby keeping an unmodified carboxylic acid for biological purposes. In addition, this molecule could be obtained stepwise on solid-phase using standard Fmoc/tBu chemistry. The peptide RLKWc would be initially assembled on the solid support, next glutamic acid (Glu) is coupled and finally tiratricol is inserted. The structure of **RLKWc-ttcol** is shown in Figure 2.

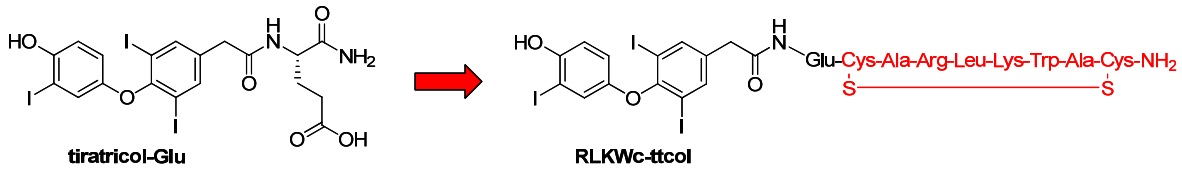


Figure 2: The conjugation of tiratricol-Glu with the peptide RLKWc renders the compound **RLKWc-ttcol**. The chemical structure of this molecule is shown.

To achieve the intramolecular disulfide bridge formation, three strategies were proposed (Scheme 1):

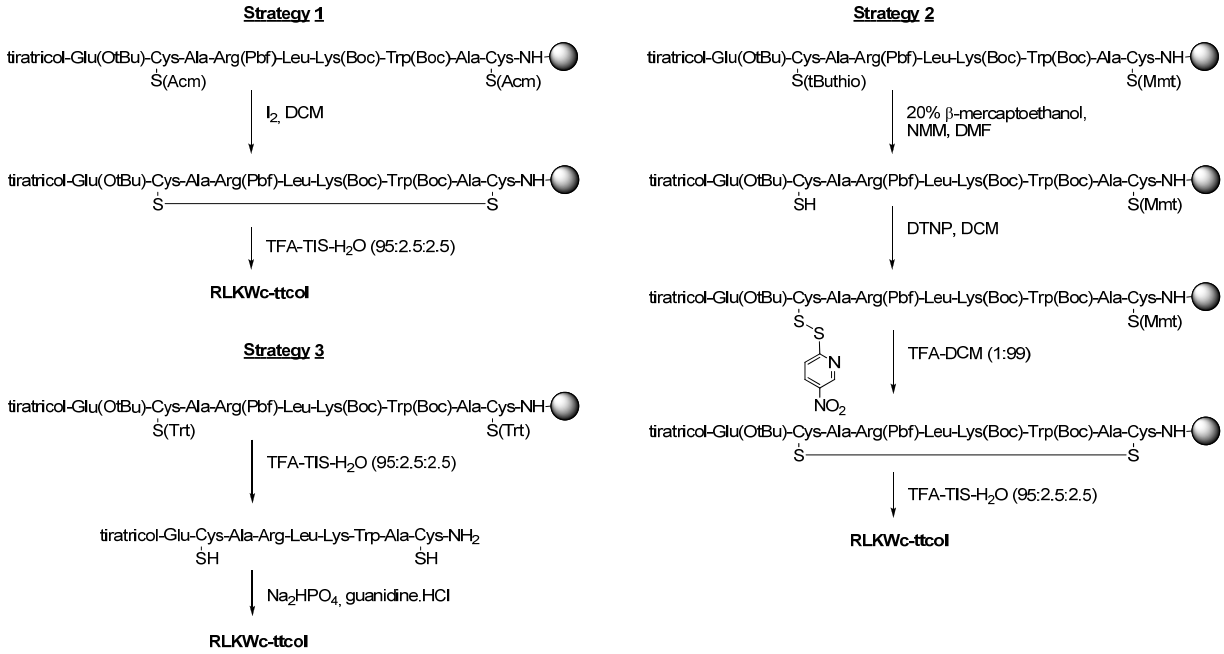
1) on resin oxidation using I_2
2) on resin disulfide bond formation using a non-oxidative method
3) cyclization in solution using air oxidation

Each method requires a particular protecting group of the Cys undergoing disulfide bond formation. The first method involves iodine-mediated oxidation of Cys protected with the acetamidomethyl (Acm) protecting group.[3] This method has been widely used

for on-resin disulfide bond formation; however, the use of iodine can lead to over-oxidation of the thiol functionality to the corresponding sulphonic acid, as well as to medication of other sensitive amino acid side chains (i.e. Trp).[4] An alternative to overcome these limitations is to use a non-oxidative method. This is the case of a recently reported strategy.[5] This strategy uses *tert*-Butylthio (S-*t*-Bu or *t*Buthio)[6] and 4-methoxytrityl (Mmt) as thiol protecting groups. The S-*t*-Bu protecting group can be selectively removed by reduction with mercaptoethanol. Next the free liberated thiol can be reprotected and activated to the 5-nitropyridin-sulfenyl (5-Npys) group after treatment with 2,2'-dithiobis(5-nitropyridine) (DTNP). A mild acidic treatment will then remove the Mmt group and initiate the cyclization. The third strategy involves the use of the trityl (Trt) group to protect both Cys. This acid-labile group is concomitantly removed during peptide cleavage, which allows disulfide bond formation in solution using mild oxidizing conditions.

Thus three syntheses of RLKWc-ttcol were performed on solid-phase using Rink MBHA resin as solid support. Fmoc-amino acids were coupled using DIC and HOAt as coupling reagents. When the peptide chain was assembled, tiratricol was inserted using PyAOP, HOAt and DIEA as described in the Experimental Section. Next, the strategies for disulfide bond formation discussed above were assayed. In the first place, Cys oxidation using iodine was carried out (Scheme 1, Strategy 1). This procedure presented several limitations. Initial treatments for 20 min with iodine were inefficient, but prolonged reaction times resulted in Cys oxidation and iodide incorporation into tiratricol aromatic rings, as observed by MALDI analyses. The second strategy involved the non-oxidative method (Scheme 1, strategy 2). The S-*t*-Bu group was treated with 20% mercaptoethanol in DMF in the presence of 0.1 M *N*-methylmorpholine (NMM) for 3 h. This treatment failed to reduce this protecting group. Thus, a longer 5-h treatment was performed. After this treatment 45% of the free thiol was observed by HPLC. The treatment was repeated for a further 12 h. However, the amount of unprotected Cys improved only slightly, to a poor 55%. S-*t*-Bu reduction has been proven to strongly depend on the Cys position in the peptide sequence as well as on the neighboring amino acids.[7] In this case, the presence of the tiratricol molecule close to this group could explain the inefficiency of the reductive treatments. This strategy was thus abandoned. Hence, the third approach was pursued (Scheme 1, Strategy 3). In this

case the peptide was cleaved with simultaneous removal of side-chain protecting groups using TFA. The unprotected peptide was then dissolved in an aqueous buffer containing 100 mM Na_2HPO_4 and 2 M guanidine·HCl at a pH of 8.5. The solution was stirred at room temperature for 12 h to allow air oxidation. The cyclization was easily monitored either by Ellman's test[8] and/or by RP-HPLC. This procedure successfully yielded the desired cyclic peptide with good yield. The peptide was finally purified by HPLC to afford **RLKWc-ttcol** with optimal purity. The three strategies are described in detail in the Experimental Section.



Scheme 1. The three strategies assayed for disulfide bridge formation are shown.

LPS-neutralizing activity of RLKWc-ttcol

The capacity of the hybrid **RLKWc-ttcol** to neutralize bacterial endotoxins was assayed using the chromogenic *Limulus* amebocyte lysate assay (LAL).[9] The compound displayed high LPS-neutralization at 100 μM (data not shown), therefore it was subjected to serial dilutions to calculate its IC_{50} (i. e. the concentration necessary to neutralize 50% of LPS *in vitro*). The LPS-neutralizing activity of **RLKWc-ttcol** was compared with that of tiratricol, tiratricol-Glu and RLKWc (see Table 1).

Table 1: LPS-neutralizing activity (IC_{50}) of **RLKWc-tiratricol** and the original analogues

Compound	IC_{50} (μM)[a]
RLKWc	30
tiratricol	20
tiratricol-Glu	7
RLKWc-ttcol	21

[a] The inhibition of the compounds was determined using the chromogenic LAL assay. The inhibitory activity is represented as IC_{50}. Standard deviation of the data was lower than 10%. The assay was performed as described in the Experimental section.

The biological activity of **RLKWc-ttcol** was similar to that of the original tiratricol molecule and slightly better than that of the peptide RLKWc. Therefore, the hybrid molecule retained the initial LPS-neutralizing activity of tiratricol and improved the biological profile of RLKWc. However, it did not reach the neutralization values observed for the most potent inhibitor, tiratricol-Glu. Therefore the incorporation of tiratricol into the cyclic peptide RLKWc resulted in an enhancement of the LPS-neutralizing activity of the peptide, but the contrary did not hold true. The LPS-neutralizing capacity of tiratricol-Glu was lowered when conjugated to RLKWc.

CONCLUDING REMARKS

Here we have presented the design and the efficient synthesis of a novel hybrid molecule, **RLKWc-ttcol**, based on two LPS-neutralizing agents: the peptide RLKWc and the molecule tiratricol. The assembly of these two compounds was accomplished on solid-phase in a facile step-wise manner. The disulfide bond formation was assayed with three distinct strategies, cyclization in solution with air oxidation being the most successful. The conjugation of these two molecules in **RLKWc-ttcol** enhanced the LPS-neutralizing activity of RLKWc, but did not imply an increase in the activity of tiratricol.

EXPERIMENTAL SECTION

Material and general methods

Materials and Instrumentation: Fmoc-Rink amide MBHA resin and protected Fmoc-amino acids were purchased from Iris Biotech GmbH (Marktredwitz, Germany), Neosystem (Strasbourg, France) and Calbiochem-Novabiochem AG (Laufelfingen, Switzerland). Tiratricol was obtained from Aldrich (Buchs, Switzerland). Coupling reagents, solvents for peptide synthesis and other reagents were purchased from commercial suppliers at the highest purity available and used without further purification. Analytical HPLC was performed using a Waters Alliance 2695 (Waters, MA, USA) chromatography system with a PDA 995 detector, a reverse-phase Sunfire C_{18} column (4.6 x 100 mm, 3.5-μm) and linear gradients of MeCN with 0.036% TFA into H_2O with 0.045% TFA. The system was run at a flow rate of 1.0 mL/min over 8 min. Semi-preparative HPLC was carried out on the same instrument using a reverse-phase Symmetry C_{18} column (7.8 x 100 mm 5-μm) and linear gradients of MeCN with 0.05% TFA into H_2O with 0.1% TFA. The system was run at a flow rate of 3.0 mL/min over 30 min. HPLC-MS was performed using a Waters Alliance 2796 with a dual absorbance detector 2487 and ESI-MS Micromass ZQ (Waters) chromatography system, a reverse-phase Symmetry 300 C_{18} column (3.9 x 150 mm, 5-μm) and H_2O with 0.1% formic acid and MeCN with 0.07% formic acid as mobile phases. Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (Applied Biosystems, Foster City, CA, USA) using ACH matrix.

Solid-phase peptide synthesis: Manual solid-phase peptide synthesis was performed in polypropylene syringes, each fitted with a polyethylene porous disk, using the Fmoc/^{t}Bu strategy. Solvents and soluble reagents were removed by suction. Washings between deprotection, couplings and subsequent deprotection steps were carried out with DMF and DCM using 10 mL of solvent/g of resin each time. The Fmoc group was removed by treatment with piperidine-DMF (1:4, v/v) and acetylation steps were performed with Ac_2O–DIEA–DMF (1:2:7). Couplings and washes were performed at 25 ºC. Couplings were monitored using standard colorimetric methods.[10]

Synthesis of RLKWc-ttcol

General procedure: RLKWc-ttcol was synthesized in three independent 10 mL-polypropylene syringes using Fmoc-Rink Amide MBHA resin (200 mg, 0.45 mmol/g). After Fmoc removal, Fmoc-$_L$-amino acids (4 equiv) were coupled using DIC (4 equiv) and HOAt (4 equiv) in DMF for 2 h. Peptide-resin samples were taken periodically, treated with TFA-H_2O-TIS (95:2.5:2.5) for 1.5 h, and analyzed by HPLC and HPLC-MS or MALDI. Finally, tiratricol (3 equiv) was incorporated with two consecutive treatments (1 x 12 h, 1 x 24 h) using PyAOP (3 equiv), HOAt (3 equiv) and DIEA (9 equiv). The efficiency of this reaction was monitored by the Kaiser test and HPLC.

First strategy: I_2 (5 equiv) was dissolved in DMF and added to the resin. The mixture was allowed to react for 20 min. HPLC analysis showed this treatment was ineffective. Hence, the treatment was repeated for an extended 40-min period. After this time a sample was taken and cleaved with TFA-H_2O-TIS (95:2.5:2.5) for 1.5 h. HPLC-MS and MALDI analysis showed the desired cyclic peptide together with the presence of side products derived from thiol oxidation to sulphonic acid and iodination of the aromatic rings (*m*/*z* calcd for $C_{60}H_{80}I_3N_{15}O_{14}S_2$, 1679.26; found, 1680.6 $[M + H]^+$, 1730.6 $[(M + 50) + H]^+$, 1805.94 $[(M + 126) + H]^+$).

Second strategy: S-t-Bu reduction was first attempted using a 20% β-mercaptoethanol solution in DMF in the presence of *N*-methylmorpholine (NMM) for 3 h. HPLC analysis did not show S-*t*-Bu removal. The same treatment was then repeated for 5 h. In this case, 45% of protecting group elimination was observed. Then, a prolonged 12 h treatment was performed. This treatment yielded a poor 55% removal of S-*t*-Bu. Due to these slow reaction rates, the strategy was abandoned.

Third strategy: For the cleavage of the compound from the solid support and concomitant deprotection of side-chain groups, the resin was washed with DCM (3 x 1 min), dried, and treated with TFA-H_2O-TIS (95:2.5:2.5) for 1.5 h. TFA was then removed by evaporation with nitrogen, and the compound was precipitated with cold anhydrous TBME, dissolved in H_2O-MeCN (1:1) and then lyophilized. The linear precursor was then dissolved in an aqueous buffer containing 100 mM Na_2HPO_4 and 2

M guanidine·HCl at a pH of 8.5. The solution was stirred at room temperature for 12 h to allow air oxidation. The cyclization was monitored either by Ellman's test[8] and/or by RP-HPLC. When cyclization was finished, the reaction mixture was treated with the aromatic adsorbent Diaion HP-20 (Supelco, Bellefonte, PA, USA) for 12 h. The peptide adsorbed was gently washed with H_2O to remove buffer salts and was then eluted with increasing concentrations of MeCN in aqueous mixtures. Lyophilization yielded the desired molecule, which was purified by HPLC: linear gradient from 5 to 100 % MeCN over 30 min, flow rate 3 mL/min. Characterization of **RLKWc-ttcol**: HPLC (from 5 to 100 % MeCN over 8 min, t_R = 4.77 min, 95 % purity), MALDI-TOF (*m*/*z* calcd for $C_{60}H_{80}I_3N_{15}O_{14}S_2$, 1679.26; found, 1680.40 $[M + H]^+$, 1702.39 $[M + Na]^+$, 1718.36 $[M + K]^+$).

LPS-neutralizing activity

All solutions used in the LPS-neutralizing activity assay were tested to ensure they were endotoxin-free and material was sterilized by heating for 3 h at 180 ºC. LPS from *E. Coli* 055:B5 and Polymyxin B were purchased from Sigma. LPS-neutralizing activity was measured using the chromogenic *Limulus* Amebocyte Lysate (LAL) test,[9] following the manufacturer's instructions (Cambrex). LAL reagent contains a clottable protein that is activated in the presence of non-neutralized LPS and is an extremely sensitive indicator of the presence of endotoxin. When activated, this enzyme catalyses the release of p-nitroaniline (pNA) from the colorless chromogenic substrate Ac-Ile-Glu-Ala-Arg-pNA. The pNA released can be measured photometrically at 405 nm. **RLKWc-ttcol** was initially incubated at 100 μM with LPS (100 pg/mL) in a 96-well microtiter for 60 min at 37 ºC. Polymyxin B (10 μg/mL) was used as positive control. LAL (12.5 μL) was added to start the reaction at 37 ºC. After 10 min, non-neutralized LPS was detected after a 5-8 min incubation with the chromogenic substrate (25 μL). Acetic acid (25 % v/v final concentration) was added to stop the reaction and the absorbance was monitored at 405 nm in a Multiskan Ascent microtiter plate reader (ThermoLabsystems). The IC_{50} value (the concentration necessary to neutralize 50% of LPS *in vitro*) for **RLKWc-ttcol** was determined by a serial dilution assay using 100 pg/mL of LPS and a range of compound concentrations (100 to 0.1 μM). All assays

were run in triplicate, and the curves were automatically adjusted by non-linear regression using "Prism 4" (GraphPad) software.

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3.3 CONCLUSIONS

The conclusions of the present chapter are the following:

1. The study of the chemical features of the toxic moiety of LPS, lipid A, is a promising approach for the design of new LPS-inhibitors. This approach allows the design of new peptide-based molecules where the amino acids required for lipid A interaction are conserved, whereas "non-essential" amino acids can be replaced by other chemical groups. In this chapter, following this approach we synthesized novel peptide-based LPS-inhibitors with enhanced LPS-neutralizing activities. The substitution of several amino acids by PEG-like chain gave the most promising compounds.

2. In addition, these LPS-inhibitors can be conjugated to dendritic platforms in order to increase their LPS-neutralizing activity and to improve their toxicity profile. In this chapter we have introduced new PEGylated dendrimers for these purposes. The conjugation of an LPS-inhibitor to one of these platforms resulted in a conjugate with higher capacity to neutralize endotoxins and a reduced toxicity profile.

3. A useful strategy to identify new hits for a certain biological activity is to study commercially available off-patented drugs known to be safe and bioavailable in humans. In this regard, in this chapter we screened the 880 compounds included in the Prestwick Chemical Library as sources of bacterial endotoxin neutralizers. The analysis of this library identified tiratricol, a non-toxic molecule that inhibits the toxic effect of LPS. In addition, the synthesis of analogues of this molecule in order to study structure-activity relationships allows the obtaining of more potent LPS-inhibitors.

4. The conjugation of an LPS-neutralizing peptide described in the previous chapter, RLKWc, with tiratricol rendered a new compound termed RLKWc-ttcol that displays an improved LPS-neutralizing activity over the parent peptide.

4. PEPTOIDS WITH ANTIPROLIFERATIVE ACTIVITY ON CANCER CELLS

4.1 INTRODUCTION

Peptoids: a peptidomimetic approach

In the previous chapters we have studied the use of peptides and peptide-based molecules as LPS-neutralizing agents. First we developed synthetic peptides derived from known LPS-binding proteins such as LALF, BPI or SAP. Next, we described several strategies to obtain inhibitors with improved biological activities and pharmacokinetic profiles. For instance, we designed peptide-based molecules with high potency where several amino acids were replaced by poly(ethylene glycol) PEG linkers and studied the effect of the conjugation of LPS-neutralizing molecules to PEGylated dendrimers. Now, in the last chapter of this Doctoral Thesis, we will study the use of peptidomimetics as another strategy to obtain effective LPS-neutralizing agents that display proteolytic stability.

Peptidomimetics are molecules designed to mimic peptides. They typically arise from the modification of an existing peptide in order to alter the molecule's properties. *N*-methylation of peptides is a common strategy for the design of peptidomimetics. Recent examples in the literature show how *N*-methylation of peptides might increase the biological activity and receptor selectivity of peptides,[1,2] and improves their enzymatic stability,[3] permeability[4] and bioavailability.[5] In this regard, an interesting class of peptidomimetics is constituted by oligomers of *N*-alkylglycines, the so-called peptoids.[6,7] The final part of this Thesis explores the use of peptoids as LPS-neutralizing compounds

4.1.1 PEPTOIDS: AN OVERVIEW

Oligomers of *N*-alkylglycines, also known as peptoids, are a very attractive family of peptidomimetics for drug discovery. Peptoids mimic the biological activity of natural peptides, but are stable to proteolysis.[8,9] This advantage can be explained by the fact that in peptoids, the side chain is located on the nitrogen atom, rather than on the α-carbon as in peptides (Figure 4.1).

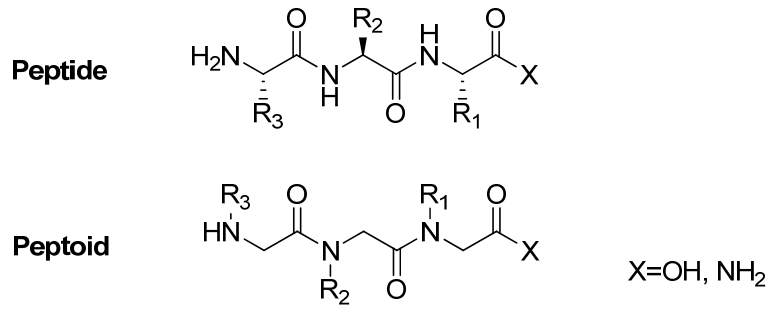

Figure 4.1: Chemical structure of peptides and peptoids

Moreover, peptoids can readily be synthesized on solid-phase using the methodology described by Zuckermann *et al*., which is based on the assembly of two submonomers: a haloacetic acid and a primary amine.[6] Since then, several additional methods have been reported to incorporate other chemical groups other than primary alkyl amines such as heterocyclic side chains[10] or amino acid side chain mimics,[11] in order to obtain more diverse structures.[12] In addition, these reactions are highly automatable, thereby facilitating the design and synthesis of peptoid libraries, whether of single compounds or compound mixtures. For these purposes, MW-assisted peptoid synthesis has recently been proposed to speed up the preparation of such libraries.[13]

Peptoid libraries have been prepared via split-and-mix[14] and positional scanning[15] chemistries and screened for a wide range of biological activities such as Tat/TAR RNA inhibition,[16,17] gene and drug delivery,[18,19] antimicrobial,[20,21] analgesic,[22] multidrug resistance,[23] and anticancer.[24,25]Recently, Pérez-Payá's group added a new biological property to this list describing the first example of peptoids with anti-endotoxin activity in the literature.[26] In that study the authors identified two peptoids, peptoids **4** and **7** (Figure 4.2), which decreased serum levels of TNF-α in an *in vivo* murine model of septicemia.

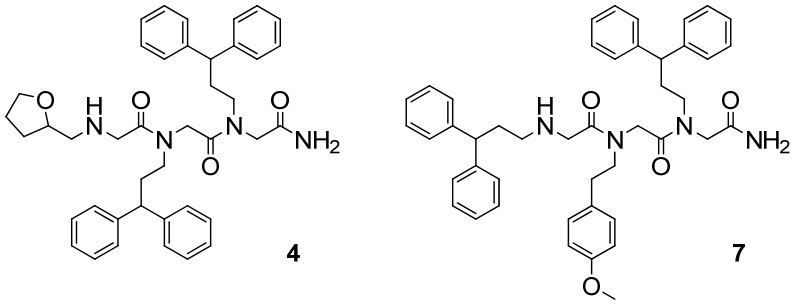

Figure 4.2: Structures of peptoids **4** and **7**. From ref [26].

4.1.2 A SERENDIPITOUS FINDING

As a continuation of this research we examined the possibility to identify shorter peptoids, monomers and dipeptoids, derived from the aforementioned tripeptoids **4** and **7**. We reasoned that smaller peptoids could overcome the problems in solubility observed for peptoid **7**,[26] have improved toxicity profiles and retain the capacity to neutralize bacterial endotoxins. We therefore synthesized a series of peptoids on solid-phase following the methodology of Zuckermann *et al*.[6] with slight modifications. Two sorts of peptoids were prepared (Figure 4.3): dipeptoids with unsubstituted carboxamides at the C-terminal (prepared with Rink resin) and dipeptoids and peptoid monomers with C-terminal *N*-substituted carboxamides (prepared with BAL resin[27,28]).

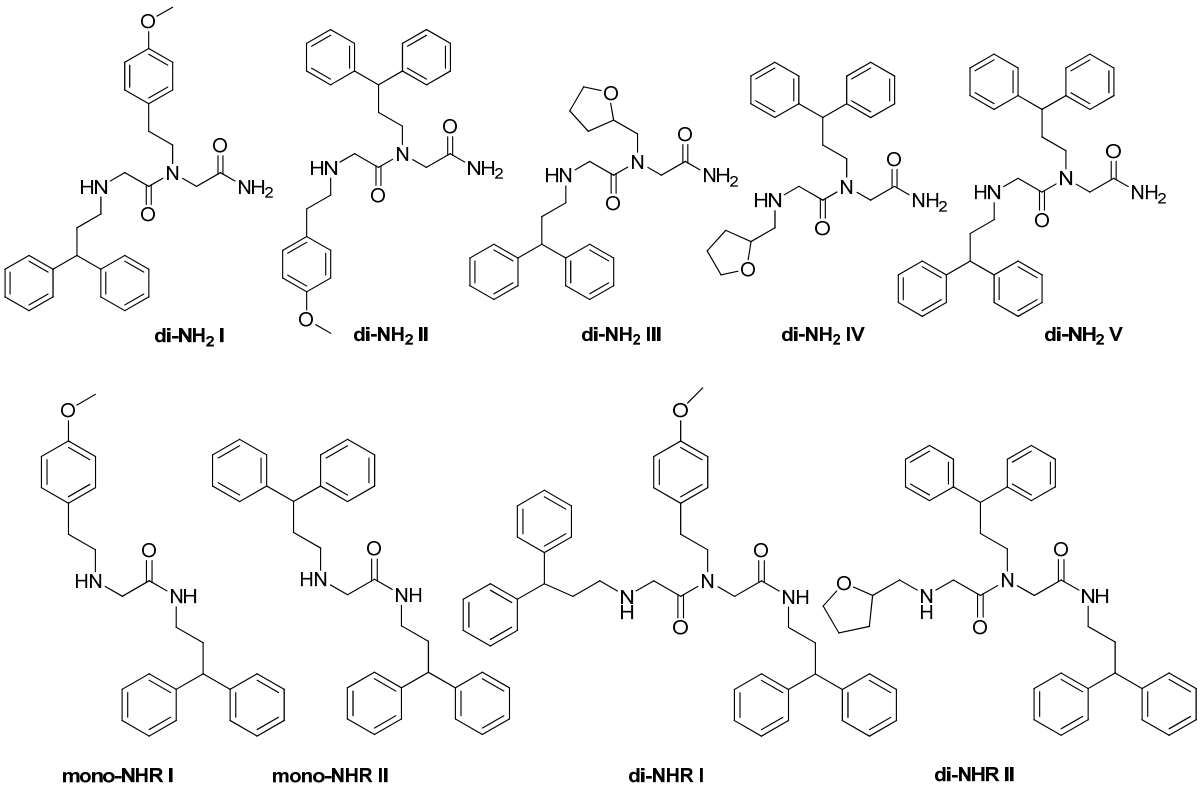

Figure 4.3: Chemical structure of the peptoids prepared. Two different kinds of peptoids were synthesized: C-terminal primary amide peptoids (top) and C-terminal *N*-alkyl amide peptoids (bottom).

Thus, peptoids were assayed for LPS-neutralizing activity using the standard LAL assay.[29] None of the peptoids showed capacity to neutralize LPS. This result was not only frustrating but surprising given the chemical similarity of peptoids **4** and **7** with, for example, peptoids **di-NHR I** and **di-NHR II** (see Figure 4.3). The exact mechanism of action of peptoids **4** and **7** is unknown; however, the authors demonstrated that they do not bind to lipid A and consequently may bind to the hydrophilic carbohydrate portion of LPS.[26] With this information, we were unable to find a reasonable explanation for the lack of activity of the compounds designed.

Hence, we went over the literature again. We found that several tripeptoid families displayed a great diversity of biological activities,[17,20,22,23,24,26] thereby suggesting a somewhat "promiscuous" behavior for these compounds. In particular, a tripeptoid family with pro-apoptotic properties on tumor cells, which was similar in chemical structure to our peptoids, was found[24] (Figure 4.4).

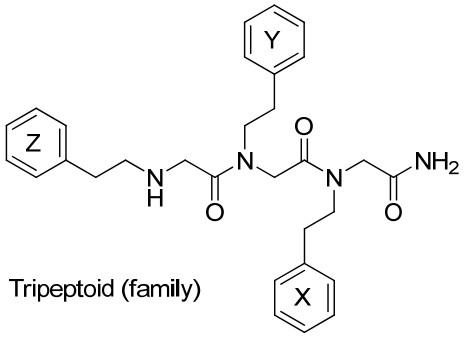

Figure 4.4: Chemical structure of a tripeptoid family with pro-apoptotic properties. From ref [24].

Therefore, we analyzed the biological activity of our peptoids for this new therapeutic application. This time results were much improved. In the following pages we will discuss the design and the synthesis of new small peptoids with promising antiproliferative activity against representative human neoplastic cell lines.

4.1.3 OBJECTIVES

The objectives of the present chapter are:

1. To assay small peptoid monomers and dipeptoids for antiproliferative activity against representative human neoplastic cell lines and to compare the biological activity of C-terminal primary amide and C-terminal *N*-alkyl amide peptoids.

2. To evaluate whether these kinds of peptoids suffer or not from diketopiperazine (DKP) formation, a common side-reaction for dipeptoids, and to correlate this behavior with their biological profiles.

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4.2 RESULTS AND DISCUSSION

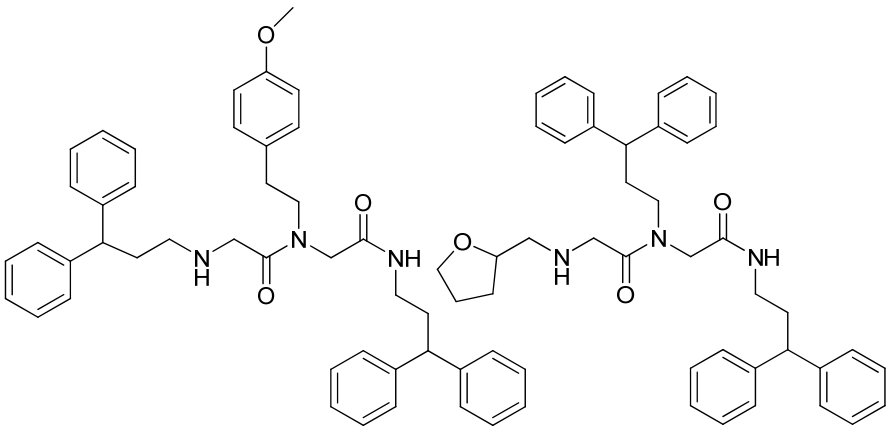
"The smallest peptoids with antiproliferative activity on human neoplastic cells"

Carlos Mas-Moruno, Luis J. Cruz, Puig Mora, Andres Francesch, Àngel Messeguer, Enrique Pérez-Payá, Fernando Albericio

Journal of Medicinal Chemistry, **2007**, vol. *50*(10), pp. 2443-2449.

RESUM

El següent treball descriu el disseny i la síntesi d'una sèrie de mono- i dipeptoids. L'activitat biològica d'aquests compostos va ser avaluada vers diferents línies cel·lulars tumorals. Els compostos C-terminal *N*-alquil amida van demostrar ser citotòxics per aquestes cèl·lules i són els peptoids de cadena més curta descrits a la literatura amb activitat anti-tumoral. Aquests compostos van ser sintetitzats en fase sòlida utilitzant la resina BAL. Gràcies a la seva estructura química aquests tipus de compostos no pateixen la formació de DKPs, una problemàtica reacció secundaria freqüentment observada en la síntesi de pèptid i peptoids.



di-NHR I **di-NHR II**

Contribucions a aquest treball:

- En Carlos Mas va realitzar el disseny, la síntesi i la caracterització de tots els compostos descrits, així com els estudis de formació de DKP i l'assaig d'electroforesi en gels d'agarosa. Va portar tot el pes de la preparació del manuscrit.

- Els assaigs biològics d'aquest treball van ser duts a terme per la Dra. Puig Mora i pel Dr. Andrés Francesch (PharmaMar).

Smallest Peptoids with Antiproliferative Activity on Human Neoplastic Cells

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Received October 8, 2006

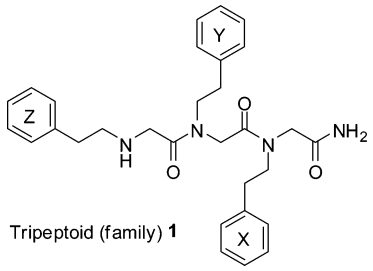
Libraries of new, small peptoid monomers and dipeptoids were synthesized and assayed for antiproliferative activity against representative human neoplastic cell lines. The C-terminal *N*-alkyl amide peptoids are cytotoxic and are the smallest peptoids reported to have such activity. These compounds were conveniently synthesized on a BAL resin. Owing to their structure, the peptoids did not suffer from DKP formation, a problematic side reaction typically observed in peptide and peptoid synthesis.

Introduction

Oligomers of *N*-alkylglycines, also known as peptoids, are a very attractive family of peptidomimetics for drug discovery. Peptoids mimic the biological activity of natural peptides, but are stable to proteolysis.[1,2] This advantage can be explained by the fact that in peptoids, the side chain is located on the nitrogen atom, rather than on the α-carbon as in peptides. Moreover, peptoids can readily be synthesized on solid-phase using the method of Zuckermann et al., which is based on the assembly of two submonomers: a haloacetic acid and a primary amine.[3] These reactions are highly automatable, facilitating the design and synthesis of peptoid libraries, whether of single compounds or compound mixtures. Peptoid libraries have been prepared via split-and-mix[4] and positional scanning[5] chemistries and screened for a wide range of biological activities such as Tat/TAR RNA inhibition,[6−8] gene delivery,[9] antimicrobial,[10−12] analgesic,[13,14] multidrug resistance,[15,16] anti-endotoxin,[17] and anticancer.[18]

In an ongoing research program performed in one of our laboratories, the tripeptoid family **1** (Figure 1) was shown to have pro-apoptotic and other activities.[18] Herein we focus on the identification of cytotoxic peptoid monomers and dipeptoids, which we hoped would have higher activity and solubility and less unspecific toxicity than some of the aforementioned tripeptoids.

To the best of our knowledge, no active dimers of *N*-alkylglycines have been described in the literature to date. In fact, the smallest peptoids reported are trimers.[12] A possible explanation for the scarcity of dipeptoids in the literature is their tendency to form diketopiperazines (DKPs).[19] Formation of DKPs, which are cyclic dipeptides, is highly influenced by amino acid structure. For example, amino acids that easily adopt an amide bond in the *cis*-configuration, such as *N*-alkylamino acids, favor cyclization.[20,21] Hence, special care should be taken to avoid this side reaction.



Tripeptoid (family) **1**

Figure 1. Chemical structure of the tripeptoid family **1**. Chemical diversity at specific positions is defined here as X, Y, and Z.

Described herein are the preparation, screening for cytotoxicity against selected representative human neoplastic cell lines, and resulting SAR of two libraries of new mono- and dipeptoids.

Results and Discussion

Design and Synthesis of the Peptoid Libraries. All libraries were synthesized following the methodology of Zuckermann et al. with slight modifications, as indicated in Scheme 1.[3] The dipeptoids with unsubstituted carboxamides at the C-terminal were prepared on Rink-resin. Alternatively, C-terminal *N*-substituted carboxamides were prepared using a similar strategy but on BAL-resin.[22,23] In this case, the first amine was incorporated onto the aldehyde-based resin by reductive amination using $NaCNBH_3$ as reducing agent. In the acylation step, the best results for the formation of the active symmetric anhydride of the chloroacetic acid were obtained with DCM. The urea byproduct formed in the pre-activation is insoluble in DCM and was thus removed by single filtration before reaction. Amines were added in the presence of triethylamine (TEA) to neutralize the hydrochloric acid formed in situ. Good yields and purity, as observed by HPLC, were obtained after two treatments (2×5 h). The final cleavage was accomplished using TFA−H_2O (95:5) for 1 h at 25 °C.

As amines, 3,3-diphenylpropylamine (**2**), 2-(4-methoxyphenyl)ethylamine (**3**), and 2-(aminomethyl)tetrahydrofuran (**4**) were used because they showed interesting biological activity in previous studies as inducers of apoptosis (Figure 2).[18]

For the dipeptoids synthesized using the Rink resin ($R_1 = H$), amine **2** was introduced either as R_2 or R_3. A total of five dipeptoids were therefore prepared and characterized (Figure 3).

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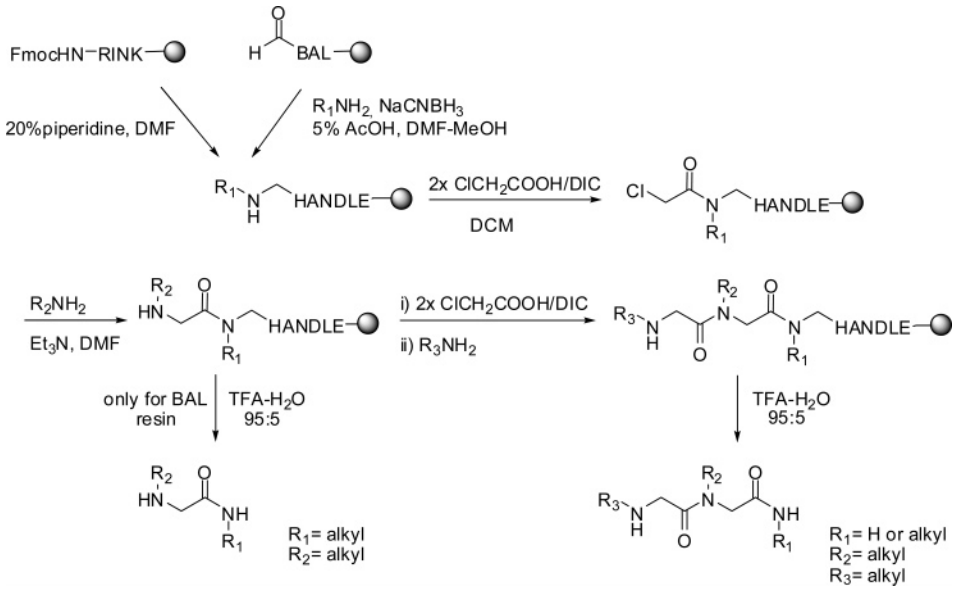
Scheme 1. Synthesis of the Peptoid Libraries



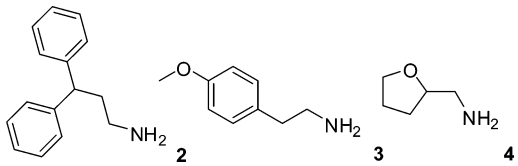


Figure 2. Primary amines used in the design and synthesis of the libraries.

As the peptoid backbone can be considered as a scaffold for aromatic systems, we sought to prepare a series of peptoids substituted at the C-terminal amide (R_1 = alkyl, Scheme 1). Thus, two dipeptoids and two *N*-alkylglycine amides (the latter are monomers and can thus be considered as *N*-substituted monopeptoids) with **2** at the C-terminal were prepared on BAL resin (Scheme 1). The structures of the dipeptoids are shown in Figure 4.

DKP Formation Studies. Finally, cyclic dipeptoids, which are *N*-alkyl-DKPs, were synthesized (Figure 5). This was easily accomplished by taking advantage of the tendency of *N*-alkyldipeptides to form DKPs via cyclization.[20]

To optimize the minimization and the formation of DKPs and to improve the understanding of the stability of these *N*-alkyl-DKPs, we first studied the formation of DKP-01. As DKPs can be formed either in basic[24] or acidic[25] media, three

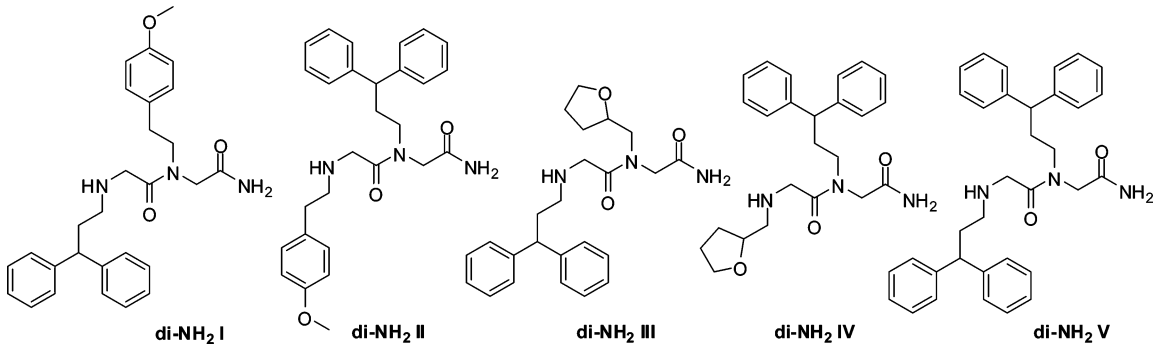


Figure 3. Chemical structures in the first dipeptoids library.

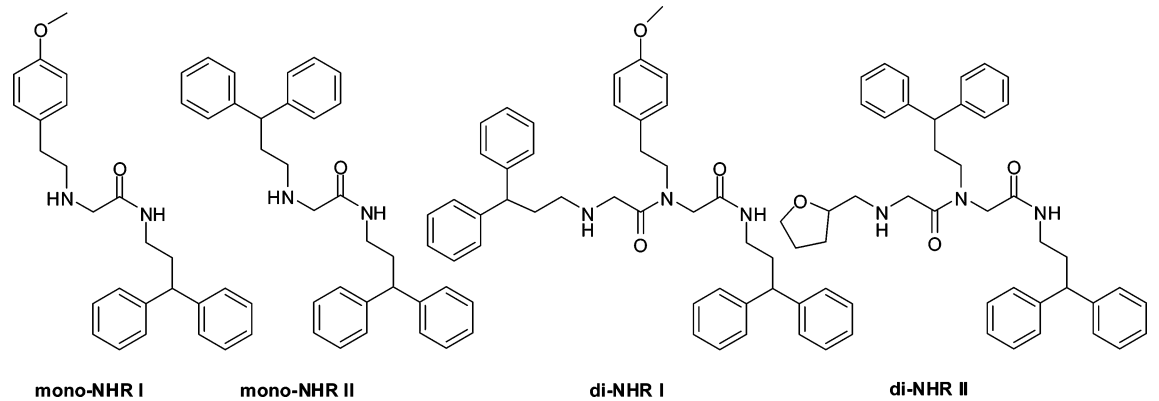


Figure 4. Chemical structures in the second dipeptoids library.

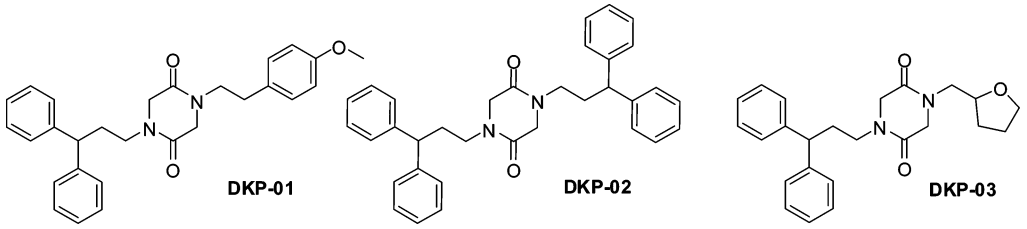


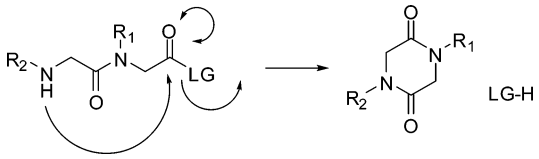
Figure 5. Chemical structures of the *N*-alkyl-DKPs synthesized.

Table 1. DKP Formation Study at $t = 0$ h and at $t = 24$ h

peptoid		DKP formation at $t = 0$ h	DKP formation at $t = 24$ h
di-NH_2 I	H_2O-CH_3CN	<2%	<2%
	10% $NaHCO_3$	37%	98%
	10% TFA	4%	53%
di-NH_2 II	H_2O-CH_3CN	<2%	<2%
	10% $NaHCO_3$	31%	100%
	10% TFA	9%	49%
di-NHR I	H_2O-CH_3CN	ND[a]	ND
	10% $NaHCO_3$	ND	ND
	10% TFA	ND	ND

[a] ND: not detected by HPLC.

Scheme 2. DKP Formation by a Nucleophilic Addition/Elimination Mechanism



conditions were tested: H_2O-CH_3CN (1:1), 10% $NaHCO_3$ in H_2O-CH_3CN (1:1), and 10% TFA in H_2O-CH_3CN (1:1). Peptoids (di-NH_2 I, di-NH_2 II, and di-NHR I) were dissolved in the above mixtures and left to stir for 24 h. DKP formation was followed by HPLC (see the Supporting Information). The results are shown in Table 1.

Peptoids di-NH_2 I and di-NH_2 II were both stable in neutral aqueous media. However, in basic or acidic media, their corresponding DKPs formed rapidly: in basic conditions, conversions were quantitative after 24 h. In contrast, no DKP formation was observed with peptoid di-NHR I in any of the conditions.

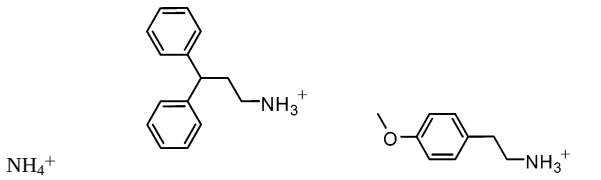
DKP formation involves nucleophilic attack of the terminal carbonyl group by the terminal amino group, followed by displacement of a leaving group (LG), as shown in Scheme 2.

Therefore, the reaction depends on the nucleophilicity of the amino group and the quality of the LG. Peptoids di-NH_2 I and di-NH_2 II, which have the same LG (NH_2) but differ in their nucleophiles (3,3-diphenylpropylamine (**2**) and 2-(4-methoxyphenyl)ethylamine (**3**), respectively), did not exhibit any differences in DKP formation. However, di-NH_2 I and di-NHR I, which contain distinct LGs but the same nucleophile, differed dramatically in DKP formation. These results support the notion that the characteristics of the LG are critical for DKP formation. The results were confirmed for the formation of DKP-03, whereby di-NH_2 III and di-NH_2 IV also gave the expected DKPs in contrast to di-NHR II, which did not react.

To analyze these results in terms of LG quality, the pK_a of each protonated LG was calculated (Table 2).

Although the pK_a values did not differ greatly, ammonia is a weaker base than the other alkyl-amines and, hence, a stronger acid. In addition, the kinetic effect should also be highlighted because the carbonyl moiety is more easily accessible in a primary amide bond, whereas it is partially masked in an alkyl-amide bond, thereby hindering nucleophilic attack. Thus, both parameters led us to conclude that NH_2 is clearly a better leaving group than the aforementioned alkylamines.

Table 2. pK_a Values of Ammonia and of Primary Amines **2** and **3** According to the pK_{alc} Module[a]



NH_4^+		
pKa: 9.24	pKa: 9.96	pKa: 9.85

[a] PALLAS, version 2.0, CompuDrug.

Table 3. In Vitro Cytotoxicity of the Peptoid Libraries in Adherent Cell Lines

	cytotoxicity GI_{50}[a] (μM)		
peptoid	MDA-MB-231	A549	HT29
Trimer I	2.72	2.57	2.27
Trimer II	1.32	1.41	1.41
di-NH_2 I	19.1	N.D.[b]	9.14
di-NH_2 II	9.36	9.36	9.14
di-NH_2 III	N.D.	N.D.	N.D.
di-NH_2 IV	N.D.	N.D.	N.D.
di-NH_2 V	3.08	3.08	1.64
mono-NHR I	2.73	3.73	1.89
mono-NHR II	3.46	3.46	3.03
di-NHR I	1.02	1.02	1.04
di-NHR II	1.06	1.06	0.94
DKP-01	N.D.	N.D.	N.D.
DKP-03	N.D.	N.D.	N.D.

[a] All assays were carried out in triplicate at 10 concentrations. The 30 points obtained were used for curve calculation and, hence, GI_{50} values determination using LIMS (Laboratory Information Management Systems) software from PharmaMar. [b] N.D. = not detected at the experimental conditions.

DKPs were then synthesized in basic medium (see above), using an adaptation of a literature procedure.[26] All reactions were monitored by HPLC, and the conversions were quantitative. However, the very hydrophobic DKP-02 was insoluble in all the aqueous media assayed as well as in $H_2O-DMSO$ mixtures and so was discarded for biological purposes.

Cytotoxicity Screening of the Peptoid Libraries. A panel of human tumor cell lines was used to evaluate the cytotoxicity of the peptoid libraries using a conventional colorimetric assay (see Experimental Section). The cell growth assays comprised three different adherent cell lines (MDA-MB-231 breast adenocarcinoma, A-549 lung carcinoma NSCL, and HT-29 colon carcinoma) and two non-adherent cell lines (Jurkat T-lymphoid and HL60 promyelocytic leukemia).

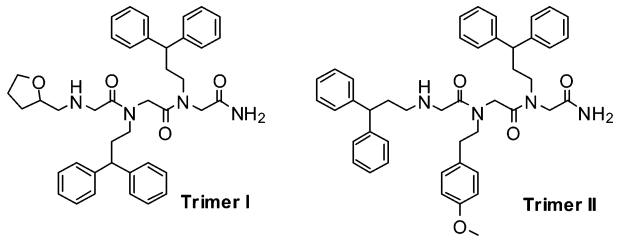
GI_{50} values (i.e., the drug concentration at which 50% of cell growth is inhibited) were determined after 72 h of continuous exposure of the adherent (Table 3) and nonadherent cell lines (Table 4) to the test molecules. The tripeptoids Trimer I and II, derived from family **1** and used in another project (for more

Table 4. In Vitro Cytotoxicity of the Peptoid Libraries in Nonadherent Cell Lines

peptoid	cytotoxicity GI_{50}[a] (μM) JURKAT	HL60
Trimer I	21.3	3.55
Trimer II	6.68	21.8
di-NH_2 I	33.9	31.1
di-NH_2 II	27.3	29.1
di-NH_2 III	N.D.[b]	N.D.
di-NH_2 IV	N.D.	N.D.
di-NH_2 V	10.5	13.2
mono-NHR I	18.0	10.6
mono-NHR II	10.2	12.6
di-NHR I	3.75	2.94
di-NHR II	5.83	23.0
DKP-01	N.D.	N.D.
DKP-03	N.D.	N.D.

[a] All assays were carried out in triplicate at 10 concentrations. The 30 points obtained were used for curve calculation and hence GI_{50} values determination using LIMS (Laboratory Information Management Systems) software from PharmaMar. [b] N.D. = not detected at the experimental conditions.

details, see ref 17, in which Trimers I and II appear as peptoids 4 and 7, repectively), were included in the test as positive controls.



As seen in Table 3, several of the test peptoids had at least moderate cytotoxicity. Among the di-NH_2 series of C-terminal primary amide dipeptoids, there was a clear relationship between aromaticity and activity: compound V was the most active, while compounds I, II, III, and IV were less or inactive. Whereas compound V contains two diphenyl groups, the others only have one, suggesting the importance of the diphenyl group for cytotoxicity. Moreover, compounds I and II, which contain a methoxyphenyl, were more active than III and IV, which instead contain a tetrahydrofuranyl.

In contrast, all of the C-terminal *N*-alkyl amide peptoids were cytotoxic. Peptoids di-NHR I and di-NHR II showed the same activity as the control tripeptoids. Compounds mono-NHR I and mono-NHR II exhibited moderate activities, similar to or even higher than their dipeptoid analogs (di-NH_2 II and di-NH_2 V, respectively), even though they are formed by only a single *N*-alkyl glycine amide. Dipeptoids di-NHR I and di-NHR II contain two diphenyl groups, which, as mentioned above, are key residues for the activity. di-NHR I also contains an extra aromatic group. Thus, the peptoids synthesized on BAL resin, which are smaller than their primary amide analogs but have the same number of substituents, had equal or greater original activities. DKPs 01 and 03 were also tested in these cell lines, but did not exhibit any cytotoxicity.

Several of the compounds synthesized also exhibited cytotoxicity in nonadherent cell lines (see Table 4). Although values of cytotoxicity for these cell lines were generally lower, the data is consistent with the results discussed above. Thus, di-NH_2 peptoids did not present any relevant activity, with the exception of compound di-NH_2 V, which was again the most active of its family. The C-terminal *N*-alkyl amides peptoids were more cytotoxic than their primary amide analogs. Interestingly, the peptoid di-NHR II was specific for JURKAT cell lines. Trimer II was also more active against JURKAT cells. In contrast, Trimer I was six times more active against HL60 cells than against JURKAT cells. DKPs 01 and 03 were also tested in these cell lines but did not show any cytotoxicity, as observed for the adherent cell lines.

Table 5. Antimitotic Activity of Select Peptoids in HeLa Cells

code	antimitotic activity IC_{50}[a] (μM)
di-NH_2 V	0.154
mono-NHR I	0.994
mono-NHR II	0.035
di-NHR I	0.025

[a] All assays were carried out in triplicate at 10 concentrations. The 30 points obtained were used for curve calculation and hence IC_{50} values determination using LIMS (Laboratory Information Management Systems) software from PharmaMar.

According to previously published results,[18] the Trimer family **1** is able to arrest G1 and induce apoptosis in several human cancer cell lines. As the peptoids in the libraries are derived from this family, we performed another assay using HeLa cells to determine if they also exhibited antimitotic activity. Several of the most active peptoids from above showed a high level of antimitotic activity (see Table 5).

The fact that none of the inactive peptoids (di-NH2 I, di-NH2 II, di-NH2 III, and di-NH2 IV) showed antimitotic activity reinforces the idea that the active ones may somehow interfere with the cell cycle and/or the apoptosis machinery. Nevertheless, further experiments are required to determine the mechanism of action of these molecules, which is outside of the scope of the present work. To discard any possible interaction with the DNA of the tumor cells used, peptoids were incubated with pBR322 DNA plasmid, and their interaction was analyzed by electrophoresis. The electrophoretic mobility pattern showed that none of the peptoids were able to bind to the DNA (see the Supporting Information).

Solubility Assay. The aqueous solubility of the peptoid libraries and the two tripeptoids was evaluated by turbidity measurements. The peptoids were dissolved in water−DMSO (4:1), and their absorbance at 620 nm was analyzed at different concentrations (0.05−5000 μM). Interestingly, the library peptoids were much more water soluble than the reference tripeptoids. All peptoids from the libraries showed greater than 10-fold solubility compared to Trimer I and greater than 20-fold solubility compared to Trimer II.

Conclusions

To the best of our knowledge, this is the first report of a dipeptoid or *N*-alkyl glycine amide with antiproliferative activity. In fact, such compounds showed to be cytotoxic and antimitotic in a panel of representative human neoplastic cell lines. The observation that C-terminal *N*-alkyl amide peptoids do not form DKPs suggests that other bioactive tripeptoid families described in the literature could also be developed into DKP-free, bioactive peptoid monomers and dipeptoids. The present results also highlight the utility of BAL resins for the synthesis of peptoid monomers and dipeptoids. The insertion of an alkyl group at the C-terminal amide provides an extra group for biological interaction without increasing the length of the peptoid chain, which also leads to marked improvements in aqueous solubility.

Experimental Section

General. Solvents, amines, and other reagents were purchased from commercial suppliers and used without further purification.

HPLC was performed using a Waters Alliance 2695 chromatography system with a PDA 995 detector, a reverse-phase Symmetry C_{18} (4.6 × 150 mm) 5-μm column, and linear gradient (MeCN with 0.036% TFA into H_2O with 0.045% TFA). The system was run at a flow rate of 1.0 mL/min from 0% to 100% over 15 min. HPLC-MS was performed using a Waters Alliance 2796 with a UV/vis detector 2487 and ESI-MS Micromass ZQ (Waters) chromatography system, a reversed-phase Symmetry 300 C_{18} (3.9 × 150 mm) 5-μm column, and H_2O with 0.1% formic acid and MeCN with 0.07% formic acid as mobile phases. Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer and high-resolution mass spectra (HRMS) were obtained at the Mass Spectrometry Service of the University of Santiago de Compostela (Spain). The ^{1}H and ^{13}C NMR spectra of peptoids were recorded using a Varian Mercury 400 apparatus (400 MHz, $CDCl_3$ or CD_3OD). The conformations of the peptoids led to the observation of complex absorption (ca), the assignments of which were confirmed by gCOSY and gHSQC experiments.

Solid-Phase Synthesis of Peptoid Libraries. Peptoids were manually synthesized on solid phase in polypropylene syringes, each of which was fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Washes between reaction steps were done with DMF and DCM, using 10 mL of solvent/g of resin per wash. The dipeptoid amide library was synthesized on Rink amide aminomethyl resin (100 mg, 0.7 mmol/g). The Fmoc group was initially removed by treatment with piperidine−DMF (1:4) for 20 min. The alkyl-amide library was synthesized on BAL aminomethyl resin (100 mg, 1 mmol/g).

Reductive Amination on BAL Resin. The primary amine (5 equiv) was dissolved in a mixture of MeOH−DMF (1:1) and added to the resin, which was pre-activated with a mixture of MeOH−DMF−AcOH (47.5:47.5:5). Solid $NaCNBH_3$ (5 equiv) was then added to the imine, and the resin was stirred overnight at room temperature. Reaction completion was confirmed using the Vazquez test.[27]

Acylation Steps. Acylation steps were accomplished after 2 × 1 h treatment at room temperature with chloroacetic acid (20 equiv) and DIC (10 equiv) in DCM. The resulting insoluble urea was filtered off before adding the mixture to the resin. The reaction was monitored by the Kaiser[28] or de Clercq[29] tests.

Insertion of Primary Amines. The chloromethyl intermediate was aminated by adding the primary amine (5 equiv) in the presence of TEA (5 equiv) in DMF (2 × 5 h treatment). The reaction was monitored using the chloranil[30] or de Clercq tests and quantified by HPLC.

Cleavage of the Peptoids. The resin was washed with DCM (3 × 1), dried, and treated with TFA−H_2O (19:1) for 1 h. TFA was then removed by evaporation, and peptoids were dissolved in H_2O−MeCN (1:1), filtered, and lyophilized. Peptoids were purified by semipreparative HPLC when purities obtained were below 80%.

Characterization. Peptoid libraries were characterized by ^{1}H NMR, ^{13}C NMR, HPLC, HPLC-MS, and MALDI-TOF and/or HRMS.

[*N*-(3,3-Diphenylpropyl)glycyl]-*N*-(4-methoxyphenethyl)glycinamide (di-NH$_2$ I). ^{1}H NMR ($CDCl_3$): 7.25−7.16 (m, 10H), 6.95 (d, J = 8.2 Hz, 2H), 6.74 (d, J = 8.2 Hz, 2H), 3.88 (ca, *CH*Ph$_2$), 3.81−3.32 (ca, 2 × CH_2CON), 3.67 (s, OCH_3), 3.32 (ca, *CH*$_2$CH$_2$-PhOMe), 2.66 (ca, CH$_2$*CH*$_2$PhOMe), 2.66 (ca, *CH*$_2$CH$_2$CHPh$_2$), 2.37 (ca, CH$_2$*CH*$_2$CHPh$_2$). ^{13}C NMR ($CDCl_3$): 171.34 ($CONH_2$), 166.33 (CO), 158.72 (C_{Ar}), 142.85 (2 × C_{Ar}), 129.85 (CH_{Ar} × 2), 129.22 (C_{Ar}), 128.76 (CH_{Ar} × 4), 127.50 (CH_{Ar} × 4), 126.74 (CH_{Ar} × 2), 114.38 (CH_{Ar} × 2), 55.16 (OCH_3), 50.62 (*CH*$_2$CONH$_2$) 49.24 (*CH*$_2$CON), 48.47 (*CH*Ph$_2$), 47.39 (*CH*$_2$CH$_2$PhOMe), 46.92 (*CH*$_2$-CH$_2$CHPh$_2$), 33.07 (CH$_2$*CH*$_2$PhOMe), 31.24 (CH$_2$*CH*$_2$CHPh$_2$). Purity of 98% (t_R = 9.53 min). HRMS [M + H]$^+$ calcd for $C_{28}H_{33}N_3O_3$, 460.2595; found, 460.2592.

[*N*-(4-Methoxyphenethyl)glycyl]-*N*-(3,3-diphenylpropyl)glycinamide (di-NH$_2$ II). ^{1}H NMR ($CDCl_3$): 7.27−7.13 (m, 10H), 7.07 (d, J = 8.2 Hz, 2H), 6.80 (d, J = 8.2 Hz, 2H), 3.88 (ca, *CH*Ph$_2$), 3.74 (s, OCH_3), 3.70−3.47 (ca, 2 × CH_2CON), 3.16 (ca, *CH*$_2$CH$_2$CHPh$_2$), 2.98 (ca, *CH*$_2$CH$_2$PhOMe), 2.94 (ca, CH$_2$*CH*$_2$-PhOMe), 2.29 (ca, CH$_2$*CH*$_2$CHPh$_2$). ^{13}C NMR ($CDCl_3$): 171.62 ($CONH_2$), 166.21 (CO), 158.93 (C_{Ar}), 143.51 (2 × C_{Ar}), 129.95 (CH_{Ar} × 2), 129.14 (CH_{Ar} × 4), 128.35 (C_{Ar}), 127.88 (CH_{Ar} × 4), 127.05 (CH_{Ar} × 2), 114.51 (CH_{Ar} × 2), 55.47 (OCH_3), 55.47 (*CH*$_2$-CONH$_2$), 49.40 (*CH*$_2$CON), 48.19 (*CH*Ph$_2$), 47.64 (*CH*$_2$CH$_2$-PhOMe), 47.61 (*CH*$_2$CH$_2$CHPh$_2$), 33.77 (CH$_2$*CH*$_2$CHPh$_2$), 31.36 (CH$_2$*CH*$_2$PhOMe). Purity of 82% (t_R = 9.56 min). HRMS [M + H]$^+$ calcd for $C_{28}H_{33}N_3O_3$, 460.2595; found, 460.2594.

[*N*-(3,3-Diphenylpropyl)glycyl]-*N*-(4-tetrahydrofurfuryl)glycinamide (di-NH$_2$ III). ^{1}H NMR ($CDCl_3$): 7.25−7.15 (m, 10H), 4.14 (ca, *CH*Ph$_2$), 4.01 (ca, OCH), 3.77−3.67 (ca, OCH_2), 3.63−3.60 (ca, CH_2CON, OCH*CH*$_2$N), 3.29 (ca, CH_2CON), 2.98 (ca, *CH*$_2$-CH$_2$CHPh$_2$), 2.59 (ca, CH$_2$*CH*$_2$CHPh$_2$), 1.92−1.39 (ca, OCH$_2$-CH$_2$*CH*$_2$), 1.85 (ca, OCH$_2$*CH*$_2$). ^{13}C NMR ($CDCl_3$): 172.09 ($CONH_2$), 167.05 (CO), 143.29 (2 × C_{Ar}), 128.98 (CH_{Ar} × 4), 127.93 (CH_{Ar} × 4), 126.91 (CH_{Ar} × 2), 76.69 (OCH), 68.40 (OCH_2), 53.46 (*CH*$_2$CONH$_2$), 50.47 (OCH*CH*$_2$N), 48.86 (*CH*Ph$_2$), 48.24 (*CH*$_2$CON), 47.34 (*CH*$_2$CH$_2$CHPh$_2$), 31.51 (CH$_2$*CH*$_2$CHPh$_2$), 29.13 (OCH$_2$CH$_2$*CH*$_2$), 25.83 (OCH$_2$*CH*$_2$). Purity of 84% (t_R = 8.63 min). HRMS [M + H]$^+$ calcd for $C_{24}H_{31}N_3O_3$, 410.2438; found, 410.2437.

[*N*-(4-Tetrahydrofurfuryl)glycyl]-*N*-(3,3-diphenylpropyl)glycinamide (di-NH$_2$ IV). ^{1}H NMR (CD_3OD): 7.34−7.13 (m, 10H), 4.09 (ca, OCH), 4.00 (ca, CH_2CONH_2), 3.97 (ca, *CH*Ph$_2$) 3.90−3.81 (ca, OCH_2), 3.35−3.25 (m, *CH*$_2$CH$_2$CHPh$_2$), 3.31 (ca, CH$_2$-CON) 3.13−2.93 (m, OCH*CH*$_2$N), 2.40−2.32 (m, CH$_2$*CH*$_2$CHPh$_2$), 2.10−1.60 (m, OCH$_2$CH$_2$*CH*$_2$), 1.95 (m, OCH$_2$*CH*$_2$). ^{13}C NMR (CD_3OD): 172.97, 172.39 ($CONH_2$), 167.33, 166.79 (CO), 145.77, 145.37 (2 × C_{Ar}), 129.85, 129.59 (CH_{Ar} × 4), 128.85, 128.80 (CH_{Ar} × 4), 127.71, 127.41 (CH_{Ar} × 2), 75.29 (OCH), 69.55 (OCH_2), 52.52 (OCH*CH*$_2$N), 50.61 (*CH*Ph$_2$), 49.59 (*CH*$_2$CONH$_2$), 48.50 (*CH*$_2$CON), 48.24 (*CH*$_2$CH$_2$CHPh$_2$), 34.56 (CH$_2$*CH*$_2$CHPh$_2$), 30.28 (OCH$_2$CH$_2$*CH*$_2$), 26.43 (OCH$_2$*CH*$_2$). Purity of 98% (t_R = 8.57 min). HRMS [M + H]$^+$ calcd for $C_{24}H_{31}N_3O_3$, 410.2438; found, 410.2438.

[*N*-(3,3-Diphenylpropyl)glycyl]-*N*-(3,3-diphenylpropyl)glycinamide (di-NH$_2$ V). ^{1}H NMR ($CDCl_3$): 7.24−7.13 (m, 20H), 3.91 (ca, *CH*Ph$_2$ (C_t)), 3.75 (ca, *CH*Ph$_2$), 3.75 (ca, CH_2CONH_2), 3.20 (CH_2CON), 3.06 (ca, *CH*$_2$CH$_2$CHPh$_2$(C_t)), 2.64 (ca, *CH*$_2$CH$_2$-CHPh$_2$), 2.41 (ca, CH$_2$*CH*$_2$CHPh$_2$), 2.22 (ca, CH$_2$*CH*$_2$CHPh$_2$ (C_t)). ^{13}C NMR ($CDCl_3$): 171.19 ($CONH_2$), 165.92 (CO), 143.18 (2 × C_{Ar}), 142.89 (2 × C_{Ar}), 128.86 (CH_{Ar} × 4), 128.81 (CH_{Ar} × 4), 127.61 (CH_{Ar} × 8), 127.48 (CH_{Ar} × 2), 126.81 (CH_{Ar} × 2), 49.19 (*CH*$_2$CONH$_2$), 48.65 (*CH*Ph$_2$ (C_t)), 47.79 (*CH*Ph$_2$), 47.39 (*CH*$_2$-CON), 47.06 (*CH*$_2$CH$_2$CHPh$_2$), 46.86 (*CH*$_2$CH$_2$CHPh$_2$(C_t)), 33.45 (CH$_2$*CH*$_2$CHPh$_2$(C_t)), 31.20 (CH$_2$*CH*$_2$CHPh$_2$). Purity of 88% (t_R = 10.72 min). HRMS [M + H]$^+$ calcd for $C_{34}H_{37}N_3O_2$, 520.2959; found, 520.2958.

***N*-(4-Methoxyphenethyl)glycin[*N*-(3,3-diphenylpropyl)]amide (mono-NHR I).** ^{1}H NMR ($CDCl_3$): 7.25−7.12 (m, 10H), 7.09 (d, J = 8.2 Hz, 2H), 6.82 (d, J = 8.2 Hz, 2H), 3.91 (ca, *CH*Ph$_2$), 3.76 (s, OCH_3), 3.74−3.71 (ca, CH_2CON), 3.17 (ca, *CH*$_2$-CH$_2$CHPh$_2$), 3.17 (ca, *CH*$_2$CH$_2$PhOMe), 2.97 (ca, CH$_2$*CH*$_2$PhOMe), 2.23 (ca, CH$_2$*CH*$_2$CHPh$_2$). ^{13}C NMR ($CDCl_3$): 164.50 (CO), 158.95 (C_{Ar}), 143.93 (2 × C_{Ar}), 129.76 (CH_{Ar} × 2), 128.59 (CH_{Ar} × 4), 127.67 (CH_{Ar} × 4), 127.17 (C_{Ar}), 126.42 (CH_{Ar} × 2), 114.48 (CH_{Ar} × 2), 55.24 (OCH_3), 49.72 (*CH*$_2$CH$_2$PhOMe), 48.83 (*CH*Ph$_2$), 48.83 (*CH*$_2$CON), 38.89 (*CH*$_2$CH$_2$CHPh$_2$), 34.58 (CH$_2$*CH*$_2$CHPh$_2$), 31.53 (CH$_2$*CH*$_2$PhOMe). Purity of 90% (t_R = 10.09 min). HRMS [M + H]$^+$ calcd for $C_{26}H_{30}N_2O_2$, 403.2380; found, 403.2381.

***N*-(3,3-Diphenylpropyl)glycin[*N*-(3,3-diphenylpropyl)]amide (mono-NHR II).** ^{1}H NMR ($CDCl_3$): 7.25−7.12 (m, 20H), 3.91−3.87 (ca, 2 × *CH*Ph$_2$), 3.53 (ca, CH_2CON), 3.11 (ca, *CH*$_2$-CH$_2$CHPh$_2$(C_t)), 2.85 (ca, *CH*$_2$CH$_2$CHPh$_2$), 2.45 (ca, CH$_2$*CH*$_2$-CHPh$_2$), 2.18 (ca, CH$_2$*CH*$_2$CHPh$_2$ (C_t)). ^{13}C NMR ($CDCl_3$): 164.71 (CO), 144.19 (2 × C_{Ar}), 142.82 (2 × C_{Ar}), 129.08 (CH_{Ar} × 4), 128.83 (CH_{Ar} × 4), 127.90 (CH_{Ar} × 4), 127.71 (CH_{Ar} × 4), 127.12 (CH_{Ar} × 2), 126.66 (CH_{Ar} × 2), 49.12 (*CH*Ph$_2$), 48.94 (CH_2CON), 48.68 (*CH*Ph$_2$), 47.68 (*CH*$_2$CH$_2$CHPh$_2$), 39.11 (*CH*$_2$CH$_2$CHPh$_2$(C_t)), 34.76 (CH$_2$*CH*$_2$CHPh$_2$(C_t)), 31.87 (CH$_2$*CH*$_2$CHPh$_2$). Purity of 89%

(t_R = 11.23 min). HRMS $[M + H]^+$ calcd for $C_{32}H_{34}N_2O$, 463.2744; found, 463.2747.

[*N*-(3,3-Diphenylpropyl)glycyl]-*N*-(4-methoxyphenethyl)glycin[*N*-(3,3-diphenylpropyl)]amide (di-NHR I). ^{1}H NMR (CD_3OD): 7.32−7.13 (m, 20H), 7.11 (d, J = 8.2 Hz, 2H), 6.81 (d, J = 8.2 Hz, 2H), 4.04−3.95 (ca, 2 × $CHPh_2$), 3.73−3.70 (br s, OCH_3), 4.00−3.47 (ca, 2 × CH_2CON), 3.51 (m, CH_2CH_2PhOMe), 3.14 (m, $CH_2CH_2CHPh_2(C_t)$), 2.78 (m, CH_2CH_2PhOMe), 2.73 (m, $CH_2CH_2CHPh_2$), 2.50−2.31 (m, $CH_2CH_2CHPh_2$), 2.27 (m, $CH_2CH_2CHPh_2$ (C_t)). ^{13}C NMR (CD_3OD): 170.33, 169.82 ($CO(C_t)$), 167.32, 166.96 (CO), 160.22, 159.89 (C_{Ar}), 145.93 (2 × C_{Ar}), 144.69 (2 × C_{Ar}), 131.55 (C_{Ar}), 131.30, 130.78 (CH_{Ar} × 2), 129.85 (CH_{Ar} × 4), 129.56 (CH_{Ar} × 4), 128.90 (CH_{Ar} × 4), 128.72 (CH_{Ar} × 4), 127.86 (CH_{Ar} × 2), 127.36 (CH_{Ar} × 2), 115.38, 115.07 (CH_{Ar} × 2), 55.79 (OCH_3), 51.47 (CH_2CON), 50.83 (CH_2CON), 50.12 ($CHPh_2$), 49.87 ($CHPh_2$), 48.52 (CH_2CH_2PhOMe), 47.97 ($CH_2CH_2CHPh_2$), 39.47 ($CH_2CH_2CHPh_2(C_t)$), 36.1 ($CH_2CH_2CHPh_2$ (C_t)), 34.10, 33.67 (CH_2CH_2PhOMe), 32.62 ($CH_2CH_2CHPh_2$). Purity of 91% (t_R = 12.05 min). HRMS $[M + H]^+$ calcd for $C_{43}H_{47}N_3O_3$, 654.3690; found, 654.3688.

[*N*-(4-Tetrahydrofurfuryl)glycyl]-*N*-(3,3-diphenylpropyl)glycin[*N*-(3,3-diphenylpropyl)]amide. (di-NHR II). ^{1}H NMR (CD_3OD): 7.31−7.10 (m, 20H), 4.15−4.07 (ca, OCH), 4.01−4.00 (ca, 2 × CH_2CON), 3.97−3.94 (ca, 2 × $CHPh_2$), 3.80−3.73 (ca, OCH_2), 3.33−3.19 (m, $CH_2CH_2CHPh_2(C_t)$), 3.17−2.83 (m, $OCHCH_2N$), 3.12 (m, $CH_2CH_2CHPh_2$), 2.37−2.26 (m, $CH_2CH_2CHPh_2$), 2.24 (m, $CH_2CH_2CHPh_2$ (C_t)), 2.08−1.57 (m, $OCH_2CH_2CH_2$), 1.93 (m, OCH_2CH_2). ^{13}C NMR (CD_3OD): 170.34, 169.93 ($CO(C_t)$), 167.30, 166.76 (CO), 145.90 (2 × C_{Ar}), 145.38 (2 × C_{Ar}), 129.88 (CH_{Ar} × 4), 129.62, 129.56 (CH_{Ar} × 4), 128.92, 128.88 (CH_{Ar} × 4), 128.85, 128.78 (CH_{Ar} × 4), 127.74 (CH_{Ar} × 2), 127.45, 127.36 (CH_{Ar} × 2), 75.29 (OCH), 69.56 (OCH_2), 52.62, 52.53 ($OCHCH_2N$), 51.00 (CH_2CON), 50.29 ($CHPh_2$), 50.06 ($CHPh_2$), 49.58 (CH_2CON), 48.25 ($CH_2CH_2CHPh_2$), 39.64, 39.44 ($CH_2CH_2CHPh_2(C_t)$), 36.12 ($CH_2CH_2CHPh_2$ (C_t)), 34.60, 33.93 ($CH_2CH_2CHPh_2$), 30.27 ($OCH_2CH_2CH_2$), 26.44 (OCH_2CH_2). Purity of 91% (t_R = 11.49 min). HRMS $[M + H]^+$ calcd for $C_{39}H_{45}N_3O_3$, 604.3534; found, 604.3531.

Solid-Phase Synthesis of the DKPs. DKP-01 and DKP-03 were obtained from peptoids di-NH_2 I and di-NH_2 III, respectively, as follows: The crude peptoids (**di-NH_2 I**, 5 mg, 0.01 mmol; **di-NH_2 III**, 4.5 mg, 0.01 mmol) were dissolved in 1 mL of a 10% solution of $NaHCO_3$ in H_2O-CH_3CN (1:1) and stirred for 24 h. The solvent was then lyophilized, and the residues were extracted with DCM (3 × 2 mL). The organic solvent was removed by evaporation, and the peptoids were dissolved in H_2O−MeCN (1:1) and lyophilized again to afford the expected DKPs as white solids (**DKP-01**, 2.2 mg, 50%; **DKP-03**, 1.3 mg, 33%).

***N*-(3,3-Diphenylpropyl)-*N′*-(4-methoxyphenethyl)-2,5-piperazinedione (DKP-01).** Purity of 85%, t_R = 12.48 min. HRMS $[M + H]^+$ calcd for $C_{28}H_{31}N_2O_3$, 443.2329; found, 443.2325.

***N*-(3,3-Diphenylpropyl)-*N′*-(4-tetrahydrofurfuryl)-2,5-piperazinedione (DKP-03).** Purity of 99%, t_R = 11.13 min. HRMS $[M + H]^+$ calcd for $C_{24}H_{28}N_2O_3$, 393.2173; found, 393.2171.

Solubility Assay. The peptoids from the library were dissolved at 10 different concentrations (0.05−5000 μM) in water−DMSO (4:1) in a 96-well plate. The turbidity of the peptoid library at the different concentrations was measured at 620 nm in a microplate reader (Thermolabsystem Multiskan Ascent).

Cell Growth Inhibition Assay. A colorimetric assay using sulforhodamine B (SRB) was adapted to perform a quantitative measurement of cell growth and viability, following a previously described method.[31] The cells were seeded in 96-well microtiter plates at 5 × 10^3 cells/well in aliquots of 195 μL of RPMI medium and were left to grow in drug-free medium for 18 h to allow attachment to the plate surface. Samples were then added in aliquots of 5 μL (dissolved in DMSO−H_2O, 3:7). After 72 h of exposure, the antitumor effect was measured by the SRB methodology: cells were fixed by adding 50 μL of cold 50% (wt/vol) trichloroacetic acid (TCA) and were incubated for 60 min at 4 °C. Plates were washed with deionized H_2O and dried, and 100 μL of SRB solution (0.4 wt %/vol in 1% acetic acid) was added to each microtiter well and incubated for 10 min at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried, and the bound stain was dissolved with Tris buffer. Optical densities were read on an automated spectrophotometer plate reader at a single wavelength of 490 nm. Data analyses were automatically generated by LIMS implementation. Assays were done in a dose−response manner at 10 different concentrations (from 10 μg/mL with 1:2.5 dilutions to 0.0026 μg/mL). Although concentrations were adjusted in mg/mL, GI_{50} values were calculated in molarity. For the nonadherent cell lines, this rank was fine-tuned and concentrations used were from 0 to 100 μM. All assays were run in triplicate, and the curves were automatically adjusted with 30 points by nonlinear regression using "Prism 3.03" (GraphPad) software.

Antimitotic Assay. The mitotic ratio of the cell cultures (percentage of cells arrested in mitosis) was estimated using a specific 96-well microplate immunoassay that quantitatively detects a specific mitotic marker. HeLa cells (h-cervix carcinoma, ATCC# CCL-2) were incubated for 18 h in the presence or absence of the peptoids being tested. Afterward, cells were washed with PBS and lysed on ice in 75 μL of freshly prepared lysis buffer (1 mM EGTA (pH 7.5), 0.5 mM PMSF, and 1 mM $NaVO_3$) for 30 min. An aliquot of the cell extract (60 μL) was transferred to a high-binding surface ELISA plate and dried in a speed-vac for 2 h at room temperature. Plates were then blocked in 100 μL of PBS-1% BSA for 30 min at 30 °C and sequentially incubated with anti-MPM2 primary mouse monoclonal antibody (Upstate Biotechnology, cat #05-368) for 18 h at 4 °C and appropriate peroxidase-conjugated secondary antibody for 1 h at 30 °C. After intensive washing in 0.02% Tween-20, the peroxidase reaction was performed using 30 μL of TMB (3,3′,5,5′-tetramethyl-benzidine) for 30 min at 30 °C. The reaction was quenched with 30 μL of a 4% H_2SO_4 solution. The assay was quantified by measuring the O.D. at 450 nm in a microplate spectrophotometer. The results are expressed as IC_{50} (i.e., the concentration of sample at which 50% of mitosis in treated cell cultures is arrested as compared to untreated cultures).

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Supporting Information Available: Chromatograms of DKP formation studies and gel electrophoresis analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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SUPPORTING INFORMATION

Smallest peptoids with antiproliferative activity on human neoplastic cells

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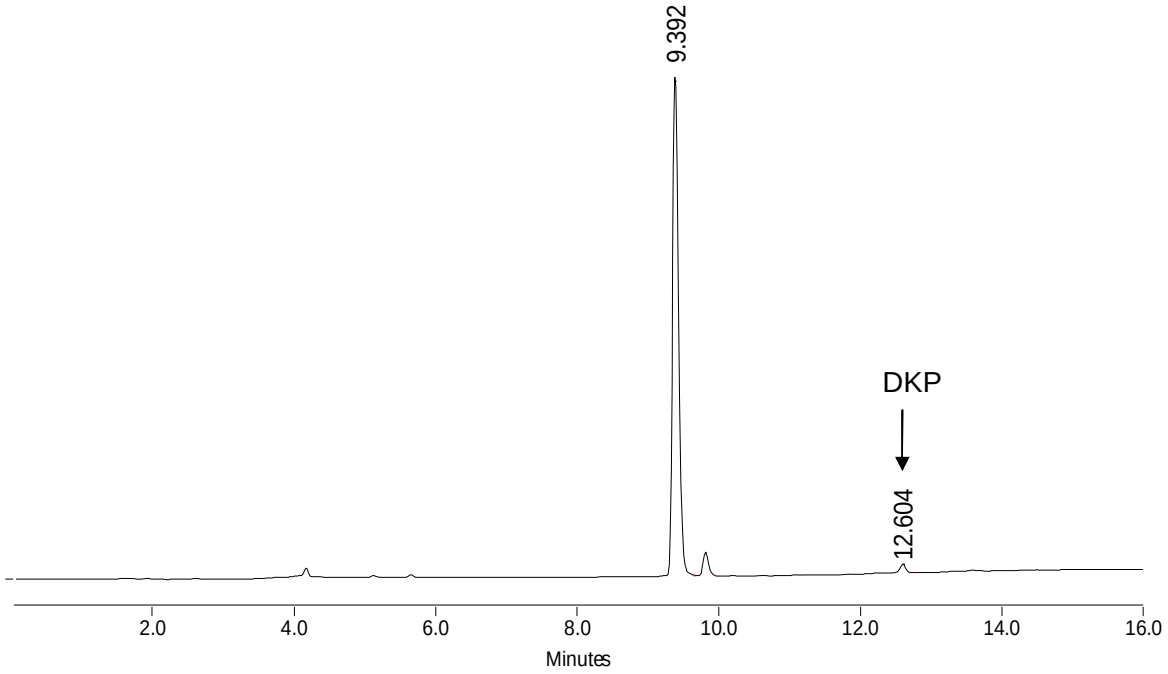
CONTENTS

DKP formation studies with peptoid Di-NH2 I (See Table 1)

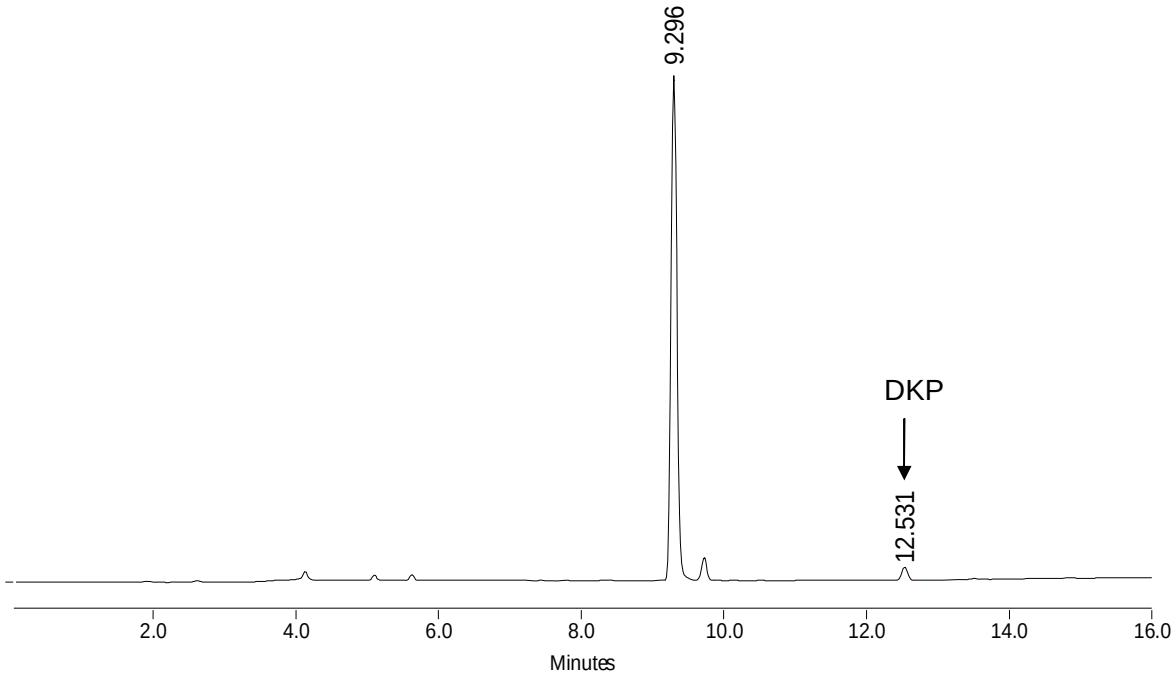
H_2O–CH_3CN, t = 0h

DKP: < 2%



H_2O–CH_3CN, t = 24h

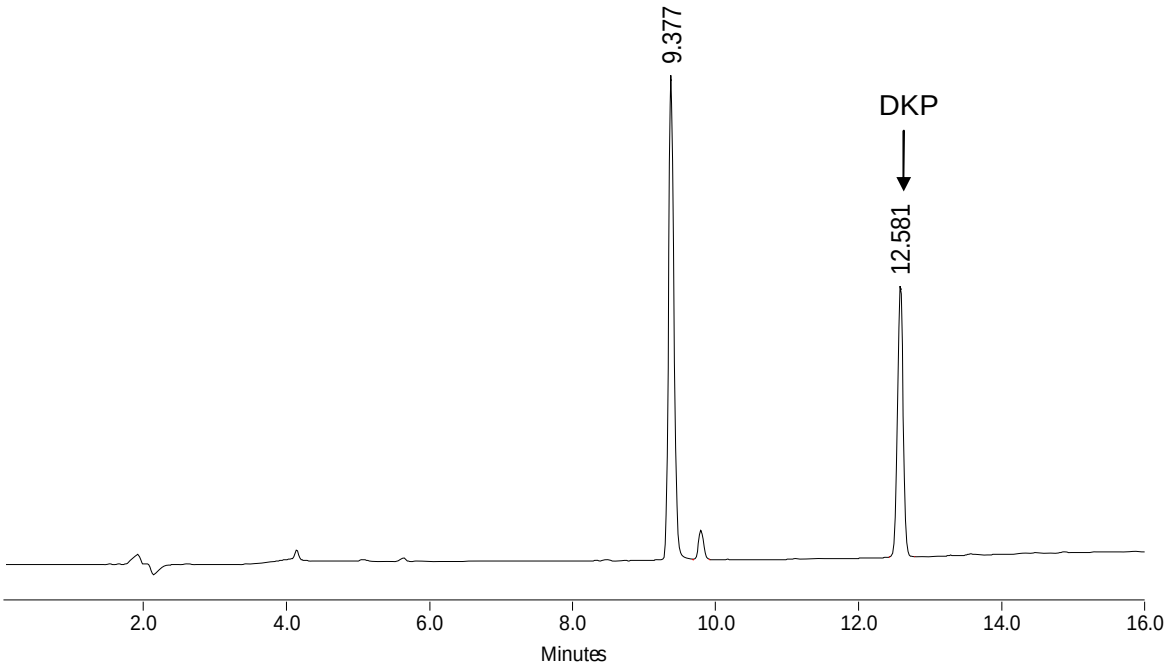
DKP: < 2%



DKP formation studies with peptoid Di-NH2 I (See Table 1)

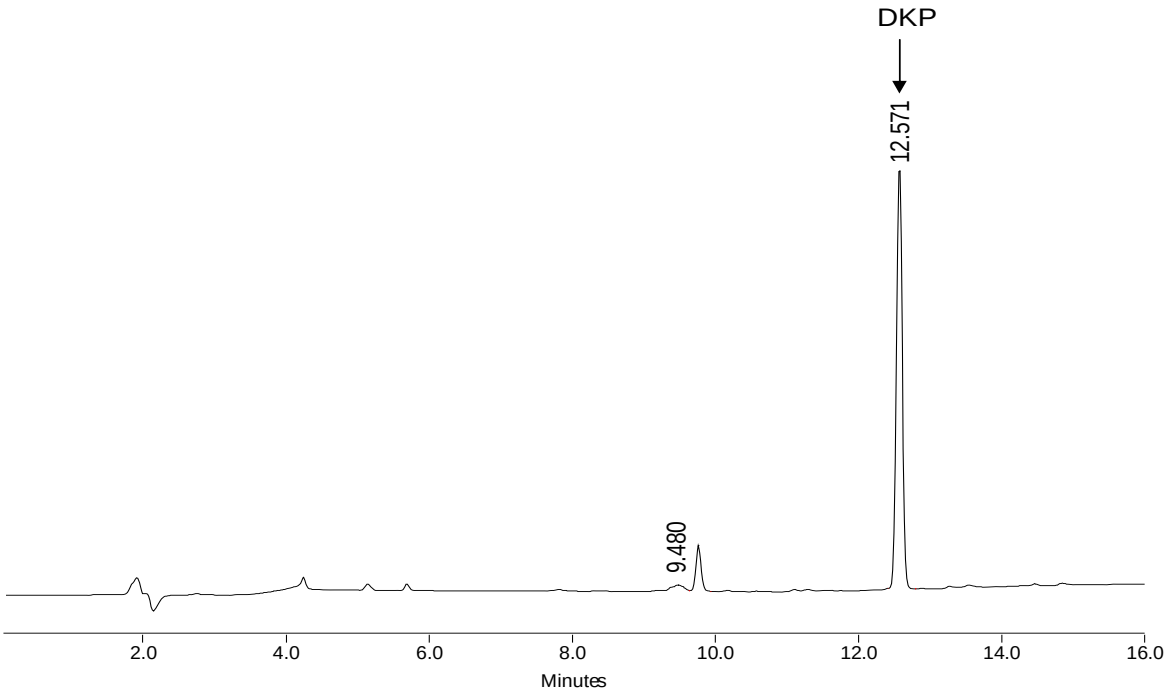
10% $NaHCO_3$, t = 0h

DKP: 37 %



10% $NaHCO_3$, t = 24h

DKP: 98 %



DKP formation studies with peptoid Di-NH2 I (See Table 1)

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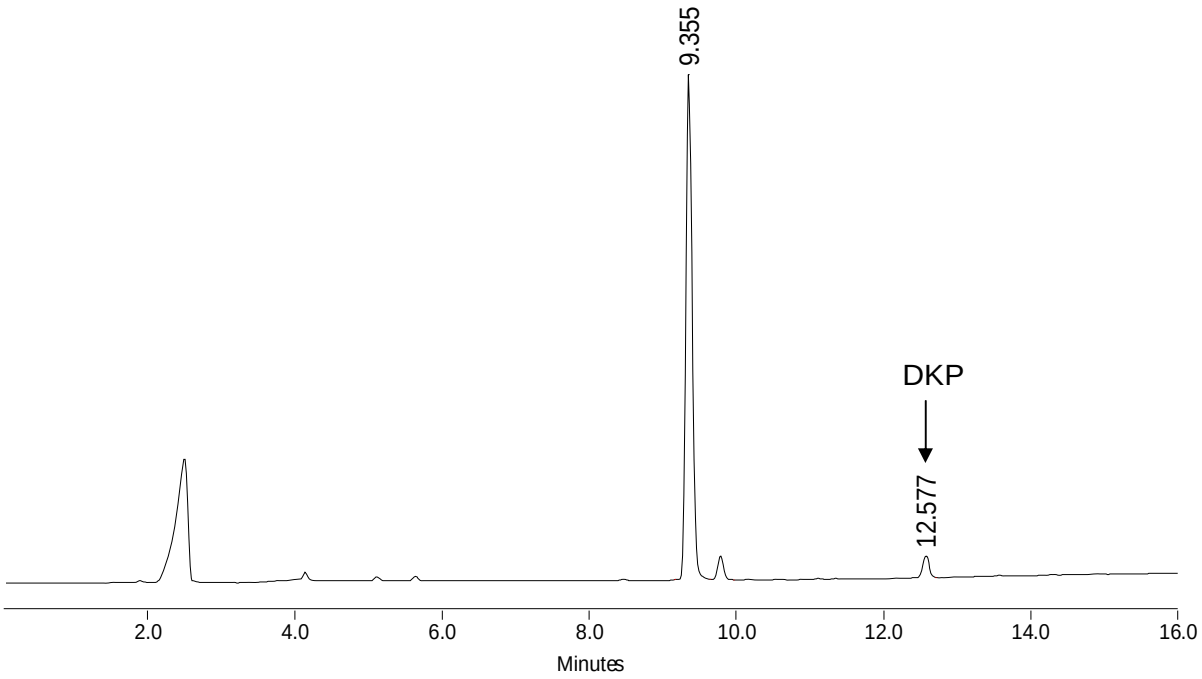
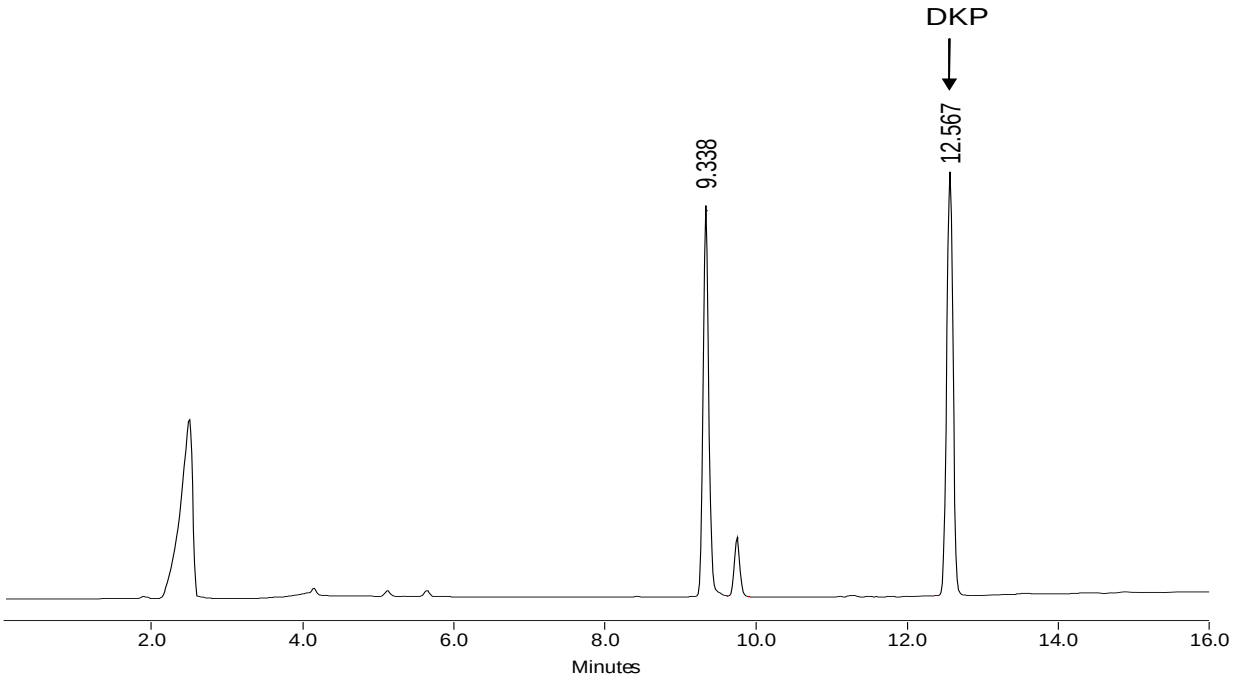
DKP: 4 %



10% TFA, t = 24h

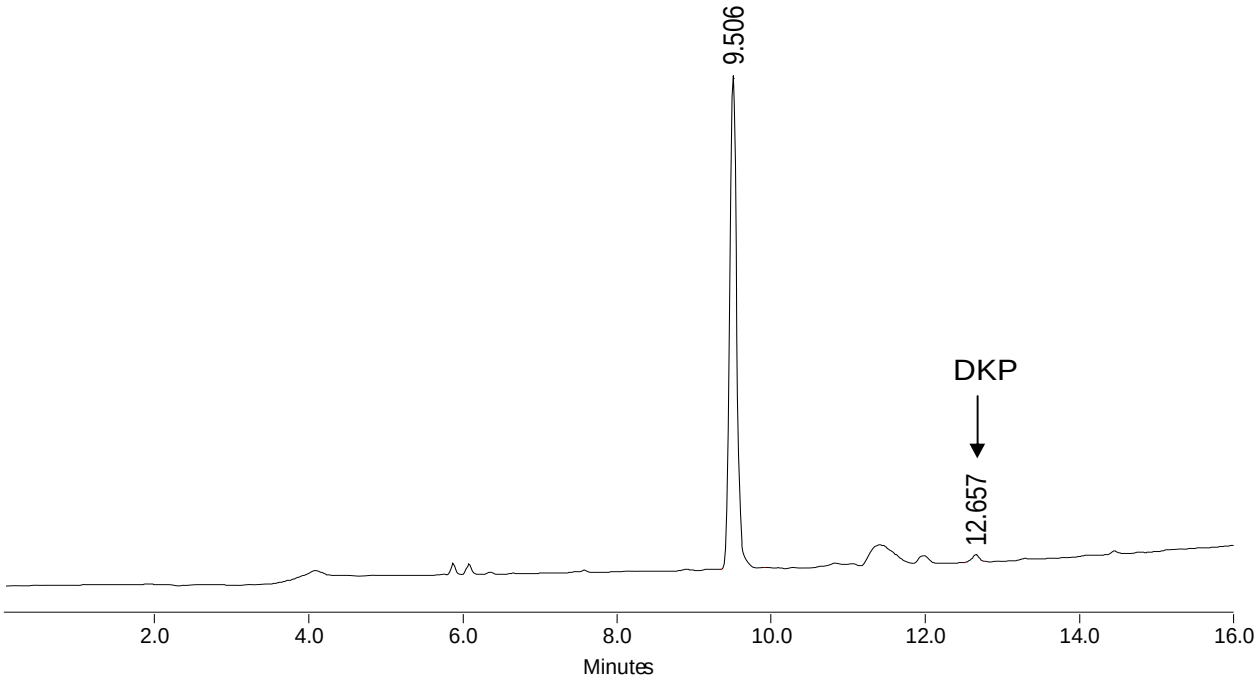
DKP: 53 %



DKP formation studies with peptoid Di-NH2 II (See Table 1)

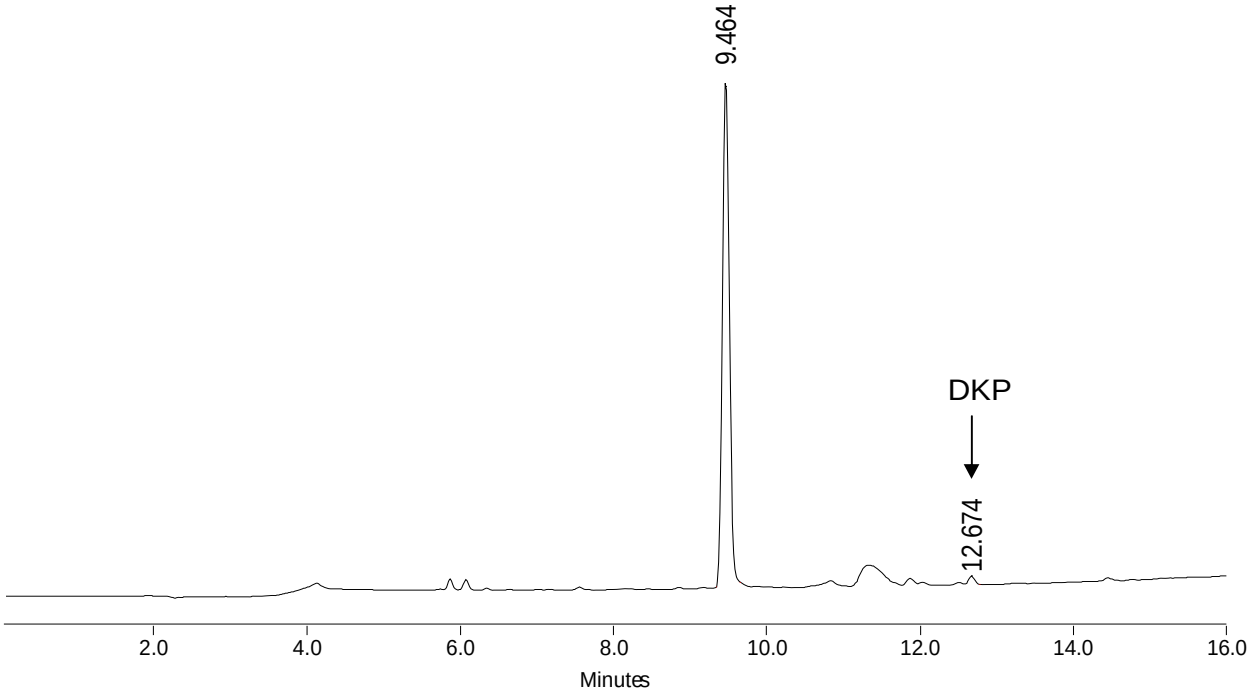
H_2O–CH_3CN, t = 0h

DKP: < 2%



H_2O–CH_3CN, t = 24h

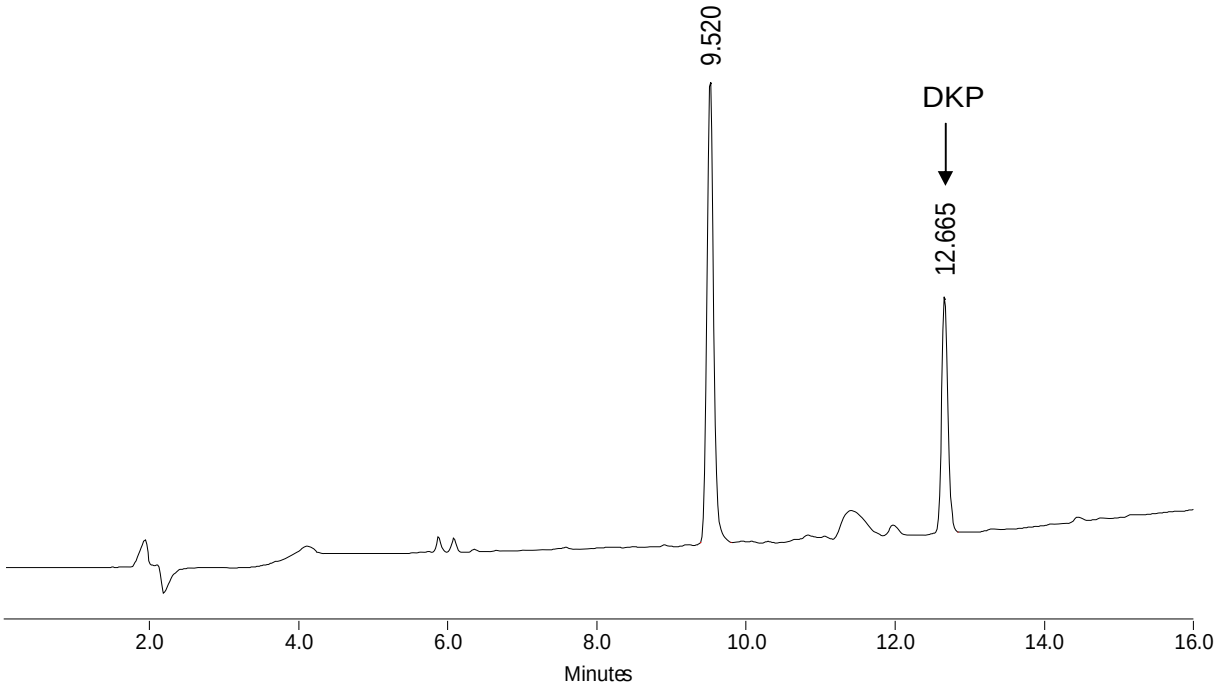
DKP: < 2%



DKP formation studies with peptoid Di-NH2 II (See Table 1)

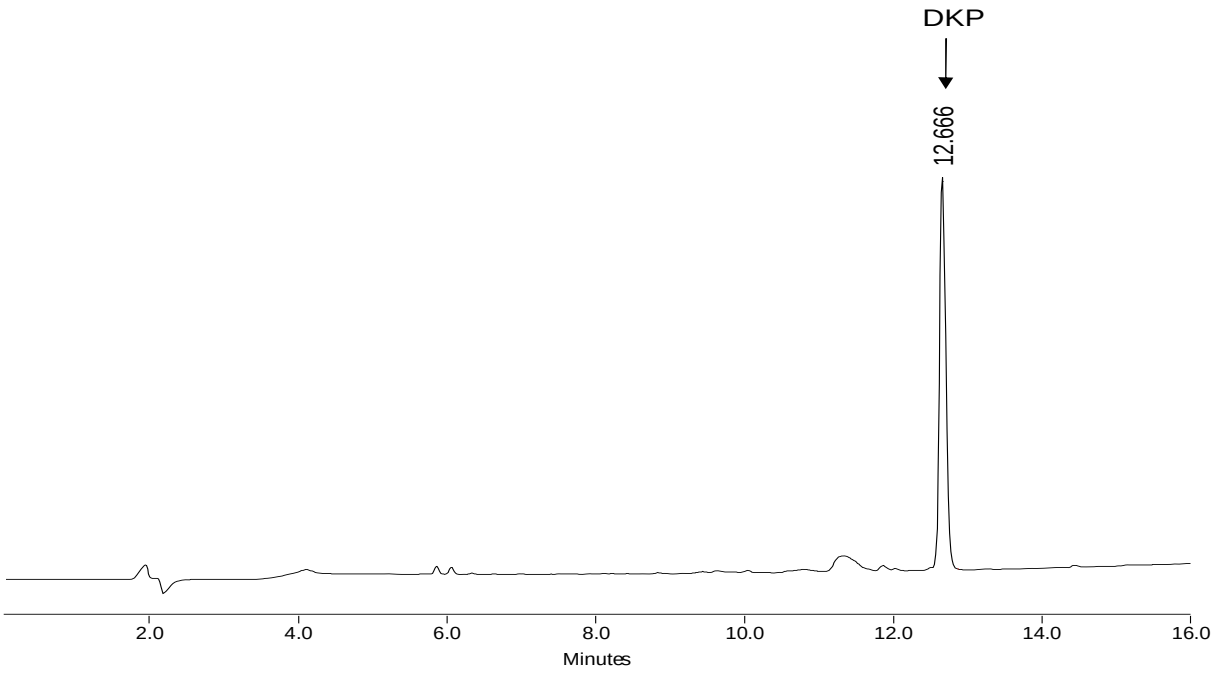
10% $NaHCO_3$, t = 0h

DKP: 31 %



10% $NaHCO_3$, t = 24h

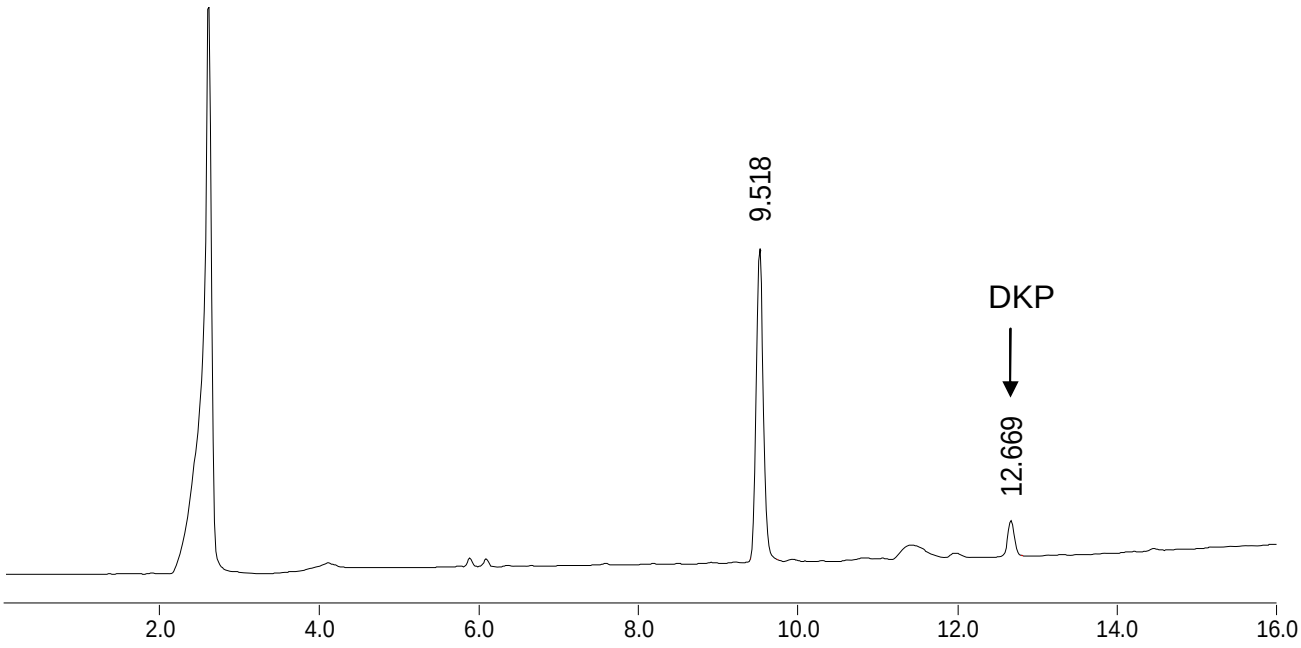
DKP: 100 %



DKP formation studies with peptoid Di-NH2 II (See Table 1)

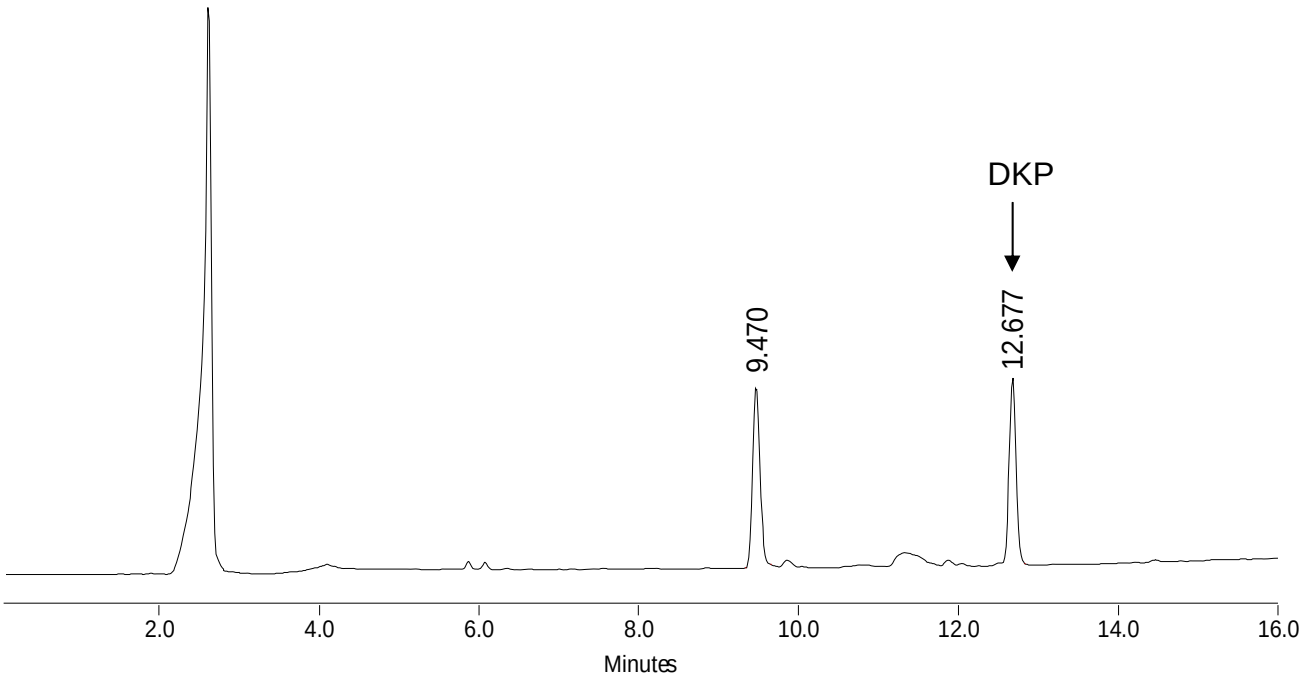
10% TFA, t = 0h

DKP: 9 %



10% TFA, t = 24h

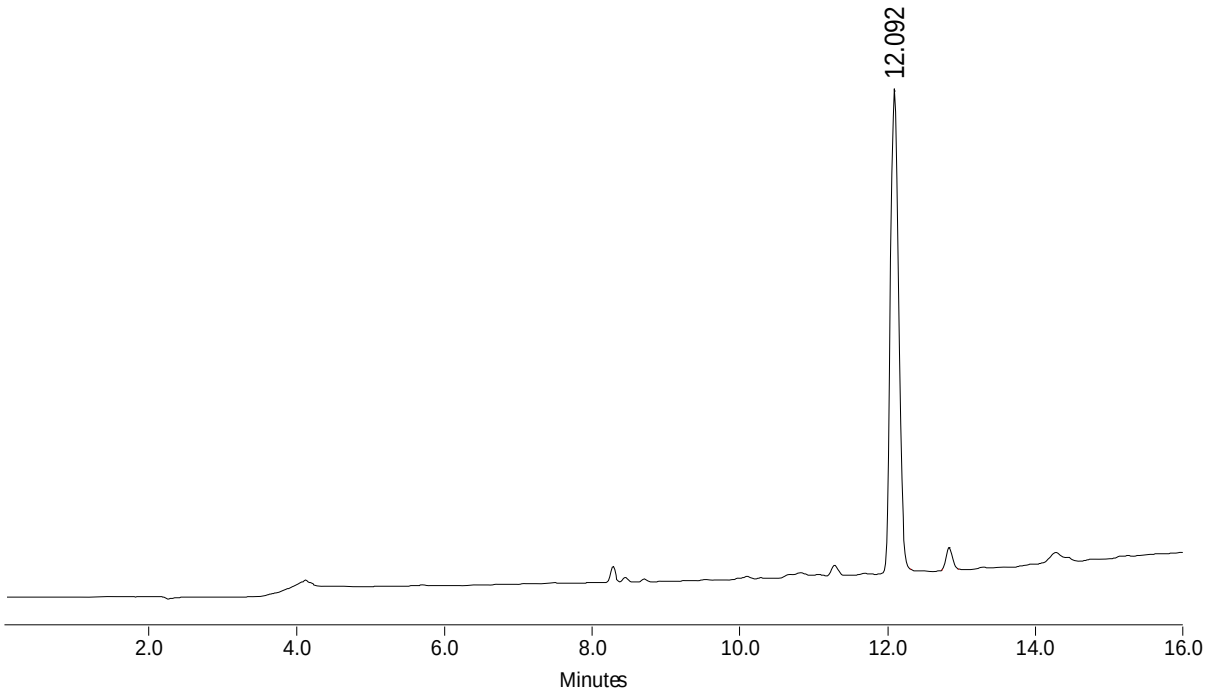
DKP: 49 %



DKP formation studies with peptoid Di-NHR I (See Table 1)

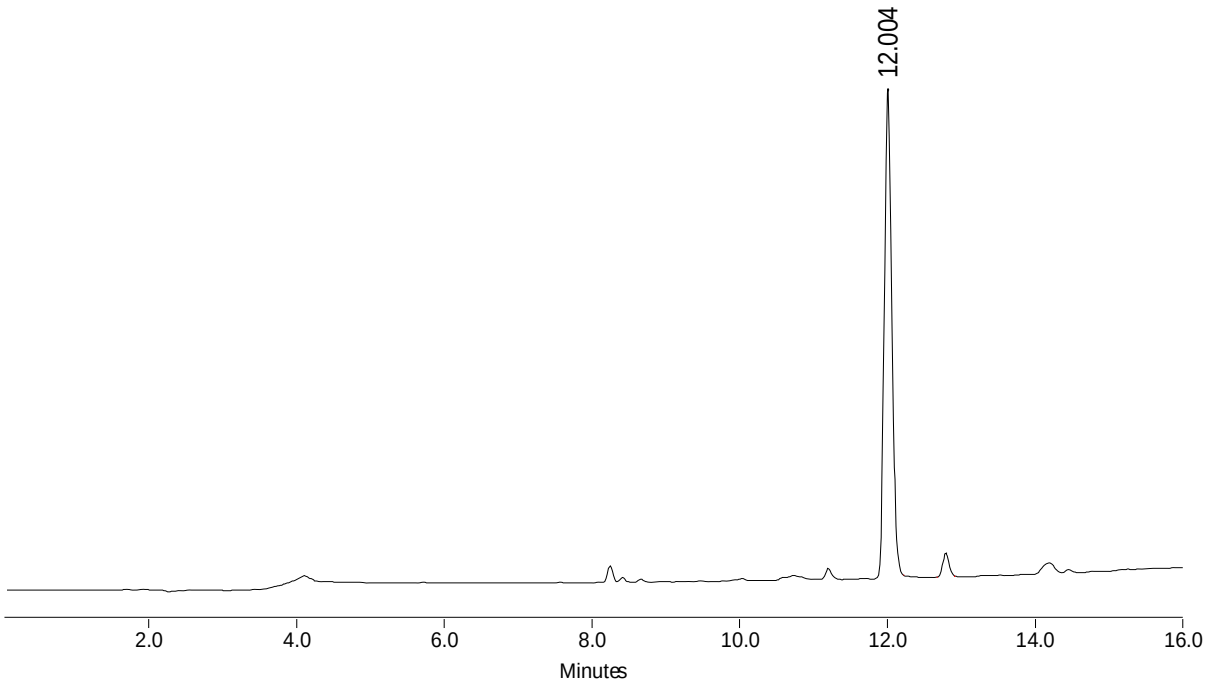
H_2O–CH_3CN, t = 0h

DKP: ND



H_2O–CH_3CN, t = 24h

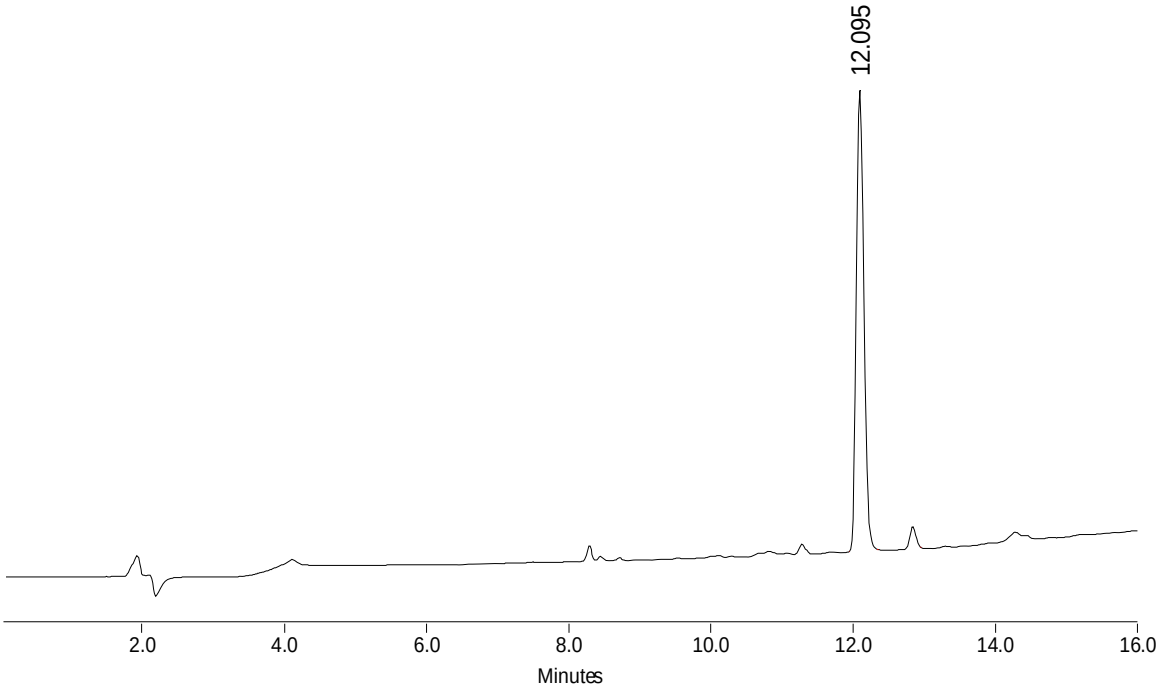
DKP: ND



DKP formation studies with peptoid Di-NHR I (See Table 1)

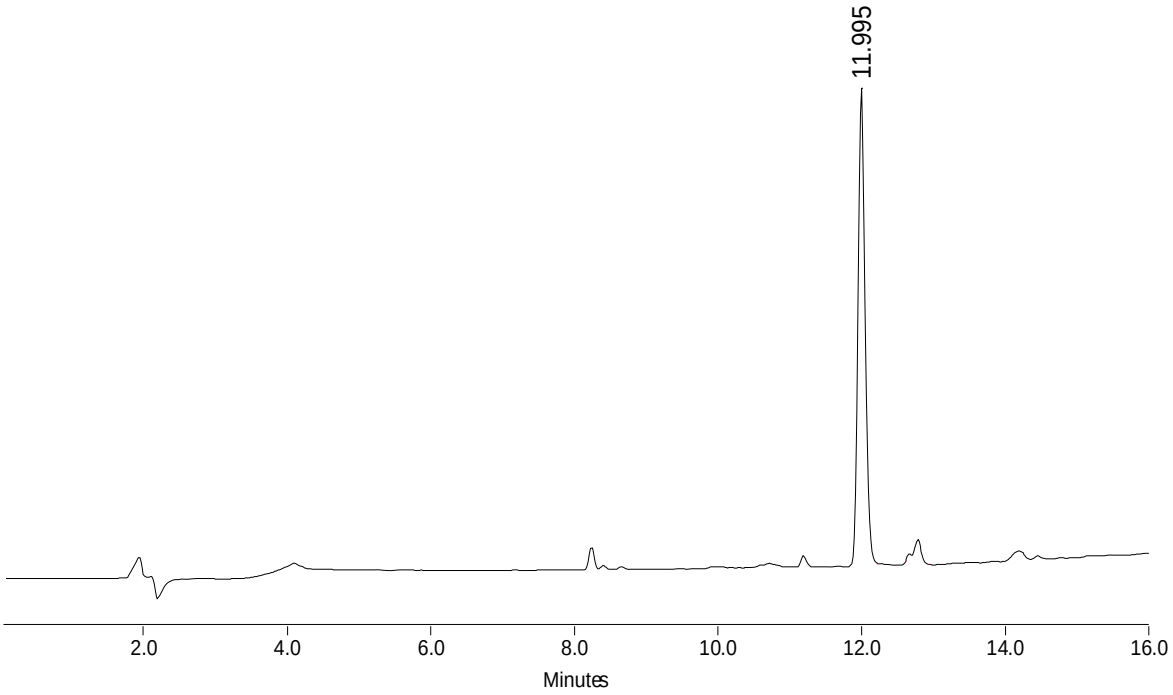
10% $NaHCO_3$, t = 0h

DKP: ND



10% $NaHCO_3$, t = 24h

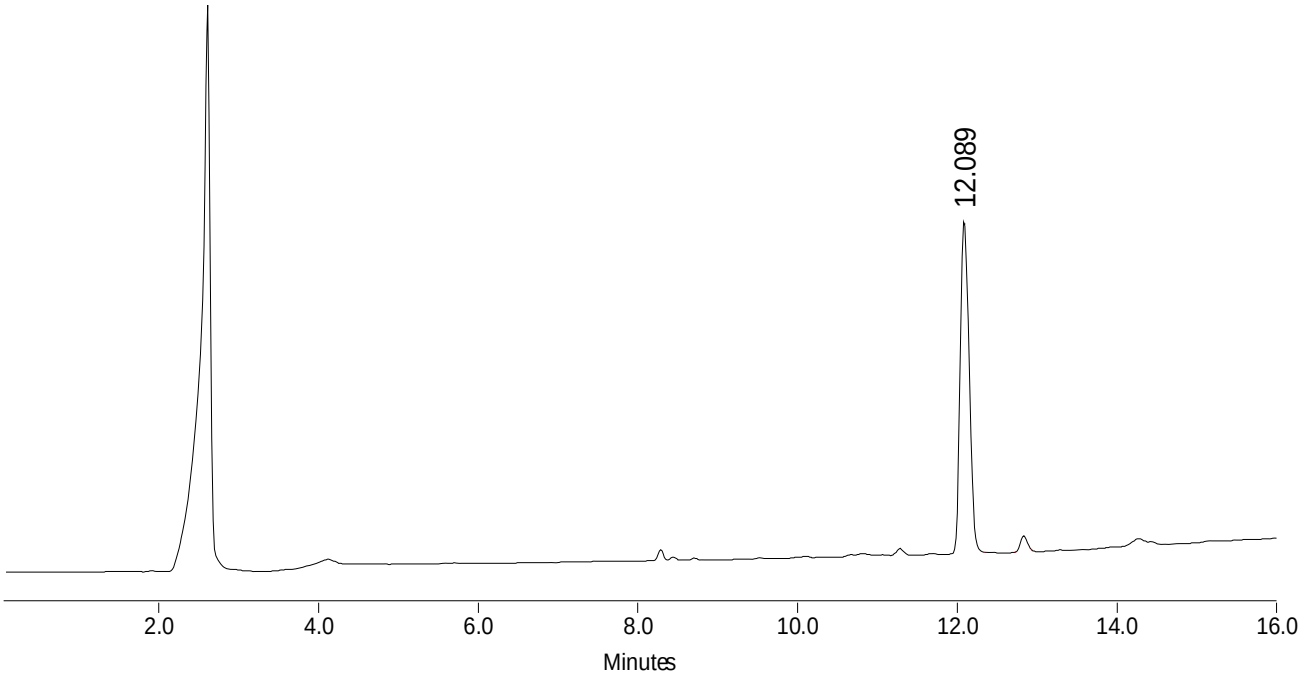
DKP: ND



DKP formation studies with peptoid Di-NHR I (See Table 1)

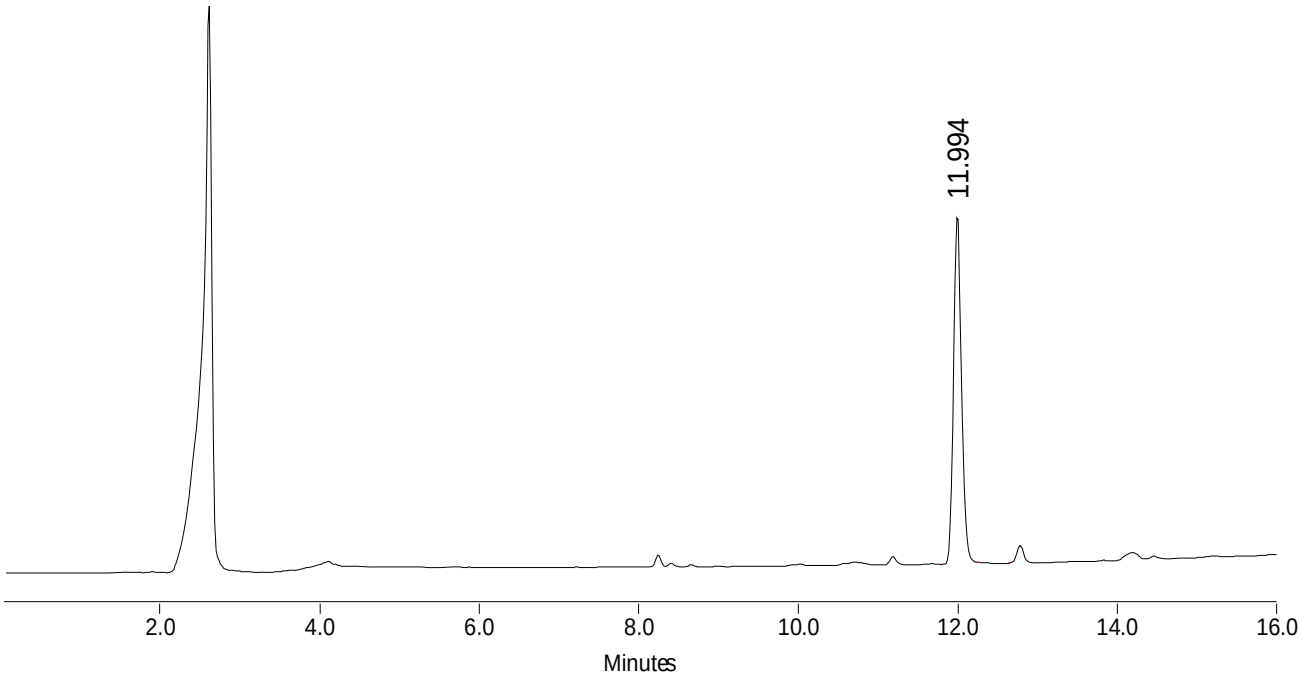
10% TFA, t = 0h

DKP: ND

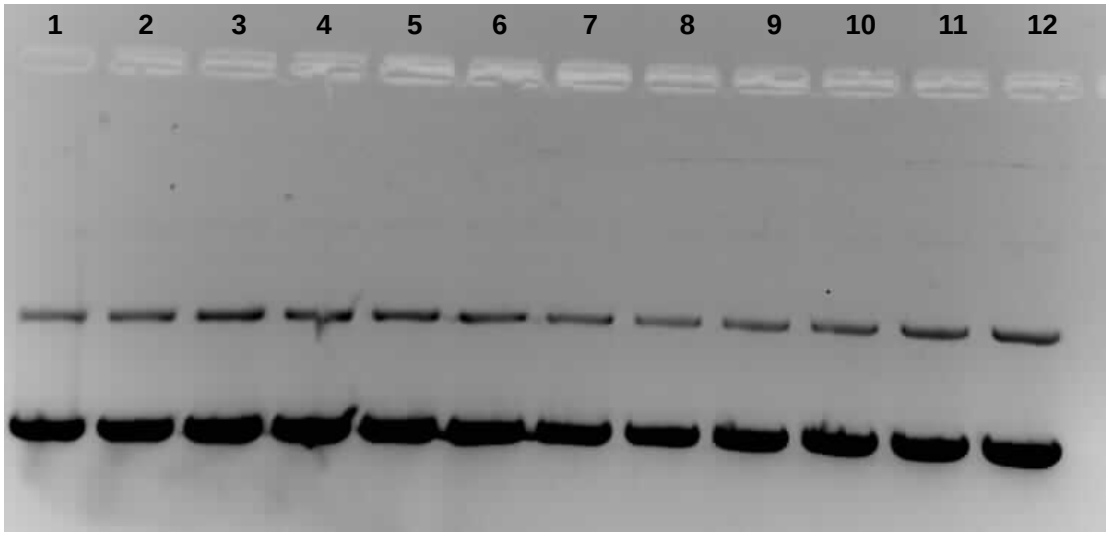


10% TFA, t = 24h

DKP: ND



Agarose gel (1.5%) electrophoresis



Lane 1: Control unmodified pBR322 DNA

Lanes 2 to 11: pBR322 incubated at 1:3000 molar ratio (DNA:peptoid) with **di-NH_2 I** (lane 2), **di-NH_2 II** (lane 3), **di-NH_2 III** (lane 4), **di-NH_2 IV** (lane 5), **di-NH_2 V** (lane 6), **mono-NHR I** (lane 7), **mono-NHR II** (lane 8), **di-NHR I** (lane 9), **di-NHR II** (lane 10), **Trimer 1** (lane 11) and **Trimer 2** (lane 12).

Gel electrophoresis analysis

Peptoids were dissolved in 10 mM Tris–HCl (Tris: tris(hydroxymethylamino)-methane) buffer and incubated with 1 μg of pBR322 DNA plasmid (4361 base pairs in length, double stranded circle, Fermentas) for 1 h at room temperature. The molar ratio used was, 1 mol DNA : 3000 mol peptoid, a concentration far enough to show interaction according to work done in our laboratories. 18 μL of plasmid DNA and peptoid-DNA mixtures altogether with bromophenol blue marker were subjected to 1.5% agarose gel electrophoresis for 2 h at 120 V in TAE buffer (Tris–acetate, 40 mM; EDTA, 2mM; pH 8.0). The DNA was stained in the same buffer containing ethidium bromide (0.5 μg/mL). The gel was photographed using a Bio Imaging System, Gene Genius model (Syngene).

4.3 CONCLUSIONS

The conclusions of this chapter are the following:

1. The novel peptoid monomers and dipeptoids derived from two tripeptoids with LPS-neutralizing activity presented in this chapter show poor LPS-inhibitory activity. However, they display cytotoxicity for cancer cells. To the best of our knowledge this is the first report of a dipeptoid or an *N*-alkyl glycine amide with antiproliferative activity. This finding represents a new promising biological application for these sorts of compounds.

2. The observation that C-terminal *N*-alkyl amide peptoids do not form DKPs suggests that other bioactive tripeptoid families described in the literature could also be developed into DKP-free, bioactive peptoid monomers and dipeptoids. The results described in this study also highlight the utility of BAL resins for the synthesis of peptoid monomers and dipeptoids. The insertion of an alkyl group at the C-terminal amide provides an extra group for biological interaction without increasing the length of the peptoid chain, which also leads to marked improvements in aqueous solubility.

5 CONCLUDING REMARKS

Sepsis is a clinical syndrome whose pathology reflects the activation of an innate host response to infection. The apparent simplicity of this definition involves a complex process that still challenges researchers and clinicians. Sepsis and related disorders represent the first cause of mortality in ICUs and a leading cause of death worldwide. Despite the great advances in this field over the last twenty years, no safe and efficacious drugs are yet available to treat this pathological condition.

To address this issue, in this Doctoral Thesis we have designed and developed a series of LPS-inhibitors. LPS is a bacterial endotoxin that origins sepsis in Gram-negative infections. Therefore, our approach was based on the assumption that the neutralization of this endotoxin during the early stages of infection (i.e. before it interacts with innate immune cells), could neutralize its toxic effects.

In the first chapter we focused on several LPS-neutralizing peptides derived from LPS-binding proteins. We have demonstrated that distinct chemical modifications of these peptides can result in enhanced LPS-neutralizing activities. In this regard, we have shown that besides cationicity, the hydrophobicity (and therefore amphipathicity) of these peptides is a major factor to exert optimal LPS-neutralization. However, a large increase in hydrophobicity may also result in higher cell toxicities, as a result of the enhanced affinity that these peptides display for cell membranes. Future work in this field should bear these considerations in mind. For instance the *N*-palmitoylation of LPS-neutralizing peptides could be replaced by shorter acyl chains, such as C8 or C10 alkyl chains.

In the second chapter we explored further modifications of these peptides in order to increase their biological activity and to improve some of their pharmacokinetic and toxicity profiles. As a result of this work, we obtained a series of LPS-inhibitors which, in the most of the cases, showed better LPS-neutralizing activities. One promising strategy is the conjugation of LPS-inhibitors to PEGylated dendrimers. This approach yielded peptide conjugates with improved biological activity and reduced toxicity. Nevertheless, we still have to evaluate the efficacy of these modifications in terms of proteolytic stability, drug permeability and oral bioavailability.

In addition, although the LAL test is a common assay to determine LPS-neutralizing values for LPS-inhibitors, *in vitro* studies in cells (such as the inhibition of NO or TNF-α LPS-induced release) are required to confirm that the molecules described in this work are *true* LPS-neutralizers. The *in vivo* biological activity of the best candidates should further be examined in animal models of septicemia.

The third chapter describes an example of how to take advantage of an unexpected side activity for a given compound. The peptoids described therein are currently being modified by means of their conjugation to therapeutic polymers in order to study their specificity and selectivity for different tumor cells, as well as to reduce unspecific toxicity in non-tumoral cells.

Finally, there is an important point to be considered. Would the compounds described in this Thesis be useful drugs for the treatment of sepsis? Or, in other words, if we were able to further improve the biological activity of one of these compounds as well as its pharmacokinetic and toxicity profiles, would a therapy directed to the inhibition of LPS be successful to treat sepsis? The major drawback of the strategy pursued here is that there are no prognostic markers for the early identification of sepsis. Hence, when the condition has been diagnosed, it may be too late to initiate an anti-LPS campaign. Nonetheless, in some cases, the risk for a patient to develop septicemia could be anticipated. This would be the case of potentially susceptible patients or hospitals with reiterative reports of sepsis cases after a specific treatment or operation. In these cases, LPS-neutralizing agents could be included in the common treatments as preventive drugs. Therefore, we would not provide a magic bullet, but we would contribute to extend the clinician's list of possible therapeutic agents to fight sepsis.

6 RESUM DE LA MEMÒRIA

1. INTRODUCCIÓ I OBJECTIUS

1.1 Introducció

1.1.1 Introducció general sobre la septicèmia

La septicèmia és un síndrome molt complex de definir, diagnosticar i tractar. La patologia de la septicèmia s'esdevé d'una resposta perjudicial de l'hoste vers una infecció. L'exposició a endotoxines bacterianes indueix una resposta sistèmica inflamatòria que té com a finalitat lluitar contra la infecció. Aquesta resposta es pot descriure amb una sèrie de símptomes clínics com serien la febre o un augment en la freqüència cardíaca i respiratòria, entre d'altres. Aquest procés, necessari per a la defensa de l'organisme, condueix a la destrucció dels patògens. Ara bé, si malgrat l'activació d'aquests mecanismes, la infecció persisteix així com l'exposició de l'organisme a productes bacterians nocius, els símptomes clínics descrits anteriorment poden agreujar-se derivant en una septicèmia. Les septicèmies en un gran nombre de casos poden progressar cap a estadis més greus, com les septicèmies severes o el xoc sèptic, que acostumen a desembocar en una fallida d'òrgans vitals i sovint la mort del pacient.

El nombre de pacients amb septicèmia per any als Estats Units supera ja els 750.000, i s'estima que aquest nombre augmentarà fins al milió de casos l'any 2020. La mortalitat dels pacients sèptics varia entre el 30 i el 70%, sent actualment la desena causa de mortalitat a aquest país. A Europa les xifres són similars, amb més de mig milió de casos anuals. Arreu del món, el nombre s'eleva a 18 milions de pacients per any. A les Unitats de Cures Intensives (UCIs), la septicèmia és la primera causa de mortalitat, on un 15% dels malalts desenvoluparan un procés sèptic.

1.1.2 Factors immunòlogics en la patogenicitat de la septicèmia

El lipopolisacàrid (LPS) és la molècula que origina la septicèmia i totes les seves variants, i és responsable d'un gran nombre de malalties infeccioses. Aquesta molècula és el component majoritari de la paret cel·lular de les bactèries Gram-negatives. L'estructura química del LPS (Figura 1.1.1) es basa en dos dominis units covalentment i

clarament diferenciats. Una part hidrofílica formada per unitats repetitives d'oligosacàrids, que proporcionen l'especificitat entre molècules de diferents soques bacterianes; i una part lipídica, coneguda com a **lípid A** que confereix la toxicitat a la molècula.

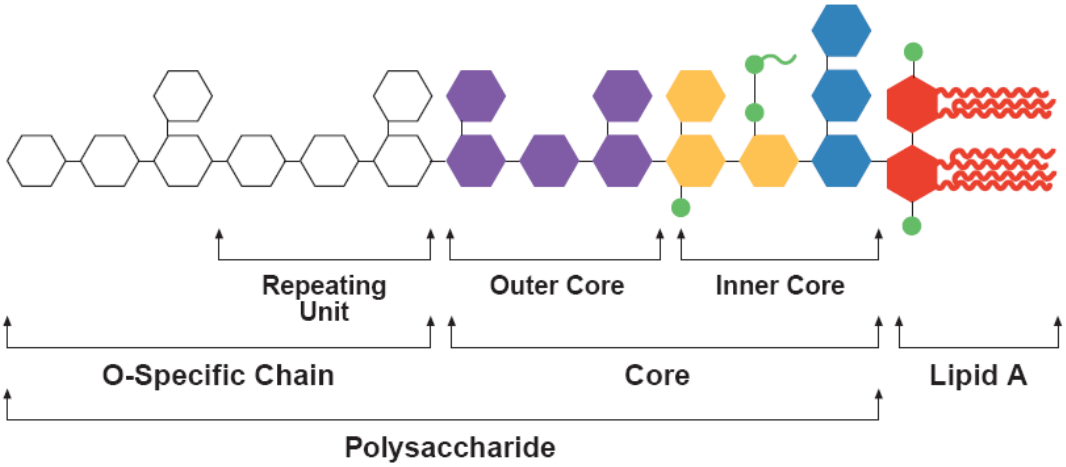


Figura 1.1.1: Estructura esquemàtica del lipopolisacàrid (LPS).

El reconeixement del LPS per part de les cèl·lules del sistema immunològic s'ha detectat com l'origen de la patologia. L'exposició continuada del LPS al torrent sanguini indueix l'alliberament descontrolat de citoquines inflamatòries que comportaran les condicions patològiques anteriorment esmentades. La cascada immunogènica comença pel reconeixement i unió del LPS a proteïnes d'unió específiques. Entre elles, la LBP (*LPS-binding protein*) uneix el LPS i el transfereix al receptor CD14. El complex LBP-CD14 haurà després d'interaccionar amb uns receptors de membrana específics, els TLR (*toll like receptors*) i la proteïna MD-2, que transduiran el senyal a l'interior de la cèl·lula fins al nucli, on s'iniciarà la transcripció dels gens immunomoduladors. La cascada de senyalització cel·lular es troba representada a la Figura 1.1.2. De manera resumida, el domini intracel·lular dels TLR, TIR (*Toll/IL-1 receptor homology domain*) s'uneix a la proteïna IRAK (*IL-1 receptor-associated kinase*). Aquest procés està mediat per dues proteïnes adaptadores, MyD88 (*myeloid differentiation protein 88*) i TIRAP (*TIR domain containing adapter protein*). Aquesta unió indueix l'activació de la proteïna TRAF6 (*TNF receptor-associated factor-6*), que en última instància comportarà la translocació nuclear del factor de transcripció NF-κB (*nuclear factor κB*), el qual iniciarà l'activació dels gens promotors de citoquines.

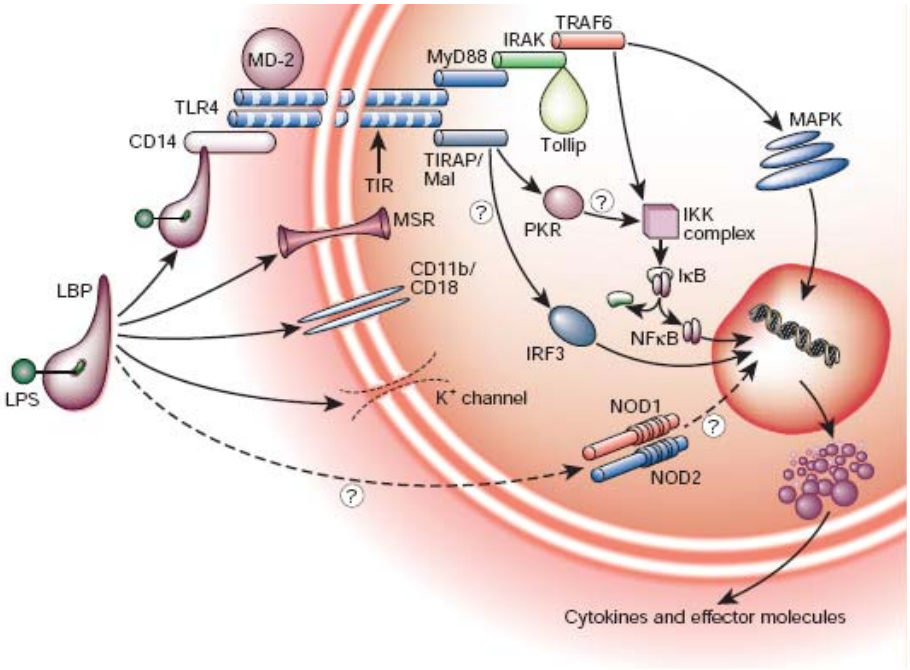


Figura 1.1.2: Cascada de senyalització de cel·lular del LPS. Hi ha diversos mecanismes proposats, però no tots ells han estat suficientment estudiats. Per aquest motiu només hem descrit el mecanisme via els TLR.

El reconeixement d'endotoxines bacterianes per part de l'hoste comporta l'activació del sistema immunològic innat. Aquest procés defineix la patogenicitat de la septicèmia i és extremadament complex. Els principals efectes observats són:

i) la producció descontrolada de citoquines pro-inflamatòries i altres mediadors amb capacitat inflamatòria als estadis inicials de la infecció,
ii) una sèries de desordres en la coagulació i en els seus mecanismes de regulació homeoestàtics,
iii) un estat d'immunosupressió i malfuncionament dels mecanismes apoptòtics de limfòcits i neutròfils,
iv) i finalment, la greu fallida d'òrgans generalitzada que representa la principal causa de mortalitat en pacients amb xoc sèptic.

1.1.3 Estratègies per al tractament de la septicèmia

A causa de l'extraordinària complexitat i heterogeneïtat del síndrome de la septicèmia, triar una diana terapèutica clínicament rellevant no és una tasca fàcil. A més, la manca d'eines per al diagnòstic de la septicèmia o la gran diversitat de pacients amb diferents malalties subjacents, afegeixen una dificultat addicional per trobar tractaments efectius. Metges i investigadors han estudiat pràcticament tots els components desencadenants de la septicèmia per a trobar dianes amb interès terapèutic. Aquestes estratègies es resumeixen a continuació:

i) La inhibició de components d'origen bacterià com el LPS
ii) La neutralització de citoquines pro-inflamatòries
iii) La inhibició d'altres mediadors inflamatoris
iv) La inhibició de membres de la cascada de coagulació
v) La reversió de l'estat d'immunosupressió i la inhibició de l'apoptosi

Lamentablement, la gran majoria de tractaments per a la septicèmia han fracassat. Només hi ha en l'actualitat un fàrmac comercialitzat per al tractament de la septicèmia, la proteïna C activada (Drotrecogin alfa, Xigris®), indicada pel tractament de pacients crítics amb casos molt severs de septicèmia. Desafortunadament, aquest fàrmac té una sèries d'efectes secundaris molt greus, com el risc d'hemorràgies, i la seva administració no s'aconsella per a pacients sèptics amb risc baix de mortalitat.

El motiu pel qual la majoria de tractaments per a la septicèmia no han funcionat són nombrosos, però hi ha una sèries de conceptes en la patogenicitat de la septicèmia que ens poden ajudar. La septicèmia és un síndrome extremadament complex que es composa d'un gran nombre de molècules del sistema immunològic i d'agents amb capacitat inflamatòria. Aquestes molècules estan involucrades en un nombre molt divers de mecanismes íntimament relacionats, que sovint es compensen els uns als altres. Per tant, és poc probable que la inhibició d'un d'aquests components comporti un efecte terapèutic. A més, hi ha un punt de no retorn en el progrés de la patologia a partir del qual tenen lloc efectes clínics irreversibles. Per la qual cosa sembla ser que seria més efectiu prevenir que tractar el síndrome de la septicèmia. En altres paraules, considerem que l'estratègia més efectiva seria aturar l'estimulació del sistema immunològic als

estadis inicials, abans que es produeixi un alliberament descontrolat de citoquines inflamatòries. Inhibir, en definitiva, l'acció tòxica del desencadenant de la resposta immunològica, el LPS.

1.2 Objectius

1. Dissenyar, sintetitzar i avaluar l'activitat biològica de pèptids neutralitzants del LPS derivats de proteïnes amb capacitat coneguda d'unió al LPS, com a futurs agents terapèutics per al tractament de les septicèmies.

2. Dissenyar, sintetitzar i avaluar l'activitat biològica de molècules derivades dels pèptid neutralitzants del LPS descrits, per tal de millorar-ne la seva activitat biològica i les seves propietats farmacocinètiques.

3. Proporcionar claus per a la millor comprensió dels mecanismes moleculars implicats en la neutralització del LPS.

2. PÈPTIDS NEUTRALITZANTS DEL LPS DERIVATS DE PROTEÏNES D'UNIÓ AL LPS

2.1 Introducció

2.1.1 Pèptids i proteïnes que s'uneixen al lípid A

Com ja hem comentat anteriorment el LPS és la molècula causant de la septicèmia en les infeccions originades per bactèries Gram-negatives. Tant en la membrana bacteriana com en fluids biològics, el LPS es troba constantment associat a proteïnes. Aquestes proteïnes tenen funcions molt diverses: poden transportar-lo, reconèixer-lo i transmetre una senyal o bé, bloquejar-lo i neutralitzar els seus efectes biològics. L'estudi d'aquestes proteïnes d'unió, representa per tant una estratègia prometedora per identificar i desenvolupar molècules amb capacitat de neutralitzar el LPS. Per aquest tipus d'estratègia els pèptids resulten candidats interessants. Gràcies a les metodologies de síntesi en fase sòlida de les que es disposa en l'actualitat, és relativament senzill poder sintetitzar pèptids que mimetitzin els dominis d'unió (seqüències actives) al LPS

de proteïnes amb una afinitat coneguda per endotoxines bacterianes. Aquests pèptids de primera generació podran ser modificats per tal d'augmentar la seva activitat biològica o millorar la seva farmacocinètica.

Existeixen un gran nombre de proteïnes amb capacitat per interaccionar amb el LPS. En aquesta introducció però només descriurem les proteïnes que s'han estudiat al llarg del capítol: les proteïnes LALF, BPI i SAP.

El ***Limulus* anti-LPS factor** (LALF) és una proteïna petita (101 aminoàcids, 11.8 kDa) bàsica que uneix i neutralitza el LPS amb gran afinitat. L'estructura cristal·lina de LALF va permetre identificar el domini d'unió d'aquesta proteïna al LPS. Es tracta d'un *loop* o gir amfipàtic amb estructura de forquilla-β que compren els residus del 31 al 52. Aquest gir amfipàtic està estabilitzant per la presència d'un pont disulfur entre les cisteines 31 52 (Figura 2.1.1). Estudis previs d'aquest domini, van descriure un pèptid cíclic de 14 amino àcids (residues 36-47), el pèptid LALF-14c, com el domini mínim d'unió al LPS de la proteïna.

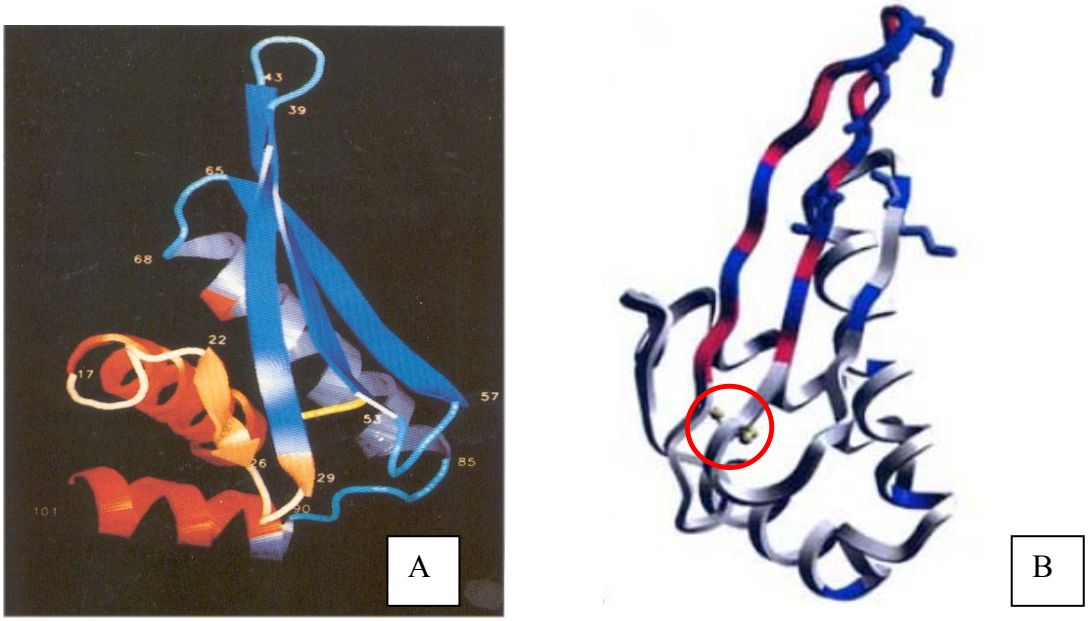


Figura 2.1.1: A. Estructura cristal·lina de LALF a 1.5 Å. **B**. Detall del gir en forquilla-β de LALF (31-52) en color vermell. El pont disulfur entre les Cys31-Cys52 es troba encerclat.

La **bactericidal permeability-increasing protein** (**BPI**) és una altra coneguda proteïna d'unió al LPS, de caràcter fortament bactericida amb un alt grau d'homologia amb la LBP. L'activitat bactericida d'aquesta proteïna es troba continguda en el seu domini *N*-terminal (Figura 2.1.2). Aquest domini també es responsable de la seva capacitat per unir-se al LPS, tal i com van demostrar estudis en els quals el domini *N*-terminal aïllat era capaç de neutralitzar eficaçment el LPS. En aquesta regió s'han identificat tres seqüències d'unió al LPS (residus 17-45, 65-99 i 142-169). El tractament d'animals amb septicèmia amb pèptids derivats d'aquestes seqüències n'ha reduït la mortalitat.

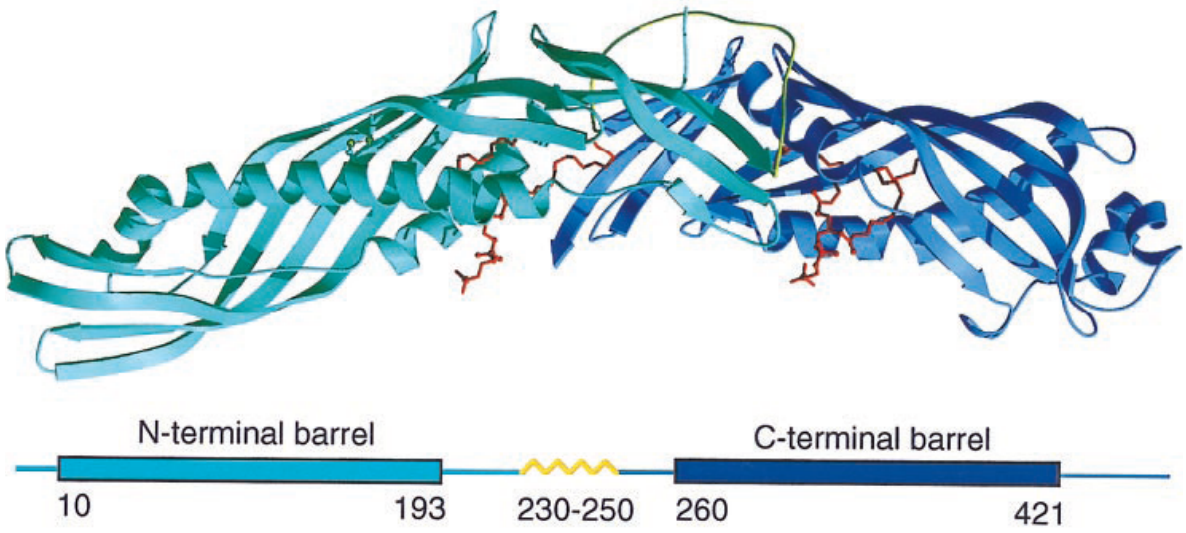


Figura 2.1.2: La figura mostra l'estructura cristal·lina de la BPI humana. El domini *N*-terminal (a l'esquerra) es troba dibuixat en blau cel. Aquest domini és responsable de l'activitat anti-LPS de la proteïna.

El **serum amyloid P** (SAP) és una glicoproteïna multiespecífica de 235 KDa present en el plasma que entre d'altres molècules s'uneix amb gran afinitat al LPS. En aquest cas, tres dominis diferents d'unió al LPS han estat detectats (aminoàcids 27–39, 61–75 i 186–200). Pèptids sintètics derivats d'aquestes seqüències han demostrat tenir activitats biològiques prometedores. Aquest és el cas, per exemple, del pèptid $SAP_{186-200}$, el qual va protegir del xoc sèptic a ratolins injectats amb LPS.

Una característica comuna d'aquestes tres proteïnes, i de la majoria de proteïnes amb capacitat d'unió al LPS descrites a la literatura, és que s'uneixen a la part tòxica del LPS, el lípid A. El lípid A està format per un 1,4'-bis-fosforilat disacàrid glucosamínic que

conté diversos àcids grassos units per enllaços ester i amida (Figura 2.1.3). Les característiques químiques d'aquesta molècula són de gran importància, ja que permeten entendre millor el mode d'interacció dels pèptids descrits. Per exemple, els pèptids neutralitzants del LPS acostumen a ser rics en residus bàsics, com la lisina o l'arginina, ja que en tenir càrrega positiva poden exercir interaccions electrostàtiques amb el fosfats del lípid A, que a pH fisiològic es troben carregats negativament. Una altra característica d'aquests pèptids, es que també tenen residus hidrofòbics en les seves seqüències, i per tant acostumen a ser altament amfipàtics. Aquesta propietat també és important a l'hora d'establir interaccions hidrofòbiques i interaccions de Van der Waals amb les cadenes lipídiques del lípid A. Les dues característiques, basicitat i amfipaticitat acostumen a ser necessàries per a una neutralització efectiva del LPS.

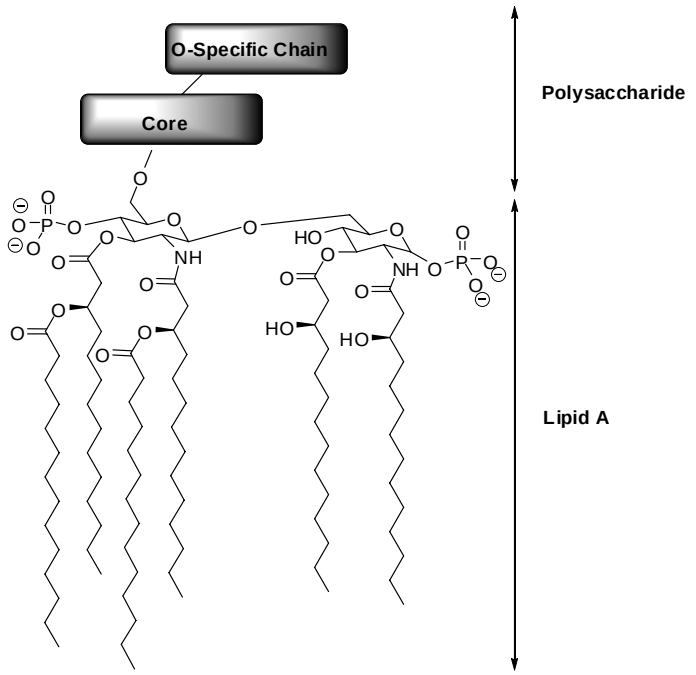


Figura 2.1.3: Estructura general del LPS on es mostra en detall l'estructura química del lípid A.

2.1.2 Objectius

1. Estudiar i modificar la seqüència activa de LALF per tal d'identificar seqüències d'unió al LPS minimitzades i obtenir pèptids amb capacitats per neutralitzar el LPS millorades.

2. Estudiar l'efecte de la incorporació de grups acil en les seqüències actives de les proteïnes LALF, BPI i SAP.

2.2 Resultats i discussió

2.2.1 Disseny d'un pèptid cíclic minimitzat que neutralitza endotoxines bacterianes

Com ja hem introduït abans, LALF-14c és un pèptid cíclic que mimetitza el gir amfipàtic d'unió al LPS de la proteïna LALF. Aquest pèptid representa el mínim domini d'unió al LPS descrit per LALF i té una capacitat per neutralitzar endotoxines bacterianes comparable a la de l'antibiòtic polimixina B (PMB). En aquest treball, vam examinar la seqüència de LALF-14c mitjançant un "mapeig" d'hexapèptids i un "scan" d'alanines. Aquests estudis van proporcionar informació valuosa sobre els aminoàcids més rellevants per a l'activitat biològica. D'aquesta manera, es va determinar la seqüència d'un tetrapèptid com el domini d'unió mínima de LALF-14c al LPS. La inserció d'aquesta seqüència en un pèptid cíclic amb cisteines va donar lloc a un nou pèptid cíclic, el pèptid RLKWc, el qual va exhibir la mateixa activitat biològica que el pèptid original. Aquests resultats, per tant, demostren que el domini d'unió al LPS de la proteïna LALF, proposat fins aleshores, era susceptible de ser minimitzat (Figura 2.2.1)

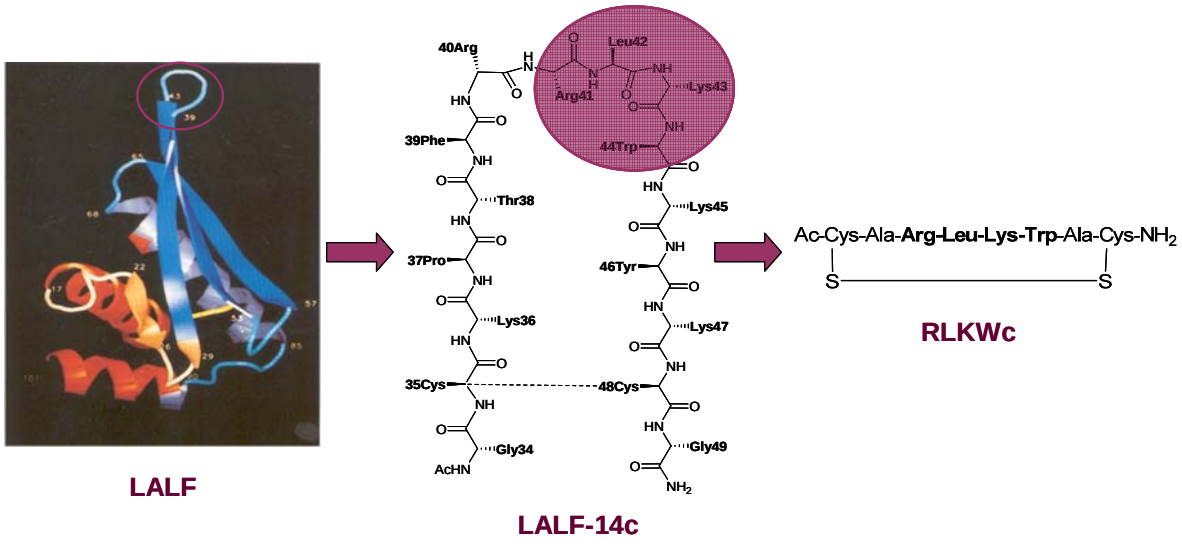


Figura 2.2.1: La figura resumeix l'obtenció del pèptid RLKWc, a partir del domini d'unió al LPS de la proteïna LALF.

2.2.2 Inserció de prolines a LALF-14c: modulació de la seva activitat neutralitzant del LPS.

A partir de la seqüència de LALF-14c, també vam estudiar l'efecte de la incorporació d'elements inductors d'estructures secundàries (girs β) i de la substitució d'un pont disulfur per un enllaç amida. D'aquesta manera es van dissenyar tres nous pèptids amb capacitat neutralitzant del LPS. Les modificacions comentades anteriorment van resultar ser excel·lents estratègies per millorar l'activitat biològica de pèptids anti-LPS.

2.2.3 La formació de nano-estructures augmenta l'activitat biològica de pèptids neutralitzants del LPS

A continuació es van preparar diferents pèptids acilats en l'extrem *N*-terminal amb activitat anti-LPS derivats de les proteïnes LALF, BPI i SAP. En tots els casos, la presència d'àcids grassos de cadena llarga va comportar un gran augment en la capacitat d'aquests pèptids per neutralitzar el LPS. L'anàlisi estructural d'aquests pèptids per microscopia de transmissió electrònica (TEM), va demostrar que la inserció de cadenes alifàtiques en aquests pèptids promou la formació de nano-estructures de tipus micelar o fibrilar (Figura 2.2.2). D'aquesta manera es va poder establir una correlació entre la capacitat que tenen els pèptids per estructurar-se i la seva activitat biològica.

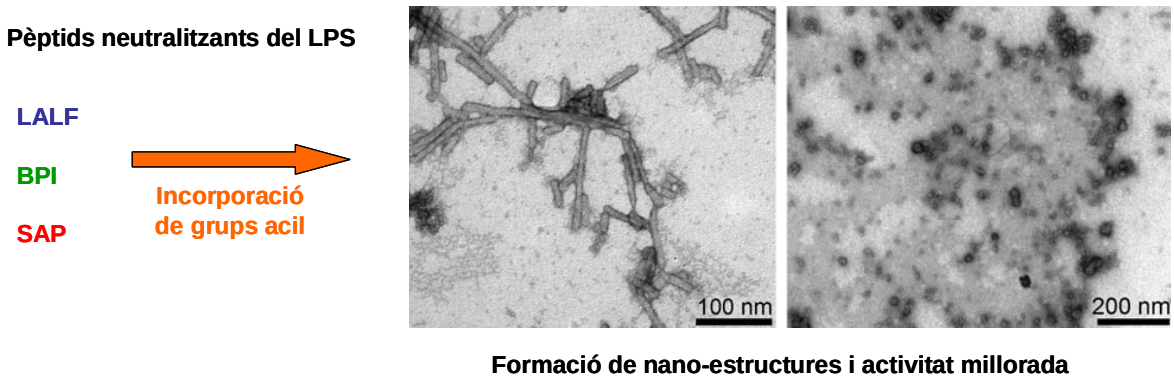


Figura 2.2.2: Imatges obtingudes per TEM de les nano-estructures que formen els pèptids anti-LPS acilats en l'extrem *N*-terminal.

2.2.4 Estudi de la interacció d'un derivat palmitoïlat de la BPI amb cèl·lules mitjançant microscopia confocal

Malauradament, els pèptids *N*-acilats descrits a la secció anterior presentaven toxicitat cel·lular. Per aquest motiu, vam sintetitzar dos pèptids derivats de la BPI, els pèptids C2- i C16-BPI conjugats amb carboxifluoresceina. L'estudi de la interacció d'aquests pèptids amb cèl·lules HeLa i macròfags es va portar a terme mitjançant microscopia confocal. En els dos tipus cel·lulars, els anàlegs acetilats van ser capaços d'internalitzar-se a altes concentracions i temps d'incubació. Aquest procés però no va comportar cap tipus de toxicitat en les cèl·lules. Els anàlegs palmitoïlats van presentar una major afinitat per les membranes cel·lulars i van ser capaços d'internalitzar-se a concentracions més baixes. En aquest cas, en canvi, aquest comportament va implicar mort cel·lular.

2.3 Conclusions

Les conclusions d'aquest capítol són les següents:

1. L'estudi de la seqüència activa d'una proteïna neutralitzant del LPS mitjançant un "scan" d'alanines i un "mapeig" d'hexapèptids és una estratègia útil per identificar els aminoàcids més rellevant per a l'activitat biològica. Aquesta informació es pot aplicar en el disseny de seqüències peptídiques minimitzades amb gran capacitat de neutralitzar endotoxines bacterianes. La seqüència peptídica proposada com a mínim domini d'unió de la proteïna LALF al LPS (el pèptid LALF-14c) és susceptible de ser minimitzada.

2. La inserció de motius estructurals que poden induir un cert grau d'estructura secundària (per ex. la inserció de prolines), o la substitució de ponts disulfur per enllaços amida, en seqüències amb capacitat d'inhibir el LPS coneguda, representen també una opció viable per augmentar l'activitat anti-LPS d'aquesta pèptids. Així, pèptids derivats de LALF-14c amb aquestes modificacions presenten activitats biològiques millorades.

3. La *N*-acilació de pèptids neutralitzants del LPS és una estratègia molt efectiva per incrementar la seva activitat biològica en vers el LPS. Aquest augment en l'activitat és causat per una banda per l'increment en les interaccions hidrofòbiques del pèptids amb els àcids grassos del lípid A; i per una altra, per la capacitat que tenen aquests pèptids per formar estructures de tipus micelar o fibrilar. Aquest fenomen ha estat observat en pèptids derivats de les proteïnes LALF, BPI i SAP.

4. Els pèptids *N*-acilats amb cadenes alifàtiques de gran llargada (per ex. un grup palmitoïl) presenten toxicitat en cèl·lules. L'estudi d'aquest tipus de pèptids, conjugats amb carboxifluoresceina, en sistemes cel·lulars mitjançant microscopia confocal és una estratègia útil per entendre aquest comportament. Els derivats palmitoïlats de la BPI tenen una major afinitat per les membranes cel·lulars respecte els derivats acetilats, així com la capacitat per internalitzar-se en les cèl·lules, la qual cosa comporta toxicitat cel·lular.

3. INHIBIDORS DEL LPS BASATS EN MOLÈCULES PEPTÍDIQUES

3.1 Introducció

A l'anterior capítol hem presentat una series de pèptids derivats de proteïnes amb capacitat per unir-se al LPS. Tot i que els pèptids són una eina molt útil per al disseny de nous fàrmacs, també presenten certes limitacions. Una d'elles és la seva ràpida degradació en els fluid biològics. En aquest capítol proposarem algunes estratègies per a l'obtenció de nous inhibidors del LPS, que tot i estar basats en molècules peptídiques, presentin diverses modificacions dirigides a millorar les seves activitats biològiques i les seves propietats farmacocinètiques.

3.1.1 Inhibidors del LPS basats en les característiques químiques del lípid A

El primer que farem serà dissenyar inhibidors del LPS basant-nos en les característiques estructurals del lípid A. Com ja hem introduït abans, el lípid A conté:

i) dos grups fosfats (amb càrrega negativa a pH fisiològic)
ii) un disacàrid 2-amino-2-deoxi-D-glucosa (glucosamina)
iii) una regió hidrofòbica que conté diversos àcids grassos units amb enllaços ester i amida

La presència de residus catiònics que puguin interaccionar amb els fosfats del lípid A és un requisit per a qualsevol inhibidor que s'uneixi al LPS via el lípid A. Diversos estudis però, han proposat que la unió al LPS és relativament independent de la seqüència dels pèptids. Per tant, aquests aminoàcids bàsics no necessàriament han de trobar-se encabits en una seqüència peptídica, i poden ser connectats per espaiadors bifuncionals de diferent naturalesa química. La utilització d'aquests espaiadors obre una gran nombre de possibilitats interessants. La seva llargada pot ser modificada per tal d'optimitzar la interacció dels centres positius amb els fosfats, també poden ser emprats per interaccionar amb el disacàrid del lípid A, i el més interessant, en funció de la seva naturalesa química poden millorar l'estabilitat i la farmacocinètica dels inhibidors. Finalment, aquests inhibidors haurien de tenir en la seva estructura cadenes alifàtiques que poguessin interaccionar amb la part hidrofòbica del LPS. Un esquema detallat dels compostos proposats es mostra a la Figura 3.1.1.

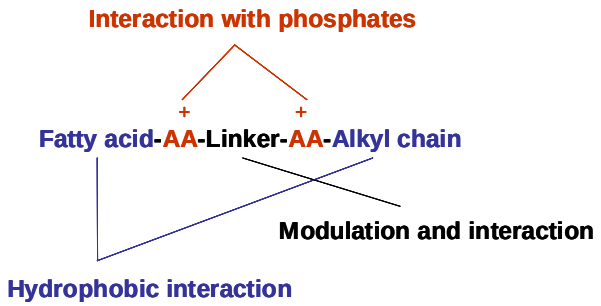


Figura 3.1.1: Representació esquemàtica de les característiques químiques que són necessàries en el disseny d'inhibidors del LPS.

3.1.2 Conjugació d'inhibidors del LPS a dendrimers basats en PEG i a molècules orgàniques

Dendrimers basats en PEG

La següent estratègia es basa en la conjugació d'alguns dels inhibidors del LPS més potents a dendrimers basats en polietilenglicol (PEG). Aquestes arquitectures són una subclasse molt interessant de dendrimers a causa de les seves aplicacions. Diversos dendrimers basats en PEG han demostrat tenir una baixa toxicitat i activitat hemolítica, tenir temps de circulació en sang prolongats i una acumulació en òrgans baixa. Algunes d'aquestes propietats es duen en part a la presència de cadenes de PEG. Aquest polímer no és tòxic i no presenta immunogenicitat o antigenicitat. És altament flexible i té la capacitat de solubilitzar compostos molt insolubles. També millora la biodisponibilitat dels fàrmacs i en general en facilita l'administració.

Tot i el gran nombre d'aplicacions biològiques que hi ha descrites per als dendrimers en la literatura, el seu ús com a agents neutralitzants del LPS és molt escàs. I fins avui, no hi ha cap exemple en la literatura de dendrimers basats en PEG amb aquesta indicació. Per aquest motiu vam decidir estudiar la conjugació d'inhibidors del LPS a aquest tipus de dendrimers per tal avaluar l'efecte en l'activitat biològica i en la toxicitat dels inhibidors.

Identificació de molècules orgàniques de baix pes molecular amb activitat neutralitzant del LPS

Finalment, vam voler també identificar molècules orgàniques de baix pes molecular amb activitat inhibidora del LPS per tal de poder-hi conjugar pèptids anti-LPS descrits en aquesta memòria, com per exemple el RLKWc. Aquesta molècula híbrida podria combinar les avantatges dels dos compostos i minimitzar-ne les limitacions.

Amb aquesta finalitat vam analitzar els 880 compostos que composen la quimioteca *Prestwick Chemical Library*. Els compostos d'aquesta quimioteca són fàrmacs amb gran diversitat estructural que no estan protegits per patents. El més interessant és que la seva efectivitat terapèutica en humans ja ha estat avaluada i per tant, tenen adients

propietats farmacològiques així com una baixa toxicitat, la qual cosa escurçaria el temps entre la recerca bàsica i l'aplicació clínica. Aquesta estratègia ha resultat ser molt fructífera en alguns camps de la química mèdica.

3.1.3 Objectius

Els objectius d'aquest capítol són:

1. Desenvolupar una metodologia en fase sòlida per tal de sintetitzar de manera senzilla potents inhibidors del LPS. Aquestes compostos seran dissenyats a partir de les característiques químiques del lípid A, i tindran diversos aminoàcids substituïts per grups de diferent naturalesa química.

2. Conjugar els inhibidors més interessants a dendrimers basats en PEG i estudiar l'efecte d'aquesta conjugació en l'activitat biològica i la toxicitat del compostos.

3. Identificar noves molècules orgàniques amb activitat anti-LPS a partir de l'anàlisi de la quimioteca *Prestwick*, i conjugar aquestes noves molècules al pèptid neutralitzant d'endotoxines RLKWc. L'activitat biològica d'aquesta molècula híbrida serà avaluada.

3.2 Resultats i discussió

3.2.1 Disseny i síntesi en fase sòlida d'inhibidors del LPS basats en pèptids que contenen funcionalitats de tipus PEG

La primera estratègia que vam dur a terme va ser la síntesi d'inhibidors del LPS mitjançant metodologies en fase sòlida. Els compostos varen ser dissenyats basant-se en les característiques químiques de la part tòxica del LPS, el lípid A. En aquest disseny es va assajar la substitució de diversos aminoàcids per altres grups químics. La presencia de cadenes de polietilenglicol (PEG) va donar lloc als compostos més actius. Aquesta estratègia també es va aplicar amb èxit en el pèptid cíclic RLKWc, tot retenint la seva capacitat per neutralitzar el LPS (Figura 3.2.1).

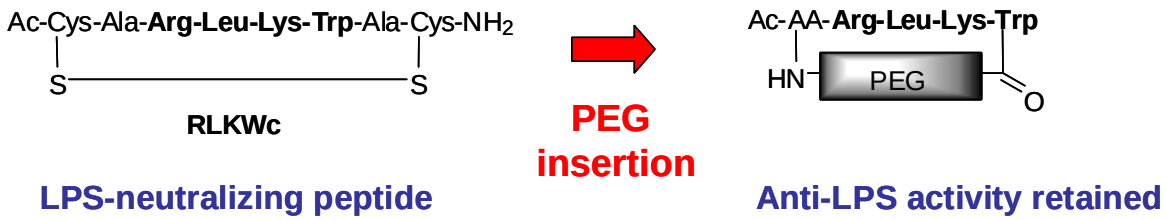


Figura 3.2.1: La substitució d'aminoàcids no essencials per a l'activitat biològica del pèptid RLKWc per cadenes de PEG va permetre obtenir un nou compost capaç de retenir l'activitat original del pèptid.

3.2.2 Conjugació d'inhibidors d'endotoxines bacterianes a plataformes dendrítiques basades en PEG: síntesis, activitat biològica i estudis de toxicitat

En el següent treball vam dissenyar i sintetitzar un nou tipus d'arquitectures dendrimèriques basades en polietilenglicol (PEG) per a la conjugació de compostos amb activitat biològica (Figura 3.2.2). La utilitat d'aquestes plataformes va ser avaluada mitjançant la conjugació d'un compost amb gran capacitat per neutralitzar endotoxines descrit prèviament en l'anterior secció (vegeu l'apartat 3.2.1) La conjugació d'aquesta molècula a les noves estructures va comportar un augment en la seva activitat neutralitzant del LPS i va reduir-ne la toxicitat. A més a més, aquestes arquitectures han demostrat ser útils per incrementar la solubilitat dels compostos conjugats.

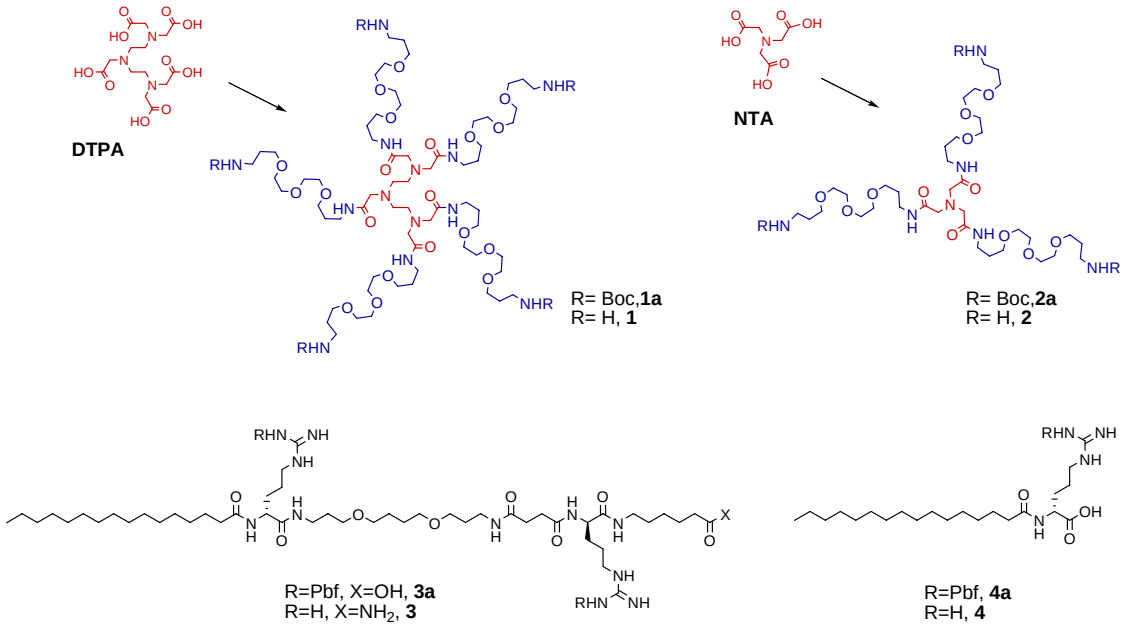
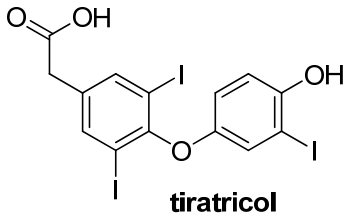


Figura 3.2.2: Es presenta l'estructura química de les plataformes dendrítiques basades en PEG dissenyades i dels inhibidors del LPS triats per a la seva conjugació.

3.2.3 La molècula tiratricol neutralitza endotoxines bacterianes i redueix la producció de TNF-α induïda pel LPS en cèl·lules.

A continuació es va explorar una nova estratègia per al descobriment de nous fàrmacs: el "screening" d'una quimioteca comercial de 880 compostos amb gran diversitat estructural i activitat farmacològica coneguda, la *Prestwick Chemical Library*. Aquests tipus de quimioteques són de gran utilitat per al descobriment de nous fàrmacs ja que els compostos que contenen tenen una bona disponibilitat oral i no presenten toxicitat. L'anàlisi d'aquesta quimioteca va permetre identificar una nova molècula amb capacitat neutralitzant del LPS, el tiratricol (Figura 3.2.3). Aquesta molècula no presenta toxicitat, ni activitat antibacteriana, i és capaç d'inhibir la producció de TNF-α induïda pel LPS en macròfags. La síntesi de diversos anàlegs del tiratricol va permetre estudiar relacions d'estructura-activitat i obtenir compostos més actius.



tiratricol

Figura 3.2.3: Estructura química del tiratricol.

3.2.4 RLKWc-ttcol: una nova molècula híbrida que neutralitza el LPS

Finalment vam dissenyar una nova molècula híbrida, el compost **RLKWc-ttcol**, basat en un anàleg del tiratricol (el tiratricol-Glu) i el pèptid RLKWc, ambdós anteriorment descrits en aquesta memòria (Figura 3.2.4). La síntesi d'aquesta molècula es va realitzar en fase sòlida. Per a l'obtenció del pont disulfur es van avaluar tres estratègies, sent la ciclació en solució mitjançant oxigen atmosfèric la més efectiva. La conjugació d'aquestes dues molècules en el RLKWc-ttcol, va representar un augment en l'activitat neutralitzant del LPS del pèptid RLKWc, però no va implicar un augment en l'activitat del tiratricol.

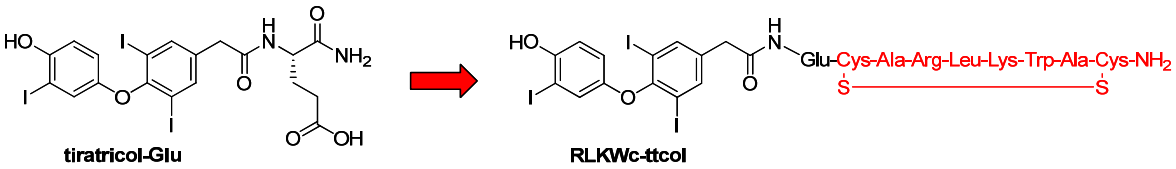


Figura 3.2.4: Estructura química del **RLKWc-ttcol**, i de l'anàleg del qual es deriva, el tiratricol-Glu.

3.3 Conclusions

Les conclusions d'aquest capítol es detallen a continuació:

1. L'estudi de les característiques químiques de la part tòxica del LPS, el lípid A, és una aproximació interessant per tal de dissenyar inhibidors del LPS. Aquesta aproximació permet dissenyar noves molècules derivades de pèptids, en les quals els aminoàcids necessaris per interaccionar amb el lípid A són conservats, mentre que els aminoàcids no essencials poden ser substituïts per altres grups químics. Aquesta estratègia ha permès sintetitzar nous inhibidors del LPS amb activitats anti-LPS millorades. La substitució de diversos aminoàcids per cadenes de PEG comporta els millors resultats pel que fa a activitat biològica.

2. A més, aquests inhibidors poden conjugar-se a plataformes dendrítiques per tal d'incrementar la seva activitat biològica i reduir-ne la seva toxicitat. En aquest capítol hem introduït nou dendrimers basats en PEG amb aquesta finalitat. La conjugació d'un inhibidor del LPS a una d'aquestes plataformes comporta un augment en la seva capacitat per neutralitzar el LPS i en redueix la toxicitat.

3. L'estudi de fàrmacs comercialment disponibles i fora de patent, els quals tenen farmacocinètiques adequades en humans, per tal d'identificar nous compostos amb una activitat biològica determinada és una estratègia útil. En aquest sentit, l'anàlisi d'una quimioteca de 880 compostos, la *Prestwick Chemical Library*, per tal d'obtenir nous inhibidors del LPS, ha permès identificar el tiratricol, una molècula no tòxica, que neutralitza els efectes nocius del LPS. A més, la síntesi de diversos anàlegs per tal de realitzar estudis d'estructura-activitat permet obtenir compostos més actius.

4. La conjugació d'un pèptid neutralitzant del LPS, el pèptid RLKWc, amb el tiratricol resulta en un nou compost, anomenat, RLKWc-ttcol que presenta una activitat biològica millorada respecte el pèptid original.

4. PEPTOIDS AMB ACTIVITAT ANTIPROLIFERATIVA EN CÈL·LULES CANCERÍGENES

4.1 Introducció

En els anteriors capítols hem descrit diversos compostos de naturalesa peptídica com a inhibidors del LPS. En aquest últim capítol ens vam plantejar l'estudi de peptidomimètics com una estratègia alternativa per obtenir inhibidors del LPS amb estabilitat proteolítica. Els peptidomimètics són molècules dissenyades per a mimetitzar els pèptids, tot millorant-ne les seves propietats. Una estratègia habitual en el disseny de peptidomimètics es la *N*-metilació de pèptids. Exemples recents en la literatura descriuen com és possible mitjançant la *N*-metilació incrementar l'activitat biològica i la selectivitat dels pèptids, així com millorar la seva estabilitat enzimàtica, la seva permeabilitat i la seva disponibilitat oral. En aquest aspecte, una interessant classe de peptidomimètics són els oligomers de *N*-alquilglicines, també coneguts com a peptoids.

4.1.1 Peptoids: una visió general

Els peptoids són una família de peptidomimètics molt interessant per al descobriment de nous fàrmacs. Aquestes molècules mimetitzen l'activitat biològica dels pèptids, però són estables a la degradació proteolítca. Aquesta avantatge s'explica pel fet que en els peptoids, la cadena lateral es troba situada a l'àtom de nitrogen, enlloc de en el carboni en α com és el cas dels pèptids (Figura 4.1.1)

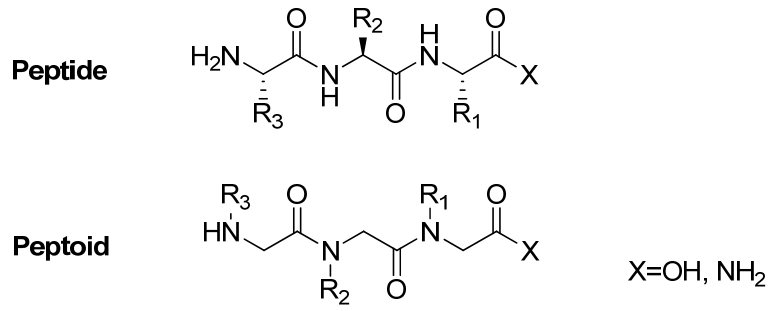


Figura 4.1.1: Estructura química dels pèptids i els peptoids.

A més els peptoids poden sintetitzar-se de manera molt senzilla i eficient en fase sòlida mitjançant la unió de dos sub-monomers: un àcid halo-acètic i una amina primària. Aquestes reaccions són fàcilment automatitzables, el que ha permès la preparació d'un gran nombre de quimioteques de peptoids. Aquestes quimioteques han estat assajades per un gran nombre d'activitats biològiques, entre les quals es poden destacar: la inhibició de Tat/TAR ARN, el transport de gens i fàrmacs i activitats anti-microbials, analgèsiques i anti-cancerígenenes. A més d'aquestes activitats, recentment s'han identificat dos peptoids, els peptoids **4** i **7**, amb activitat neutralitzant d'endotoxines *in vivo* (Figura 4.1.2).

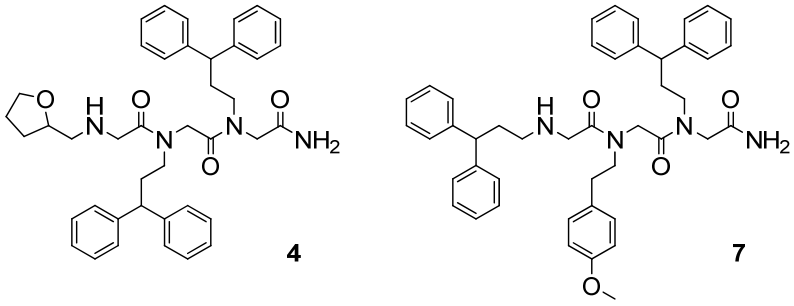


Figura 4.1.2: Estructura química dels peptoids **4** i **7**.

4.1.2 Un descobriment atzarós

Per continuar amb aquesta recerca ens vam plantejar la possibilitat d'identificar peptoids de cadena més curta derivats dels peptoids anteriors, per tal de comprovar si aquestes modificacions podrien tenir efectes positius en l'activitat biològica, toxicitat i solubilitat dels peptoids descrits. Per aquest motiu vam preparar una sèrie de peptoids (monomers i dipeptoids) en fase sòlida. Dos tipus de peptoids van ser sintetitzats. Per

una banda peptoids amb les carboxamides del C-terminal no substituïdes; i per altra, peptoids amb les carboxamides del C-terminal alquilades (Figura 4.1.3).

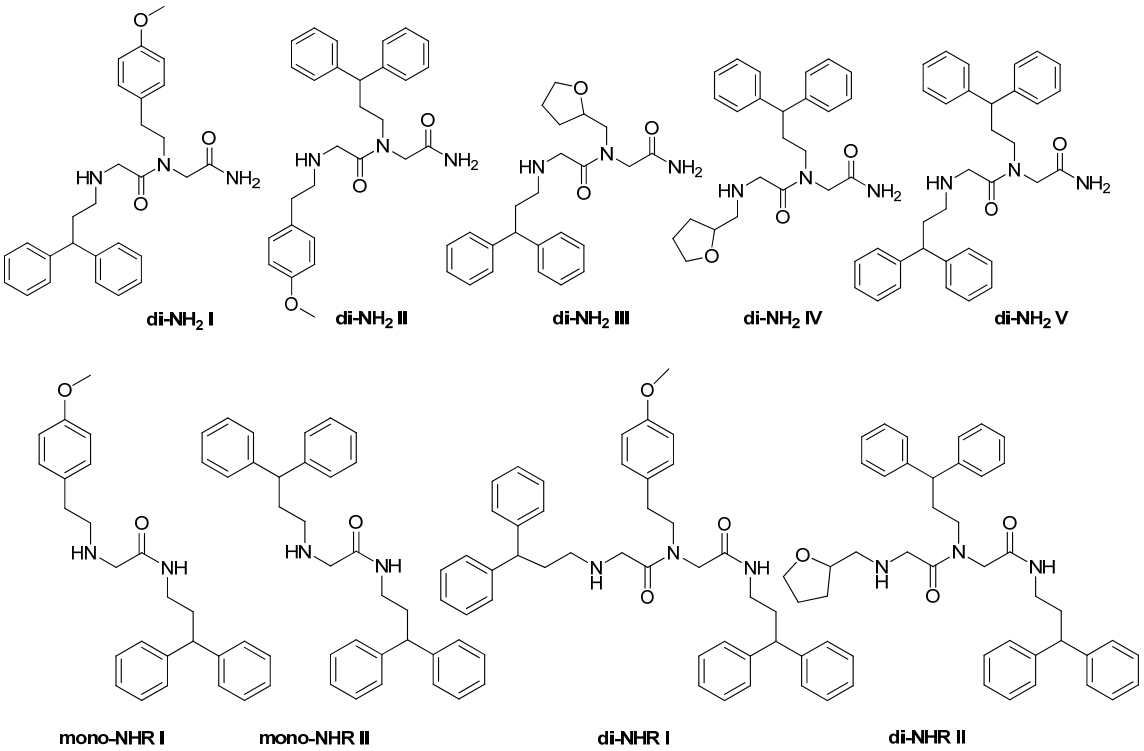


Figura 4.1.3: Estructura química de les dues famílies de peptoids sintetitzades.

Aquests peptoids desafortunadament no van mostrar capacitat per neutralitzar el LPS. Per aquest motiu es va realitzar una cerca en la literatura d'altres tipus d'activitats biològiques associades a peptoids amb aquest tipus d'estructures. Així, vam trobar una família de tripeptoids amb propietats pro-apoptòtiques en cèl·lules tumorals. Per tant, vam analitzar els peptoids per aquesta nova indicació terapèutica. Aquesta vegada els resultats van ser sorprenents, tal i com es detallarà a continuació.

4.1.3 Objectius

Els objectius d'aquest capítol són:

1. Avaluar l'activitat anti-proliferativa de peptoids de cadena curta (monomers i dipeptoids) front un nombre representatiu de línies cel·lulars tumorals humanes, i

comparar l'activitat biològica dels peptoids amb diferent substitució en les carboxamides de l'extrem C-terminal.

2. Avaluar si aquests diferents tipus de peptoids pateixen la formació de DKPs, una reacció secundària molt comuna en els peptoids, i relacionar aquest comportament amb les activitats biològiques que s'observin.

4.2 Resultats i discussió

4.2.1 Els peptoids més petits amb activitat anti-proliferativa en cèl·lules cancerígenes humanes

El aquest treball vam descriure el disseny i la síntesi d'una sèrie de mono- i dipeptoids. L'activitat biològica d'aquests compostos va ser avaluada vers diferents línies cel·lulars tumorals. Els compostos C-terminal *N*-alquil amida (Figura 4.2.1) van demostrar ser citotòxics per aquestes cèl·lules i són els peptoids de cadena més curta descrits a la literatura amb activitat anti-tumoral. Aquests compostos van ser sintetitzats en fase sòlida utilitzant la resina BAL. Gràcies a la seva estructura química aquests tipus de compostos no pateixen la formació de DKPs, una problemàtica reacció secundaria freqüentment observada en la síntesi de pèptid i peptoids.

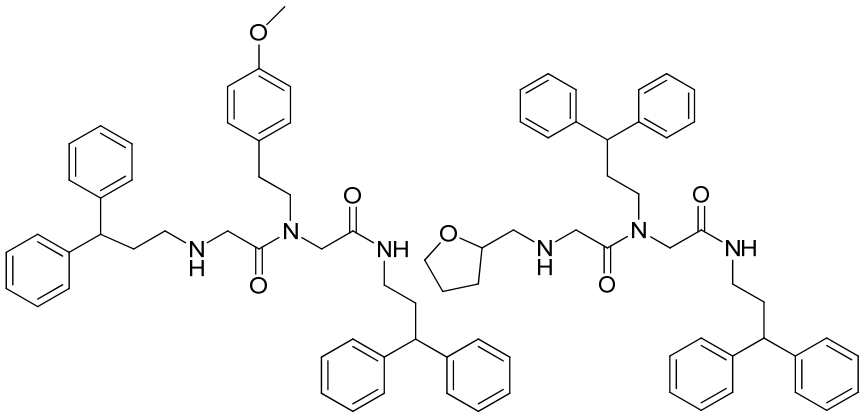


Figura 4.2.1: Estructura química de dos dipeptoids C-terminal *N*-alquil amida amb gran activitat citotòxica en diferents línies cel·lulars tumorals.

4.3 Conclusions

Les conclusions de l'últim capítol són les següents:

1. Una sèrie de nous peptoids, monomers i dipeptoids, derivats de dos tripeptoids amb activitat neutralitzant del LPS van tenir una pobre activitat inhibitòria del LPS, però van resultar ser altament citotòxics en cèl·lules cancerígenes. Aquest és el primer exemple a la literatura en el qual un dipeptoid o una *N*-alquilglicinamida presenten activitat anti-proliferativa en un nombre representatiu de línies tumorals.

2. Els peptoids de tipus C-terminal *N*-alquil amida no pateixen la formació de DKPs. Això podria suggerir que altres famílies de tripeptoids descrites a la literatura podrien ser modificades per obtenir nous peptoids més pèptids, amb activitat biològica i sense risc de formar DKPs. La resina BAL és de gran utilitat per a la síntesi d'aquest tipus de peptoids. La inserció d'un grup alquil a l'amida C-terminal mitjançant aquesta resina permet disposar d'un grup extra per a l'activitat biològica, sense que això representi un augment en la llargada de la cadena del peptoid. La qual cosa a més, comporta una millora en la solubilitat d'aquests compostos.