NEUROPATIA INDUÏDA PER BORTEZOMIB. CARACTERITZACIÓ I FACTORS DE RISC EN UN MODEL EXPERIMENTAL

Jordi Bruna Escuer

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Jordi Bruna Escuer

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Aquesta Tesis Doctoral ha estat realitzada sota la direcció de:

Dr. Xavier Navarro Acebes la Dra. Esther Udina Bonet

en el programa de doctorat de Neurociències, en el Departament de Biologia Cel·lular, Fisiologia i Immunologia, a la Universitat Autònoma de Barcelona

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A la Júlia i als bessons que estan en camí, per la seva estimació, el seu riure narcotitzant i la seva infinita paciència

AGRAÏMENTS

Probablement aquesta és una de les pàgines del present treball que m'ha portat més mal de caps i m'han requerit un major esforç de síntesi. Per una banda, perquè sé que és una de les pàgines més llegides en tota tesi doctoral, donada la natural curiositat de l'ésser humà, i per l'altra, per la por d'oblidar-me i manllevar el reconeixement públic a algú que m'hagi ajudat en algun moment del meu treball. Totes dues raons, dignes de ser remarcades. La primera, la curiositat, les ganes de saber, és a fi de comptes, el motiu principal de l'existència d'aquest treball i la principal eina adaptativa al medi de la nostre espècie. Per tant, un neguit, una sensació, un sentiment a estimular i potenciar. La segona raó, l'agraïment, és una acció que hem d'acostumar-nos a practicar més sovint, ja que és un dels pilars de la convivència i la solidaritat. Desgraciadament, els temps que ens toquen viure, on l'erosió accelerada de les estructures democràtiques i el ressorgiment d'oligocràcies són un fet, posen en perill aquests valors i de retruc s'incrementa el risc d'un segrest interessat de la ciència amb la seva pèrdua de valor social i col·lectiu. Per tant, començaré amb el meu exercici particular d'agraïment:

En primer lloc, no tinc paraules suficients de reconeixement cap als meus pares, gràcies al seu sacrifici personal, econòmic i a l'ambient familiar que van generar, m'han ofert les millors oportunitats per permetre'm tirar endavant amb èxit els meus estudis. Així mateix, han col·laborat a proveir-me d'una arma que no té preu en aquest món, la capacitat de pensar per un mateix. Òbviament, no puc oblidar en aquest punt, l'ajuda i la venerable estimació que professo envers els meus avis i al record dels germans d'aquets. Aquest treball és també en part la culminació dels seus esforços.

En segon lloc, m'agradaria expressar la meva plena gratitud, molt més enllà del seu paper en la direcció d'aquesta tesis, a en Xavier Navarro. És una obvietat que sense directors que pilotin el vaixell d'una tesi, aquesta no hauria arribat mai a bon port i estaria a la deriva en algun dels múltiples atzucacs que es presenten en el desenvolupament d'un treball d'aquestes característiques. Però en Xavier és per a mi més que un director, no

només ha orientat aquesta tesi sinó que m'ha servit de referent des de que vaig posar els peus en la facultat, en el sentit més clàssic de la paraula m'ha fet de Mestre. Em va obrir generosament les portes del seu laboratori quan era un estudiant perdut, em va ensenyar una forma única de pensar la medicina i em va transmetre en definitiva, la passió per la ciència. En part, gràcies a ell, estic on estic. En aquest punt, no em puc oblidar de l'Esther Udina, tota nau ha de tenir un excel·lent i tenaç contramestre per a que tots els engranatges funcionin adequadament i aquest paper l'ha realitzat l'Esther. Coneixent-me, no deu ser gaire fàcil dirigir-me, però l'Esther ho ha aconseguit de forma brillant. Qui hagués dit, a la que vaig titllar una mica àcidament de 'reina del muscle and nerve' quan estava fent la seva tesi, que anys més tard em dirigiria i salvaria la meva.

Tampoc puc deixar de fer esment a l'Albert Alé, pel seu suport, ajuda i entusiasme incondicional a totes les idees de bomber que se m'acudien en el laboratori, així com a l'Abel i l'Elena pel seu humor i ironia tan desestressants, o al Jordi, la Jessica, la Marta, el Rubèn i tots els companys del laboratori que m'ha aguantat i ajudat pacientment. També han de tenir menció especial, la Roser Velasco i la Marta Simó, les meves altres companyes del hospital, gràcies a la seva feina i paciència m'han facilitat el poder atendre les necessitats del laboratori.

Al marge de les persones que han col·laborat amb mi, haig d'expressar el meu reconeixement i estima al meu germà Pere, per estar sempre present quan el necessito. Així mateix a tots els amics, Jaumes, Xose, David, Ferran, Isra, Furi... que m'han acompanyat al llarg d'aquests anys fent més agradable i passatger el dia a dia. També recordar la memòria del Dr. Josep Petit, excel·lent hematòleg i extraordinària persona que ens ha abandonat prematurament i que gràcies als seus primers contactes van permetre'ns aconseguir el bortezomib.

Per acabar, retre homenatge a la Soca Swis OF1, pel seu sacrifici, sense el qual no hagués estat possible realitzar aquest treball.

ÍNDEX D'ABREVIATURES

AMPA: Alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate

AUC: Area under the curve

CTCAE: Common Terminology Criteria for Adverse Events

CGRP: Calcitonin gene-related peptide

FDA: Food and Drug Administration

GRP: Gangli raquidi posterior

GTP: Guanosine triphosphate

LD50: Lethal dose 50

MAP: Proteïnes associades a microtúbuls

MM: Mieloma múltiple

NCI: National Cancer Institute

NIB: Neuropatia induïda per bortezomib

NIQ: Neuropatia induïda per quimioteràpia

SNP: Sistema nerviós perifèric

PANCS: potencial d'acció nerviós compost sensitiu

PAMC: Potencial d'acció muscular compost

PGP9.5: Protein gene product 9.5

RGP: Retinoblastoma gene product

ÍNDEX

INTRODUCCIÓ

NEUROPATIA INDUÏDA PER AGENTS ANTINEOPLÀSICS

L'afectació del sistema nerviós perifèric (SNP) induïda per l'administració d'agents citostàtics és probablement la complicació neurològica més freqüent dels pacients amb càncer. La majoria d'antineoplàstics utilitzats en primera i segona línia de tractament, tant de neoplàsies sòlides com hematològiques molt prevalents, presentaran aquesta complicació com a efecte secundari a la seva administració. Malgrat la manca d'estudis epidemiològics rigorosos al respecte, és assumible que la neuropatia induïda per quimioteràpia (NIQ), és la neuropatia tòxica exògena amb una major incidència i prevalença en el nostre medi.

Aquesta complicació comporta dos problemes greus: per una banda, els símptomes generats per la neuropatia tenen un impacte important sobre la qualitat de vida dels pacients i, per altra banda, la seva aparició durant el tractament condiciona reduccions de dosis o fins i tot la suspensió del tractament antineoplàsic amb el conseqüent efecte sobre les probabilitats de supervivència del pacient.

Els agents citostàtics actuen sobre totes les cèl·lules de l'organisme, però repercutiran especialment sobre aquelles que presentin cicles proliferatius ràpids. Per tant, no només afectaran les neoplàsiques sinó també les de teixits sans amb alts índex proliferatius, com serien les de la mucosa intestinal, les hematopoètiques i les dels ganglis limfàtics, provocant prominents símptomes digestius, citopènies hematològiques i immunosupressió. Paradoxalment, cèl·lules postmitòtiques quiescents com les neurones són susceptibles a l'acció d'aquests fàrmacs. Per aquest fet, la neuropatia perifèrica, a l'igual que els altres efectes secundaris, es converteix en un factor dosi-limitant de l'administració de la majoria de quimioteràpics en pacients amb càncer. Els motius que explicarien la susceptibilitat de les neurones del SNP a aquests agents vindria donada per diverses raons. Al sistema nerviós central, l'existència de la barrera hematoencefàlica, permet aïllar les neurones i les cèl·lules glials de la resta del organisme. Per tant, els somes de les motoneurones espinals, que es troben a nivell medul·lar, estarien protegits per aquesta barrera. En contrast, les neurones autonòmiques i sensorials del SNP, localitzades en els ganglis vertebrals, viscerals i raquidis posteriors (GRP), no estan protegides a l'accés de substàncies exògenes per aquesta estructura anatomico-funcional. Així mateix,

tots els axons que porta un nervi perifèric, ja siguin motors, sensorials com autonòmics, es veuen igualment exposats. Els *vasa nervorum* que aporten el flux sanguini al SNP estan constituïts per capil·lars d'endoteli fenestrat amb menor densitat de molècules d'unió estreta entre elles respecte a la barrera hemato-encefàlica, permetent el pas de molècules entre la circulació i l'espai extracel·lular dels ganglis i dels nervis perifèrics, independentment de les característiques de solubilitat d'aquestes (Mizisin et al. 2011). Per altra banda, la longitud dels axons que composen el SNP també determina la seva susceptibilitat als citostàtics. A més, molts dels fàrmacs antineoplàsics interfereixen sobre el metabolisme energètic o provoquen la disrupció dels microtúbuls del fus mitòtic, afectant conseqüentment també el transport axonal. Finalment, les neurones presenten vies d'activació de mort cel·lular programada particularment sensibles al dany del DNA, acció característica d'alguns dels citostàtics. Malgrat que tots aquests factors juguen un paper destacable en la susceptibilitat de les neurones als agents antiproliferatius, aquests no expliquen completament el quadre presentat pels pacients. Per exemple, hi ha una divergència entre la intensitat de l'afectació entre axons sensitius i motors de fàrmacs com els alcaloides de la vinca i els taxans, que actuarien alterant el transport axonal. Així mateix, l'absència d'afectació autonòmica dels platins, que actuarien sobre el DNA del soma neuronal, tampoc estaria justificada.

Epidemiologia

La incidència i prevalença de la NIQ és molt variable, comprenent amplis rangs en funció dels estudis (Argyriou et al. 2012). Aquesta variabilitat es deu principalment als següents factors: a) el tipus de quimioteràpic usat; b) la dosis a la qual s'administra el fàrmac; c) l'existència de règims consistents en combinacions de diferents agents citostàtics neurotòxics; d) les característiques intrínseques dels pacients, com l'eficiència de diferents polimorfismes genètics de molècules implicades en els mecanismes de reparació i detoxificació cel·lular; i e) el criteri i tècnica diagnòstica d'avaluació de la NIQ (Velasco et al. 2010; Argyriou et al. 2012). A més, altres factors contribueixen a l'heterogeneïtat interpretativa de les xifres d'incidència. Moltes de les dades s'han extret d'assajos clínics, on els pacients participants poden ser una mostra excessivament seleccionada de la població general a la que s'aplica el tractament. Aquest fet pot provocar un biaix alhora de poder extrapolar els resultats de seguretat a una població general en la que coexisteixen altres factors mòrbids que poden ser criteris d'exclusió dels assajos. Per altra banda, hi ha estudis on s'observa que la NIQ podria estar subestimada tant pels propis

facultatius com pels pacients, els quals minimitzarien els símptomes per por a una potencial reducció o suspensió del tractament (Markman 1996; Shimozuma et al. 2009). Finalment, alhora de valorar la prevalença de la NIQ, també s'ha de tenir en compte el temps transcorregut des de la suspensió del fàrmac, ja que existeix cert grau de recuperació espontània i progressiva, variable en funció de la intensitat de la neuropatia que s'hagi arribat a desenvolupar. Tenint en compte tots aquests matisos, en la Taula 1 (pàgina 4) es mostren els rangs d'incidència i prevalença de la neuropatia induïda pels principals agents citostàtics neurotòxics d'us habitual.

Per altra banda, l'increment de la supervivència dels pacients amb càncer, gràcies a l'optimització dels règims terapèutics, als nous fàrmacs i a tècniques de diagnòstic i seguiment més sensibles, contribueix a augmentar el nombre de pacients que pateixen els efectes crònics de la neuropatia establerta. A més, en cas de recaigudes, actualment és possible l'aplicació de segones i terceres línies de tractaments que poden seguir mantenint un perfil neurotòxic d'efectes adversos. Alhora, molts dels nous fàrmacs antineoplàsics apareguts en els darrers anys, com el bortezomib o les ixabepilones, segueixen presentant efectes tòxics sobre el SNP. Tampoc s'ha d'oblidar la implementació, al llarg del temps, d'estratègies cada cop més eficients per minimitzar altres tipus de toxicitats associades als citostàtics que poden ser dosi limitants. Així, l'administració concomitantment de factors estimulants del creixement de colònies hematopoètiques, d'antiemètics i la hiperhidració i diüresis forçada, redueixen la toxicitat hematològica, digestiva i renal i permet incrementar el nombre de pacients als quals se'ls pot dispensar dosis complertes de quimioteràpia. Això, no obstant, incrementa el risc potencial de desenvolupar neurotoxicitat en pacients que haurien manifestat abans un altre tipus de toxicitat. Tots aquests factors contribueixen al creixent nombre de pacients amb NIQ.

Taula 1. Principals citostàtics neurotòxics i característiques de la neuropatia que indueixen.

Agent citostàtic	Indicacions	Dosis acumulada neurotòxica	Incidència (rangs)*	Tipus de neuropatia	Evolució
Platins					
Cisplatí	Pulmó, testicular, ovari, bufeta, cap i coll, esofagogàstric	>250-350 mg/m^2 >600 mg/m^2	30-40% 100% (16-100%)	Sensitiva pura	20-40% als 30 i 15 anys
Carboplatí	Ovari, mama, pulmó, endometri, bufeta, cap i coll	AUC>6	10-20%	Sensitiva pura	40% al any en règims combinatoris, no dades com a únic fàrmac
Oxaliplatí	Colon i gàstric	<765mg/m^2 >765-1000 mg/m^2 >1000 mg/m^2	10% 30-50% >50% (12.5-94%)	Crònica: Sensitiva pura Aguda: Neuromiotonia Hiperestèsia al fred	35% als 5-6 anys
Taxans					
Paclitaxel	Mama, ovari, pulmó, pròstata, cap i coll i esòfag	>200 mg/m^2 >1000 mg/m^2	70% 95% (14-67%)	Sensitivo-motora	Reversible en la majoria de casos, no dades a llarg terme per als 2 compostos
Docetaxel	Mama, pròstata, pulmó, bufeta, gàstric, cap i coll	>370 mg/m^2	50% (17-70%)	Sensitivo-motora	

Alcaloides de la vinca					
Vincristina	Limfomes i leucèmies	2-6 mg/m^2 >8 mg/m^2	(26-80%)	Sensitivo-motora i autonòmica Simptomes motors prominents	24% als 3 anys
Vinorelbina	Mama, pulmó no oat-cell i ovari	25-30 mg/m^2 **	(6-29%)	Sensitvo-motora Predomina símptomes sensitius i autonòmics	Reversible en la majoria de casos als 6 mesos
Epotilones Ixabepilona	Mama	40 mg/m^2 **	71% (41-93%)	Sensitivo-motora	Desconeguda a llarg terme
Talidomida	Mieloma	>20g	(14-70%)	Sensitiva pura	Desconeguda a llarg terme
Bortezomib	Mieloma, limfoma del mant	30-40 mg/m^2	(31-64%)	Sensitiva pura	23-36% als 4 mesos

**Incidències, independentment del grau de severitat, dels diferents estudis amb avaluació neurològica específica i d'assajos oncològics fase III. Esquemes d'administració, no disponibles estudis de dosis acumulada .

Avaluació de la neuropatia induïda per citostàtics

Els mètodes d'avaluació de la severitat de la NIQ no són homogenis, encara que en la majoria d'assajos clínics oncològics s'utilitza l'escala del *National Cancer Institute – Common Toxicity Criteria* (NCI-CTC) (Taula 2). Ara bé, malgrat que té les avantatges d'una ràpida administració, no requereix instrumentització i el seu ús és molt generalitzat, presenta una sèrie de problemes. Existeix una notable discrepància entre observadors (Postma et al. 1998), ja que grada els símptomes manifestats pels pacients amb termes subjectius com 'lleu', 'moderat' o 'sever' i usa el concepte genèric de 'limitant de les activitats de la vida diària', no aporta dades objectives o quantificables ni de localització. Contràriament, en els estudis de cohorts de pacients focalitzats en l'avaluació de la NIQ o en assajos de neuroprotectors habitualment s'usen altres escales, producte de la composició de símptomes i signes que aporten dades quantificables, localitzadores i generalment de major qualitat, sent la *Total Neuropathy Scale* la més estesa i en la que s'han avaluat més adequadament les seves propietats clinimètriques en pacients amb NIQ (Cavaletti et al. 2010). No obstant, totes aquestes escales compostes, presenten el problema de consumir força temps i del requeriment d'un entrenament específic per part del avaluador. Aquest conjunt de problemes, contribueix a incrementar la variabilitat de les incidències reportades de NIQ en els diferents estudis.

Taula 2. Escala NCI-CTCAEC de toxicitat sobre el nervi perifèric utilitzada en els assajos clínics en oncologia.

Efecte Advers	Grau 1	Grau 2	Grau 3	Grau 4
Neuropatia perifèrica sensitiva	Pèrdua de reflexes o parestèsies que no interfereixen en les AVD	Alteracions sensitives o parestèsies que interfereixen en la funcionalitat però no causen alteracions de les AVD	Alteracions sensitives o parestèsies que interfereixen en les AVD	Alteracions sensitives incapacitants o que amenacen la vida
Neuropatia perifèrica motora	Asimptomàtic, debilitat observada únicament a l'exploració	Debilitat simptomàtica que interfereixen en la funcionalitat però no causen alteracions de les AVD	Símptomes severs que impedeixen les AVD o requereixen suports al deambular	Alteracions incapacitants o que amenacen la vida

AVD: activitats de la vida diària

Quadre clínic i alteracions electrofisiològiques

La NIQ es presenta de forma progressiva i subaguda al llarg de l'administració del tractament quimioteràpic, predominantment durant els darrers cicles d'aquest, encara que en alguns casos s'han reportat presentacions molt precoces, durant els primers cicles. Per altra banda, en certs fàrmacs com el platins, la vincristina i els taxans s'han descrit empitjoraments progressius setmanes i mesos després de la suspensió de l'agent causal, fenomen conegut com efecte *coasting*. Els pacients referiran símptomes positius i/o negatius, predominantment sensitius, secundaris a la disfunció del SNP amb algunes característiques particulars en funció del mecanisme d'acció del citostàtic usat. La distribució dels símptomes i signes segueix el patró clàssic de les polineuropaties, amb una afectació inicial de les extremitats inferiors a nivell distal, simètrica i progressivament ascendent, que s'extendrà també a les extremitats superiors en el mateix sentit durant la continuació del tractament. Així mateix, els signes clínics més precoços en la majoria de pacients són la disminució de la sensibilitat vibratòria i la pèrdua dels reflexes aquilis. Entre els símptomes sensitius positius destaquen sobretot les parestèsies, acompanyades en ocasions de disestèsies, al.lodínia i hiperalgèsia. El símptoma sensitiu negatiu predominant és la hipoestèsia tàctil, que pot comportar, si aquesta és severa, incapacitat funcional per a activitats quotidianes. Altres símptomes negatius deguts a la hipoestèsia propioceptiva, com l'atàxia de la marxa, rarament són incapacitants. Els símptomes disautonòmics més reportats són els còlics intestinals, l'ili paralític, la impotència sexual i l'hipotensió ortoestàtica, si bé cal tenir en compte que no sempre són avaluats de forma sistemàtica. Per altra banda, no tots els fàrmacs antineoplàsics els provoquen (Velasco et al. 2010; Argyriou et al. 2012).

Els principals citostàtics neurotòxics es poden agrupar en diferents famílies de compostos: els derivats dels platins (cisplatí, oxaliplatí i carboplatí), els derivats dels alcaloids de la vinca (vincristina, vinblastina, vinflunina i vinorelbina), els taxans (docetaxel i paclitaxel), les epotilones (ixabepilona), els inhibidors del proteasoma (bortezomib) i la talidomida. Les característiques fonamentals d'indicació, dosis neurotòxica limitant habitual, característiques peculiars clíniques i electrofisiològiques i la seva evolució estan sintetitzades en la Taula1 (pàgina 4).

Platins: A nivell distintiu, generen una neuropatia sensitiva pura, predominantment de fibra gruixuda, estant la sensibilitat termo-algèsica, l'artrocinètica i el tacte fi menys afectats. En els casos més greus es pot observar una marxa atàxica i moviments pseudoatetòsics dels dits de mans i peus. Tampoc és inusual observar un

fenomen de Lhermitte secundari al dany sobre els cordons posteriors degut a la degeneració retrògrada de les arrels posteriors dels GRP (Argyriou et al. 2007; Park et al. 2009). Electofisiològicament s'observa una reducció de l'amplitud dels potencials d'acció nerviosos composts sensitius (PANCS) amb preservació de les velocitats de conducció, de l'amplitud dels potencials d'acció musculars compostos (PAMC) i de les latències de les ones F (Roelfols et al. 1984; Thomson et al. 1984; Daugaard et al. 1987; Mollman et al. 1988; Krarup-Hansen et al. 2007; Argyriou et al., 2008a). Com a tret remarcable, l'oxaliplatí genera un tipus de neuropatia aguda autolimitada que cursa amb disestèsies i al·lodinia tèrmica pel fred a mans, peus i orofaringe. S'inicia amb la infusió del fàrmac i dura entre 48-72h, encara que s'han descrit quadres més perllongats, si bé sempre inferiors als 15 dies. Un nombre reduït de pacients també presentarà disàrtria, dolor mandibular durant la masticació, sensacions subjectives de disfàgia, dificultat respiratòria, rampes durant la deambulació, fasciculacions i miotonia. Electrofisiològicament, s'observen descàrregues neuromiotòniques i de potencials d'unitats motores repetitius, juntament amb una hiperexcitabiliat i reducció dels paràmetres de refractorietat de la conducció (de Gramont et al. 2000; Wilson et al. 2002; Lehky et al. 2004; Krishnan et al. 2005; Park et al. 2009).

Alcaloides de la vinca: els pacients presenten una neuropatia sensitivo-motora, si bé a nivell clínic l'afectació motora només s'observa a altes dosis. La hiporeflèxia es una troballa molt precoç, que pot ser prèvia a l'aparició de símptomes de neuropatia establerta. És freqüent la disfunció autonòmica en forma de còlic abdominal i ili paralític de diferents intensitats, que sol aparèixer als pocs dies d'iniciar el tractament, i també la impotència. A nivell electrofisiològic es registra primer una disminució de les amplituds dels PANCS seguida de la dels PAMC, amb fibril·lacions i reducció del patró d'interferència durant la contracció de la musculatura distal (Sandler et al. 1969; Casey et al. 1973; Haim et al. 1994; Verstappen et al. 2005).

Agents estabilitzadors dels microtúbuls: **els taxans** provoquen els símptomes i signes clàssics d'una neuropatia de predomini sensitiu, amb afectació motora únicament a altes dosis. La troballa electrofisiològica predominant es la disminució de l'amplitud dels PANCS. S'han descrit també fenòmens de pruït, miàlgies i bradicàrdies asimptomàtiques associades al tractament amb paclitaxel. Les **epotilones**, presenten un perfil clínic similar al dels taxans, encara que no disposem actualment d'estudis electrofisiològics seriats (Thomas et al. 2007a; Thomas et al. 2007b; Argyriou et al. 2008b; Krzakowski et al. 2010).

Talidomida: indueix una neuropatia sensitiva pura, que sol desenvolupar-se de forma tardana a altes dosis acumulades. Electrofisiològicament presenta l'esperada reducció dels PANCS amb preservació dels PAMC i de les velocitats de conducció (Cavaletti et al. 2004).

Inhibidors del proteasoma: Les característiques clíniques i electrofisiològiques de la neuropatia induïda per bortezomib seran descrites amb detall en un apartat posterior.

Pronòstic i factors de risc

El principal factor de risc conegut dels agents quimioteràpics és la dosis total acumulada rebuda pel pacient i aquesta dependrà del citostàtic administrat. En funció d'aquest, el risc de neuropatia severa varia i, conseqüentment, les probabilitats de recuperació complerta. Un altre factor de risc important són els règims terapèutics que combinen diferents fàrmacs neurotòxics. De totes maneres, la suspensió del tractament comportarà una estabilització de la neuropatia i, en força casos, una millora parcial o complerta d'aquesta, en uns temps variables en relació a la severitat de la neuropatia que s'ha generat (Argyriou et al. 2012).

Platins: amb el cisplatí, la neuropatia pot aparèixer a partir de dosis acumulades de 250-350 mg/m^2 (Thompson et al. 1984; Glendenning et al. 2010), i serà present en quasi tots els pacients que arribin a dosis de 500-600 mg/m^2 (Roelofs et al. 1984). La seva recuperació habitualment és incomplerta, persistint els símptomes en un 30% dels pacients a 15 anys i en un 10% d'aquests a 30 anys després de la seva suspensió. En un 10% dels pacients amb neuropatia crònica, aquesta serà incapacitant (Strumberg et al. 2002; Glendenning et al. 2010). El carboplatí és el platí amb menys capacitat d'induir neuropatia a dosis convencionals, observant-la a dosis d'AUCs>6 (Cavaletti et al. 1998). L'oxaliplatí provocarà neuropatia severa en un 10% dels pacients a partir de dosis acumulades de 510-765 mg/m^2, i en el 50% d'aquests a dosis de 1000 mg/m^2 o superiors (de Gramont et al. 2000; Souglakos et al. 2002). S'observa una persistència de la neuropatia fins al 35% dels pacients a 5-6 anys després de finalitzar el tractament (Pietrangeli et al. 2006; Brouwers et al. 2009). Altres factors que poden facilitar l'aparició de neuropatia en els platins són la hipomagnesèmia en el cas del cisplatí (Bokemeyer et al. 1996; Glendenning et al. 2010) o la combinació d'oxiplatí amb l'agent antiangiogènic bevacizumab, que per si mateix no produeix cap efecte advers sobre el SNP (Giantonio et al. 2007; Allegra et al. 2009). Per altra banda, els polimorfismes de diferents gens

implicats en la seva biotransformació i en els mecanismes de reparació del DNA (Dzagnidze et al. 2007; McWhinney et al. 2009) han de tenir-se en compte alhora de valorar el potencial risc de desenvolupar neuropatia per platins.

Alcaloides de la vinca: la majoria de pacients que rebin dosis acumulades entre 2-6 mg/m^2 de vincristina presentaran símptomes i signes de neuropatia sensitiva i a partir de 8 mg/m^2 també símptomes motors (Sandler et al. 1969) i dolor (Dougherty et al. 2007). Tres anys després del tractament, fins a un 24% dels pacients mantenen símptomes de neuropatia sensitiva (Postma et al. 1993), mentres que els símptomes motors i disautonòmics es resolen en tots els pacients (Sandler et al. 1969; Casey et al. 1973; Haim et al. 1994). Aquells pacients que acaben presentant una recuperació total, triguen entre 34-40 mesos a assolir-la (Postma et al. 1993; Haim et al 1994). Altres alcaloides de la vinca, com la vinorelbina, presenten generalment una neuropatia reversible als 6 mesos de la suspensió del tractament (Pace et al 1996). Altres factors de risc sorgits de l'experiència clínica empírica són el presentar una forma indolent no reconeguda de neuropatia hereditària (Graf et al. 1996; Kalfakis et al. 2002; Trobaugh-Lotrario et al. 2003) i la insuficiencia hepàtica (Sandler et al. 1969).

Estabilitzadors de microtúbuls: els règims que contenen paclitaxel s'associen a un major risc d'incidència de neuropatia respecte els de docetaxel (Chon et al. 2009). S'observen neuropaties severes a partir de dosis acumulades de 1000 mg/m^2 de paclitaxel i de 371 mg/m^2 de docetaxel (Lee et al. 2006). La recuperació d'aquesta neuropatia és bona als 3-6 mesos de la suspensió del tractament, encara que la severa persistirà en el temps (Argyriou et al. 2008b). No s'han identificat consistentment altres factors de risc independents a la dosis acumulada i a la combinació amb altres fàrmacs neurotòxics. Pel que fa a les ixabepilones, existeixen pocs estudis sistemàtics sobre l'evolució, els factors de risc i fins i tot hi ha dades contradictòries en relació a la incidència de la seva neuropatia. Aquest fet ve donat probablement perquè es un fàrmac relativament nou, no aprovat en tots els països i que té com a indicació principal les neoplàsies de mama metastàtiques, és a dir en pacients moltes vegades tractades prèviament amb altres fàrmacs neurotòxics (Argyriou et al. 2012).

Talidomida: l'únic factor de risc conegut és la dosis total acumulada (Cavaletti et al. 2004), esperant-se la seva aparició a altes dosis (>20g). Així mateix, poques dades es tenen sobre la seva evolució, observant una millora amb la suspensió del seu tractament (Isoardo et al. 2004).

MECANISMES NEUROPATOGÈNICS DELS CITOSTÀTICS

Compostos derivats del platí

Malgrat l'existència de diferents derivats del platí amb propietats químiques diferencials, l'acció neurotòxica de tots ells recau en el dany que exerceixen sobre els somes de les neurones dels GRP (Thompson et al. 1984; Krarup-Hansen et al. 1999; Cavaletti et al. 2001). Així, la concentració de nivells de platí al GRP es correlaciona amb la severitat de la neuropatia induïda (Gregg et al. 1992; Dzagnidze et al. 2007). L'etiopatogènia d'aquesta neuropatia s'explica per dos mecanismes. Per una banda, els platins formen adductes creuats intra i intercadena amb el DNA, alterant d'aquesta forma la seva estructura terciària (McDonald et al. 2005; Ta et al. 2006). Aquest efecte sobre el DNA provoca pertorbacions en la cinètica normal del cicle cel·lular, ja que comportarà una sobre-regulació de l'expressió de ciclina D1 i una hiperfosforilació del *retinoblastoma gene product* (RGP). Això provocarà un intent aberrant de re-entrada al cicle cel·lular de les neurones diferenciades postmitòtiques del GRP, que posteriorment activarà els mecanismes d'apoptosis (Gill et al. 1998), modulada per l'activació de p38 i ERK1/2 (Scuteri et al. 2009) i per un increment de l'activitat de p53, que acabarà amb l'alliberació final del citocrom-c mitocondrial (Mcdonald et al. 2002). Paral·lelament, encara que de significat incert, també s'ha observat una reducció de les subunitats pesades fosforilades del neurofilament (pNF-H) a nivell dels GRP però no en els axons (Jamieson et al. 2009).

Com a mecanisme complementari neurotòxic, s'ha proposat l'estrès oxidatiu generat pel tractament i la malaltia de base. Aquest estrés facilitaria la disfunció mitocondrial de la neurona, que actuaria com a desencadenant dels mecanismes d'apotosis neuronals (Leonetti et al. 2003; Zhang et al. 2007).

De manera excepcional, l'oxaliplatí també genera una neuropatia aguda transitòria relacionada amb cadascuna de les infusions del fàrmac. Aquesta acció aguda, a diferència de la neuropatia crònica, es desenvolupa sobre l'axó i és causada per la formació d'oxalat, producte de la metabolització de l'oxaliplatí. L'oxalat, un quelant del calci i del magnesi, actua provocant una alteració de la regulació dels canals de sodi voltatge-depenents a l'interferir amb els fluxos i concentracions del calci (Adelsberger et al. 2000; Grolleau et al. 2001). Aquest fet tindr· com a conseq∏¿ncia final la reducciÄ de la refractorierat

axonal i la predisposició cap a la generació d'activitat ectòpica per part de l'axó (Krishnan et al. 2005; Park et al. 2009).

Derivats dels alcaloides de la vinca

La seva acció neurotòxica és deguda a la formació de complexes estables entre els alcaloides de la vinca i el domini GTPasa de la β-tubulina. Aquest fet inhibeix la hidròlisi del GTP, impedint així la polimerització dels dímers solubles de tubulina als microtúbuls i causant una disrupció d'aquests que es manifestarà en alteracions de la seva longitud i orientació. Tot això acaba provocant alteracions del diàmetre axonal i de la microestructura de les fibres mielíniques i amielíniques (Shelanski et al. 1969; Sahenk et al. 1987; Tanner et al. 1998; Topp et al. 2000). La disfunció de la dinàmica normal dels microtúbuls del citoesquelet afecta, en darrera instància, al transport axonal, conduint a la degeneració de l'axó (Schlaepfer et al. 1971).

El perfil de severitat de neurotoxicitat entre els diferents derivats de la vinca s'ha relacionat amb la seva afinitat per la tubulina (vincristina > vinblastina > vinorelbina > vinflunina) (Lobert 1996), correlacionant aquesta afinitat amb la intensitat de la neuropatia que poden arribar a generar aquests compostos.

Estabilitzadors dels microtúbuls

Els mecanismes involucrats en la neurotoxicitat d'aquests fàrmacs són menys coneguts i contradictoris. Es considera un element bàsic en la gènesis de la neuropatia la interferència en la dinàmica normal dels microtúbuls i la conseqüent alteració del transport axonal (Cavaletti et al. 1995; Persohn et al. 2005). Els taxans no només actuen sobre els microtúbuls axonals sinó també en el soma de la neurona i fins i tot en les cèl·lules de Schwann (Cavaletti et al. 1995). A part d'unir-se a dominis de la β-tubulina específics diferents als dels derivats de la vinca, també suprimirien les corrents de calci intracel·lulars provinents dels receptors AMPA, induint la despolimerització dels microtúbuls (Furukawa et al. 1995) i provocant una reconfiguració polar de l'orientació d'aquests, que comporta una alteració del transport d'organeles (Shemesh et al. 2010). Malgrat que s'observa una acció en diferents dianes del SNP, es creu que l'acció principal dels taxans recau sobre el GRP (Sahenk et al. 1994), tant a nivell neural com de les cèl·lules satèl·lit. També s'ha observat una activació de macròfags a ganglis i nervi, juntament amb una activació de la micròglia a nivell de la medul·la espinal (Peters et al.

2007), si bé es desconeix si aquest fet és conseqüència o causa de la neuropatia induïda per aquests fàrmacs.

Les epotilones, d'estructura i propietats químiques ben diferents als taxans, tenen igualment la capacitat d'estabilitzar els microtúbuls (Patrick et al. 2008). El domini de la tubulina sobre el que s'uneixen es sobreposa amb el dels taxans i, a l'igual que aquests, causen una polimerització dels dímers de β-tubulina i una estabilització dels microtúbuls preformats en absència de GTP i proteïnes associades del microtúbul (Bollag et al. 1995). En aquest cas, s'observa únicament un efecte axonopàtic, sense alteracions en els GRP, encara que també s'aprecia una disminució de les concentracions de RNA missatger de proteïnes de la mielina (MBP, P0 i MAL) en els extractes de nervi (Chiorazzi et al. 2009).

Talidomida

Malgrat ser un fàrmac conegut i usat des de fa més de 30 anys, la patogènia de la NIQ induïda per talidomida és de les més desconegudes, probablement degut a la manca de bons models animals per a estudiar-la. Les hipòtesis no comprovades al voltant del seu mecanisme d'acció neurotòxic estarien relacionades amb la seva activitat antiangiogènica, immunomoduladora i reguladora de la síntesis de citoquines, que afectarien primordialment el soma neural (Giannini et al. 2003; Isoardo et al. 2004; Lepper et al. 2006).

Altres mecanismes potencialment implicats

Independentment de les dianes específiques de la neurona sobre les quals actuen els citostàtics, explicatives del seu efecte neurotòxic, diferents estudis *in vivo* advoquen cap a una contribució extraneural. Aquests estudis defensen, com a mecanisme primari o concomitant de la neuropatia, una etiologia isquèmica del nervi. En suport a aquesta hipòtesis, s'ha observat que el cisplatí indueix l'apoptosis de les cèl·lules endotelials dels *vasa nervorum* (Kirchmair et al. 2005) i que el tractament mitjançant la transfecció de plàsmids que codifiquen per a diferents formes de *vascular endothelial growth factor* (VEGF) reverteix els efectes d'aquesta neuropatia i la induïda per taxans i talidomida (Kirchmair et al. 2007). Paral·lelament, a nivell clínic s'ha observat que els règims que combinen l'administració d'oxaliplatí amb anticossos contra el VEGF, com el

bevacizumab, presenten una incidència de neuropatia superior a la d'oxaliplatí habitual (Giantonio et al. 2007; Allegra et al. 2009). El problema d'aquesta hipòtesis és que la neuropatia isquèmica hauria de ser sensitivo-motora i amb afectació autonòmica. En contrast, la que manifesten els pacients és sensitiva pura sota règims de platins i talidomida i de gran predomini sensitiu en el cas dels taxans; sense presentar, els platins, indicis de disfunció autonòmica.

BORTEZOMIB

El bortezomib (Velcade®), un àcid dipeptidil boriònic modificat (Figura 1A), és el primer fàrmac desenvolupat d'una nova classe d'agents antineoplàsics coneguts com a inhibidors del proteasoma. El seu mecanisme d'acció consisteix en inhibir de forma reversible i dosis-depenent l'activitat tripsina-*like* de l'unitat 20S del proteasoma (Adams 2003). Administrat endovenosament presenta una vida mitja de 9-15h i es metabolitza pel sistema d'isoenzims del citocrom P450 hepàtic a través de la deboronació, formant-se dos metabòlits deboronats que són inactius com a inhibidors del proteasoma.

Estructura i funció del proteasoma

El proteasoma és un complex enzimàtic multicatalític d'estructura cilíndrica que s'encarrega de degradar la majoria de proteïnes intracel·lulars, tant citoplasmàtiques com nuclears (Glickman et al. 2002), i s'estima que elimina entre 80-90% de les proteïnes cel·lulars (Nandi et al. 2006). La segona via de degradació de proteïnes és la via dels agresomes-lisosomes, encarregada de degradar proteïnes extracel·lulars i transmembrana.

La forma activa del proteasoma, la unitat 26S, està constituïda per la unió ATP-depenent de les subunitats 19S als extrems de la subunitat 20S, que conté el *core* catalític del complex (Adams 2004). Les proteïnes que han de ser degradades són marcades mitjançant una cadena polipetídica d'ubiquitina gràcies a l'unió covalent als residus de lisina d'aquestes. La subunitat 19S reconeix la cadena d'ubiquitina de les proteïnes destinades a ser degradades, lleva la cadena i desnaturalitza la proteïna per mitjà de l'intervenció de 6 ATPases. Posteriorment, introdueix la proteïna desplegada en el centre de l'estructura cilíndrica de la subunitat 20S. Aquesta subunitat està formada per quatre anells, 2α i 2β subunitats, en el centre de la qual les unitats β presenten una activitat enzimàtica tripsina-*like*, quemotripsina-*like* i post-glutamil pèptid hidrolasa. En aquest

punt, el substrat proteic és progressivament degradat, i a la fi del procés s'alliberen cadenes curtes de pèptids (Figura 1B).

El proteasoma, per tant, mitjançant la degradació proteica, juga un paper essencial en la eliminació i regulació de la concentració intracel·lular de diverses proteïnes.

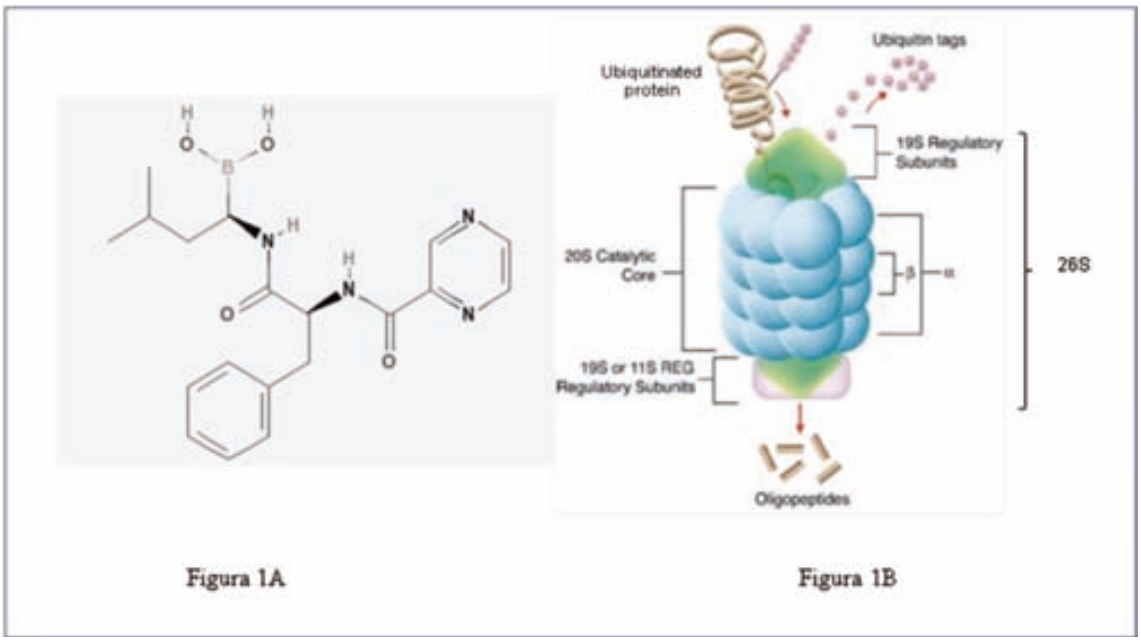

Figura 1A: Estructura molecular del bortezomib. **Figura 1B**: Representació esquemàtica del proteasoma (26S), composat per les unitats 20S i la 19S o 11S REG (PA28). La 20S està formada per quatre anells apilats (2α i 2β), cadascun comprès per set subunitat. Els extrems distals del complex cilíndric 20S estan coberts per la unitat 19S que s'encarrega de la unió, trencament i alliberació de la cadena ubiquinada, dirigint la proteïna desplegada cap al *core* proteolític de la unitat 20S. Aquesta portarà a terme la degradació final de la proteïna desnaturalitzada, alliberant posteriorment els pèptids resultants.

Acció antineoplàsica de la inhibició del proteasoma

El proteasoma juga un paper crític en la degradació de proteïnes de senyalització intracel·lular involucrades en la transducció de senyals que regulen el creixement i la proliferació cel·lular, com les ciclines A, B, D, E, p21 i p27 i el supressor tumoral p53. Alhora, també modula l'activació del factor de transcripció NF-κB i l'expressió de molècules d'adhesió (Sanchez-Serrano et al. 2006). Malgrat que no s'ha definit amb precisió l'efecte específic que genera el bortezomib sobre aquests reguladors de l'homeòstasi cel·lular, es creu que a través de la inhibició de l'acció del proteasoma, es produiria un desplaçament del balanç de proteïnes antiapoptòtiques cap a un perfil més

proapoptòtic, conduint finalment a la mort de la cèl·lula neoplàsica (Voorhees et al. 2003; Ludwig et al. 2005). El motiu pel qual el bortezomib causa la mort a les cèl·lules neoplàsiques de forma preferencial respecte a les de la resta del organisme no ha estat completament determinat. Fins ara s'han identificat quatre potencials vies per les quals la inhibició del proteasoma induiria la mort de la cèl·lula neoplàsica (Chari et al. 2011).

La primera estaria en relació a la regulació de la concentració de molècules implicades en el control del cicle cel·lular, com les ciclines i la proteïna supressora tumoral p53. Les transicions entre les diferents etapes del cicle cel·lular depenen de l'activació dels complexes ciclina/ciclina-quinasa dependents que són activats en fases específiques durant la progressió del cicle cel·lular. L'expressió i/o l'activació d'aquests complexes en cadascuna de les fases del cicle està regulada pel proteasoma. A més, els complexes ciclina/ciclina-quinasa dependents són regulats per un seguit de factors inhibidors, com el p21 i p27, que impedeixen la seva formació i aturen la progressió del cicle cel·lular. Aquests dos factors inhibitoris són també substrat del proteasoma. Per altra banda, la proteïna p53 atura el cicle en la fase G1 i inhibeix la seva progressió cap a la fase S; l'acció d'aquesta vindria facilitada per la seva pròpia sobrexpressió i la de p21 a l'estar inhibida per l'acció del proteasoma (Waldman et al. 1995). A més, la p53 promou l'apoptosis de les cèl·lules danyades tot induint l'acció dels factors proapoptòtics Bax, que al mateix temps són també substrat del proteasoma. El factor Bax inhibeix les proteïnes antiapoptòtiques Bcl-2 i Bcl-x_L en la mitocòndria, provocant l'alliberació del citocrom c i la conseqüent activació de la cascada de caspases (Voorhees et al. 2003). Així, l'estabilització de les proteïnes p53, p21 i Bax degut a la inhibició del proteasoma provocaria la desregulació de la progressió del cicle cel·lular i, en darrera instància, l'apoptosis de la cèl·lula.

Una segona via, relacionada parcialment amb l'anterior, implicaria el factor proapoptòtic NOXA. NOXA s'indueix per diversos senyals d'estrès i per p53. La inhibició del proteasoma provoca una inducció de NOXA restringida a les cèl.lules tumorals perquè és directament depenent de l'oncogen c-myc (Nikiforov et al. 2007). L'apoptosis mediada per l'acció de NOXA sembla molt específica de bortzomib, ja que no s'ha observat en altres citostàtics, ni tan sols en altres inhibidors del proteasoma en fase experimental preclínica (Chari et al. 2010). NOXA, segrestant el factor anti-apoptòtic Bcl-xL, desequilibra el balanç de les proteïnes pro/antiapoptòtiques cap a la mort cel·lular. No obstant, la inhibició del proteasoma també provoca un increment de la

proteïna anti-apoptòtica Mcl-1, que hauria de compensar l'efecte apoptòtic de NOXA. Però s'ha demostrat que un increment de Mcl-1 no influeix en l'apoptosis mediada per bortezomib (Hagenbuchner 2010). NOXA, que té gran afinitat per Mcl-1, s'hi uneix i desplaça la unió de Bak amb Mcl-1, de manera que Bak s'allibera, permeten la seva oligomerització i l'activació de la via de les caspases (Willis et al. 2005). El bortezomib, incrementant els nivells tant de NOXA com de Mcl-1 podria by-passejar l'acció antiapoptòtica de Mcl-1 i permetre l'activació de la maquinària apoptòtica via Bak.

La tercera via implicada seria la modulació del factor de transcripció NF-κB (Adams 2002; Voorhees et al. 2003). Aquest factor juga un paper important en la tumorogènesis tot estimulant la proliferació cel·lular, bloquejant l'apoptosis i induint la angiogènesis (Karin et al. 2002). En les cèl·lules quiescents, la proteïna inhibidora NF-κB(IκB) està unida al factor NF-κB en el citoplasma, impedint així la seva translocació al nucli. Diferents estímuls d'estrès conduiran a la fosforilació dels residus de serina del IκB, provocant la seva ubiquinació i posterior degradació proteasomal. Aquest procıs permet l'alliberació del NF-κB i la seva translocació al nucli, on indueix l'expressió de gens que codifiquen citoquines inflamatòries, eicosanoids, molècules d'adhesió cel·lular i factors antiapoptòtics com el bcl-2 (Adams 2002; Adams 2004). El mateix factor indueix també la seva pròpia transcripció, amplificant i mantenint d'aquesta manera els seus efectes. Així, mitjançant el bloqueig de la degradació del IκB s'impedeix l'acció protumoral del NF-κB (Orlowski et al. 2002).

Finalment, una quarta via que explicaria perquè el bortezomib actua de forma específica sobre les cèl·lules del mieloma està relacionada amb la resposta a l'estrès del reticle endoplasmàtic o resposta cel·lular a proteïnes no plegades. Aquesta resposta consisteix en la sobreexpressisó de chaperones que repleguen l'excés de proteïnes mal plegades o les destrueixen a través de l'acció del proteasoma. Les cèl·lules plasmàtiques sintetitzen una gran quantitat de proteïnes, que poden no estar ben plegades. Per tant, la inactivació del proteasoma provocarà una acumulació de proteïnes mal plegades que portarà a la cèl·lula cap a la mort mitjançant la via de degradació dels agresomes-lisosomes. (Obeng et al. 2006)

Indicacions i posologia

El bortezomib va rebre l'aprovació accelerada de la Food and Drugs Administration (FDA) el 2003 gràcies als resultats positius dels assajos fase I i II en

pacients amb mieloma múltiple (MM) refractari a la primera línea de tractament. Posteriorment, es varen confirmar aquests resultats positius en preceptius assajos de fase III tant en recaiguda com en primera línia de tractament (Richardson et al. 2004; Richardson et al. 2005; Jagannath et al. 2005; San Miguel et al. 2008). A més, també ha rebut la indicació per al tractament del limfoma no-Hodgkin de cèl·lules del mant. En paral·lel, també s'està testant el seu efecte en altres neoplàsies hematològiques i en neoplàsies sòlides de cap i coll, pròstata, colorectals, gàstriques, pancreàtiques, de mama, pulmonars de cèl·lula no petita i renals metastàtics (http://clinicaltrials.gov). Alhora, també s'està avaluant en fase I el seu potencial en combinacions amb platins, ja que el bortezomib podria interferir en un dels mecanismes de resistència cel·lular a aquests fàrmacs (Howell et al. 2010). Actualment, aquest fàrmac és una de les pedres angulars del tractament del MM, combinat amb dexametasona o formant part de la majoria de règims combinatoris amb altres fàrmacs, utilitzats tant en tractaments d'inducció previ al transplantament de moll d'ós, com en pacients que no són candidats al transplantament. Els tractaments combinats amb bortezomib han aconseguit el major percentatge de respostes i, juntament amb el trasplantament del moll d'os, del qual es poden beneficiar un percentatge menor de pacients, ha contribuit a canviar la història natural del MM. Abans, la mediana de supervivència global dels pacients amb MM era de 3 anys, mentres que la ratio actual de supervivència als 3 anys és d'un 75-80%.

La dosis recomanada és de 1,3 mg/m^2 administrada endovenosament. Cada cicle de tractament dura 21 dies i consisteix en l'administració de bortezomib dos cops per setmana durant 2 setmanes (dies 1, 4, 8 i 11) seguits d'un període de descans de 10 dies (dies 12-21). El tractament complert per al MM consisteix en 8 cicles de tractament (Chu et al. 2009). Els règims de tractament poden administrar el bortezomib en combinació amb dexametasona, amb melfalan i prednisona, amb talidomida i dexametasona, amb ciclofosfamida i dexametasona o juntament amb lenalidomida i dexametasona (Rajkumar 2011).

Efectes adversos

Els principals efectes adversos que presenta l'administració de bortezomib són l'astènia, la febre, la toxicitat gastrointestinal en forma de nàusees, vòmits i diarrea, la trombopènia i la neuropatia perifèrica. La febre, que pot aparèixer fins en el 40% dels pacients, és fàcilment manejada amb antitèrmics (Chu et al. 2009). La toxicitat gastrointestinal, observada sobre tot durant els primers cicles, si bé pot ser severa en un

25% dels pacients, és fàcilment previnguda o atenuada mitjançant l'ús de fàrmacs antiemètics o astringents. De la mateixa manera, la plaquetopènia severa que pot afectar fins al 31% dels pacients durant la primera meitat dels cicles del tractament, es recupera espontàniament en la segona meitat de cada cicle, no presenta una toxicitat acumulativa sobre el moll d'ós i rarament serà causa de transfusió o suspensió del tractament (Chari et al. 2010). En canvi, la neuropatia perifèrica ha estat identificat com el principal efecte advers limitant de dosis (Richardson et al. 2005; Richardson et al. 2006; San Miguel et al. 2008;). Per aquest motiu, s'han establert un seguit de recomanacions de modificacions de dosis en funció de l'aparició i de la severitat de la neuropatia (Taula 3; Richardson et al. 2009).

Taula 3. Recomanacions de modificació de dosis en funció del grau de neuropatia desenvolupada.

SEVERITAT DE LA NEUROPATIA	**MODIFICACIÓ DE DOSIS**
Grau 1	No canvis
Grau 1 amb dolor neuropàtic o Grau 2	Reduir a 1 mg/m^2
Grau 2 amb dolor neuropàtic o Grau 3	Suspensió del tractament fins a millora i reiniciar a dosis de 0.7 mg/m^2
Grau 4	Suspensió definitiva del tractament

Gradació de la neuropatia en funció de l'escala NCI Common Toxicity criteria (Veure taula 2)

Acció neuropàtica de la inhibició del proteasoma

Els mecanismes implicats en la patogènia de la neuropatia induïda per bortezomib (NIB) resten en gran mesura per dilucidar i existeixen pocs treballs focalitzats en aquest tema. El primer model animal que va intentar reproduir i abordar el problema de la NIB observava, només qualitativament, certes alteracions morfològiques consistents en vacuolitzacions i engrandiments del reticle endoplasmàtic de les cèl·lules de Schwann i de les satèl·lit, acompanyades d'una disminució de la velocitat de conducció sensitiva que es recuperava després d'un període de descans. No es van detectar alteracions en el nombre, densitat o morfologia dels axons mielínics, únicament un increment de la raó g. Tampoc s'observà cap canvi patològic remarcable en els axons amielínics, ni en la morfometria de neurones del GRP i les medul·lars (Cavaletti et al. 2006). Les diferents troballes, per tant,

suggerien, en contra de les evidències clíniques i electrofisiològiques en pacients, un origen primari desmielinitzant d'aquesta neuropatia.

Posteriorment a la publicació d'aquests resultats, tres mecanismes diferents han estat investigats a nivell molecular. Per una banda, un treball que utilitza cultius de neurones adultes de GRP provinents de rates tractades, postula que la disfunció neural és producte de l'acumulació nuclear de proteïnes i poly(A)RNAs que provoquen una reducció de la transcripció i una alteració de la translocació del RNA missatger cap al citoplasma (Casadefont et al. 2010). Potencialment, els dèficits causats per aquesta alteració en la transcripció de proteïnes i de factors tròfics conduiria a la disfunció i, en darrera instància, a la mort de la neurona. D'altra banda, en un estudi realitzat en línies cel·lulars neurals de còrtex (HCN2) i de neuroblastoma (SY5Y i KCNR) es proposa que el mecanisme etiopatogènic implicat estaria en relació a les alteracions produïdes sobre el citoesquelet. En aquestes línies cel·lulars, s'observa l'aparició d'agresomes intracel·lulars, indicatius d'una degradació deficient de proteïnes ubiquitinades. També s'observa un augment de la fracció acetilada de l'α-tubulina que suggeriria un efecte estabilitzador sobre els microtúbuls. Aquesta afectació dels microtúbuls acabaria alterant el transport axonal i la funcionalitat i viabilitat de les neurones. Per tant, es proposa que l'acció final neuropàtica seria similar a la dels taxans. Ara bé, donat que el bortezomib és incapaç, per si sol, d'afavorir la polimerització en solucions de tubulina purificades, s'advoca per un mecanisme indirecte no relacionat amb l'unió a aquests. Concretament, s'implica una alteració en el balanç de les proteïnes associades a microtúbuls (MAP) secundària a l'alteració de la seva degradació. Aquestes proteïnes s'encarreguen de modular l'estabilitat i composició dels microtúbuls. In vitro, el bortezomib incrementa els nivells de la MAP2, sense afectar la MAP4 o la tau (Poruchysnky et al. 2008).

Degut a que el primer model animal apuntava a una afectació primàriament glial en la NIB, un estudi posterior avalua els efectes del bortezomib sobre línies cel·lulars de schwannoma, amb les conseqüents crítiques sobre l'extrapolabilitat dels seus resultats en els pacients. Aquest estudi descriu també la formació d'agresomes, que són transportats cap als centres organitzadors de microtúbuls adjacents al nucli mitjançant les dineines i observa que poden ser eliminats per la via de l'autofàgia (Watanabe et al. 2010). Aquest fet podria explicar la ràpida recuperació de la neuropatia en molt dels pacients. Finalment, un altre estudi que combina un model animal amb cultius de cèl·lules de Schwann provinents d'aquest, confirma les observacions de l'engrandiment del reticle endoplasmàtic en les cèl·lules de Schwann i la sobreexpressió de proteïnes indicatives

d'estrès de reticle. També observa alteracions morfològiques dels paquets axònics de Remak, infiltració de macròfags en els nervis degut a un increment de la síntesi de citoquines quimotàctiques per part de les cèl·lules de Schwann i una *down-regulation* en la transcripció de gens relacionats amb la mielina (P0, krox20, Sox10). És interessant remarcar que, al igual que l'anterior estudi, també observen que l'administració mantinguda del bortezomib a llarg terme, atenua tots aquests efectes gràcies a l'increment de diferents chaperones relacionades amb l'autofàgia (Shin et al. 2010).

NEUROPATIA INDUÏDA PER BORTEZOMIB

Incidència i conseqüències

La incidència de la NIB, reportada en diferents estudis que inclouen assajos de fase II i fase III abarca un rang del 31 al 64% (Velasco et al. 2010b). El tractament amb bortezomib com a primera línia, a dosis de 1,3 mg/m^2 per cicle, indueix neuropatia de grau 1 i 2 (lleu-moderada) segons l'escala NCI-CTCAE (Taula 2, pàgina 6) en un 31% dels pacients. Si aquests pacients han rebut prèviament altres tractaments citostàtics, el percentatge de neuropatia no severa és semblant, entre un 23-28%. El risc es redueix a un 15% si reben dosis de 1 mg/m^2. La neuropatia severa es dóna en un 13% dels pacients tractats en primera línia amb dosis de 1,3 mg/m^2 per cicle. El percentatge és similar (8-14%) si els pacients prèviament havien rebut algun altre tipus de tractament per al MM. Globalment, tenint en compte tots els graus de neuropatia observats en els assajos de fase II i III que varen portar a l'aprovació accelerada de l'ús del bortezomib pel tractament del MM, no existeixen grans diferències en incidència entre els pacients pretractats respecte als que se'ls administra bortezomib com a primera línia de tractament, un 36% contra 44% (Richardson et al 2006; Richardson et al 2005; San Miguel et al 2008; Richardson et al 2006b). Ara bé, la incidència de dolor neuropàtic en els pacients pretractats és més alta i es resol més tardanament que en els que reben bortezomib com a primer tractament, i també requereixen reduccions o suspensions del tractament amb més freqüència (Corso et al. 2010).

Per altra banda, recentment s'ha testat en un assaig de fase III una formulació subcutània com a alternativa a la clàssica endovenosa, on es demostra una eficàcia similar i una incidència menor de neurotoxicitat (Moreau et al. 2011). En la mateixa línia, un altre assaig de fase III en que s'avalua l'eficàcia i la seguretat d'una administració setmanal respecte a la clàssica bisetmanal, observa menor incidència de neuropatia, tan globalment

com de grau sever (Bringhen et al. 2010). No obstant, en ambdós estudis, malgrat que la incidència global de neuropatia és menor a la estàndar, les xifres dels dos tractaments alternatius es mouen en el mateix rang d'incidència que s'observa en l'anàlisi del conjunt d'assajos comentats anteriorment.

S'estima que del global de pacients que reben bortezomib, un 12% necessitaran una reducció de la dosis o suspensió del seu tractament. Ara bé, si ens centrem únicament en els pacients que desenvolupen neuropatia de *novo* o bé un empitjorament d'una neuropatia ja pre-existent, la reducció i/o suspensió es produeix en un 34.4% d'ells (Richardson et al. 2006b). Aquestes modificacions del tractament tenen com a conseqüència una dramàtica reducció de la ràtio de resposta del MM al bortezomib (Jagannath et al. 2005; Jagannath et al. 2008; Richardson et al. 2009) i de la duració d'aquesta resposta (Jagannath et al. 2004; Richardson et al. 2005; Freimann et al. 2007). A part, la neuropatia per bortezomib presenta un impacte molt negatiu sobre la qualitat de vida dels pacients, sobre tot perquè es habitual que s'acompanyi de dolor neuropàtic de forma molt més freqüent que amb altres citostàtics neurotòxics (Richardson et al. 2006b; Cata et al. 2007; Prommer et al. 2009; Velasco et al. 2010c).

Quadre clínic i alteracions electrofisiològiques

Els pacients afectes de NIB presenten una neuropatia axonal sensitiva pura, molts cops dolorosa, de predomini distal amb distribució típica de guant i mitjó. Aquesta afecta tan a fibra gruixuda com a fibra petita i es pot acompanyar de disautonomia en forma d'hipotensió ortostàtica i constipació en un 12% i 50% dels pacients respectivament (Velasco et al. 2010b). El dolor neuropàtic és més freqüent en aquells pacients que ja han rebut algun tractament previ a l'administració de bortezomib, presentant-se en un 39% respecte un 15% dels pacients que el reben com a primera línia (Jagannath et al. 2005; Richardson et al. 2006b). Electrofisiològicament, s'observa una reducció de l'amplitud dels PANCS, amb velocitats de conducció i registres motors conservats (Velasco et al. 2010b). Mitjançant tests quantitatius sensorials s'objectiven alteracions en les fibres Aβ, Aδ i C (Cata et al. 2007).

Pronòstic i factors de risc

La NIB apareix habitualment en els primers 5 cicles de tractament, en funció de la dosis acumulada, però a partir d'aquest cicle (aproximadament a 30 mg/m^2) s'arriba a un *plateau* on la incidència de neuropatia només s'incrementa un 4% fins arribar als 8 cicles

totals de tractament (Richardson et al. 2009). No existeixen diferències en la incidència i el pronòstic entre els pacients prèviament tractats i en els que reben el bortezomib com a primera línia. Ara bé, s'ha observat que els pacients pretractats amb altres fàrmacs requereixen més freqüentment reduccions de dosis (73% contra 36%) (Corso et al. 2010). Els pacients pretractats amb altres fàrmacs que desenvolupen neuropatia severa o de grau menor que requereixin suspensió del tractament experimenten una resolució o millora parcial en el 64-71% dels casos, en un temps que oscil·la entre els 47-110 dies de mediana, segons els estudis (Richardson et al. 2006b). Els pacients que únicament requereixen una disminució de dosis presenten unes taxes de recuperació en un temps similar. Els pacients que reben el bortezomib com a primer tractament del MM, resolen o milloren la seva neuropatia en una mediana de 60 dies, en un 56% i un 18% dels casos respectivament (San Miguel et al. 2008). Per altra banda, els pacients amb neuropaties lleu i moderada es recuperen abans (mediana de 3-4 mesos) que els que tenen neuropatia severa (mediana de 8 mesos) (Badros et al. 2007). No hi ha estudis observacionals publicats de l'evolució de la neuropatia per bortezomib a llarg termini, i probablement serà difícil que apareguin, ja que els fàrmacs utilitzats en línies de rescat per a les recaigudes o progressions del MM són també neurotòxics (talidomida, vincristina) i la malaltia sol ser incurable en la majoria de pacients. Ara bé, amb les dades que disposem en l'actualitat, es pot afirmar que el 26-29% dels pacients que desenvolupen la neuropatia per bortezomib no experimentaran cap millora durant els següents 2-4 mesos, període d'observació curt per a valorar la recuperació o regeneració en el SNP.

Altres factors de risc discutits són l'edat i l'existència de neuropatia de base com a factors que augmenten la susceptibilitat per a desenvolupar aquesta neuropatia. Respecte l'edat, en sèries no controlades de pacients, s'ha observat que els majors de 50 anys presenten un increment de risc d'un 6% per any afegit (Corso et al. 2010). Un altre estudi, en canvi, apunta que l'edat de tall on s'observava un major risc era als 75 anys (Mateos et al. 2006). Contràriament, l'anàlisi conjunta de les dades dels grans assajos de fase II i III no indica que l'edat sigui un factor de risc (Richardson et al. 2006b; Dimopoulos et al. 2011). En quant a l'existència de neuropatia de base com a factor de risc, persisteix certa controvèrsia. Per una banda, alguns estudis clínics mostren que si es parteix d'una neuropatia de base lleu o moderada és més fàcil desenvolupar una neuropatia severa davant l'exposició a un fàrmac neurotòxic (Richardson et al. 2006b; Lanzani et al. 2008; Dimopoulos et al. 2011). Altres estudis també suggereixen, indirectament, que l'exposició a tractaments previs neurotòxics constitueixen un factor de risc (El-Cheikh et al. 2008;

Velasco et al. 2010b). Ara bé, caldria saber si, proporcionalment, hi ha major grau de l'alteració estructural o de pèrdua funcional del nervi entre els pacients amb neuropatia de base per confirmar que aquesta és un factor de risc. Seria important aclarir aquest punt, ja que molts pacients amb MM presenten una neuropatia de base. Per tant, es podrien beneficiar d'una línia de tractament amb un fàrmac menys neurotòxic, encara que impliqui una menor taxa de respostes, com seria la lenalidomida, que presenta un 70% de respostes globals respecte al 90-100% de respostes del bortezomib (Rajkumar 2011) o bé, d'una monitorització neurològica durant el tractament (Velasco et al. 2010b). Finalment, un estudi ha intentat identificar una signatura genètica mitjançant l'anàlisis de polimorfismes, capaç de predir quins pacients són més susceptibles de desenvolupar neuropatia per bortezomib. Aquest estudi postula que certs polimorfismes dels gens CTLA4, CTSS, GJE1, PSMB1, TCF4 i DYNC11 estarien relacionats amb el temps en que triga a aparèixer la neuropatia. No obstant, fracassa en la demostració del seu objectiu primari i, usant una mostra diferent de pacients, tampoc valida les seves troballes (Favis et al. 2011).

MODELS MURINS EN L'ESTUDI DE LES NEUROPATIES INDUIDES PER CITOSTÀTICS

Utilitat dels models in vivo

Des de fa uns 40 anys, poc temps després de l'inici de l'implementació clínica dels primers citostàtics com els alcaloides de la vinca i els platins (Johnson et al. 1963; Lippman et al. 1973), s'han anat desenvolupant models animals per intentar comprendre i analitzar els mecanismes subjacents a l'aparició d'un dels seus efectes adversos més freqüents i habitualment limitant de dosis, com és la neuropatia perifèrica. Malgrat les crítiques i les dificultats que en els darrers anys s'estan posant a la recerca en models animals des de certs sectors socials, la seva utilitat segueix fora de dubte. En el cas de les neuropaties tòxiques induïdes per agents antineoplàsics podríem agrupar l'interès de disposar de bons models en tres grans punts.

En primer lloc, el model animal ens permet un coneixement exhaustiu, tant a nivell funcional com morfològic, que no és viable en els estudis amb pacients. La valoració neurofisiològica possibilita la monitorització de la funció del nervi perifèric de forma no invasiva i poder determinar el diagnòstic de neuropatia establerta, permetent posteriorment la seva confirmació i la seva caracterització histològica tan a nivell

estructural com molecular. Conèixer aquests canvis seran d'utilitat per generar hipòtesis sobre els mecanismes implicats en la disfunció neural. En canvi, a nivell clínic, l'obtenció de mostres seriades per a l'estudi de la neuropatia és impensable i l'existència de material anatomo-patològic provinent de necròpsies és anecdòtic, restringit únicament als primers citostàtics usats. A més, en la majoria d'ocasions, les mostres són obtingudes en condicions no òptimes degut tant a l'heterogeneïtat dels temps entre l'administració del fàrmac i l'extracció de la mostra, com al fet que els pacients puguin haver rebut altres tractaments de rescat per a la seva neoplàsia de base (Bruna 2011a; Bruna 2011b). Actualment, malgrat que s'optimitzessin els estudis necròpsics, l'existència de segones i terceres línies de tractament per a la majoria de neoplàsies, farien difícilment valorables les troballes. A nivell funcional, si bé és factible obtenir dades electrofisiològiques dels malalts que reben tractaments amb citostàtics, aquests estudis són difícils de realitzar donada la fragilitat dels pacients amb càncer i al discomfort que representa realitzar tests neurofisiològics seriats amb certa freqüència.

En segon lloc, la disponibilitat d'un bon model *in vivo* de neuropatia és necessari i complementari als models *in vitro* de cultius de neurones adultes. Aquests models són d'utilitat per estudiar els múltiples mecanismes moleculars potencialment involucrats en el desenvolupament de la neuropatia, de forma més ràpida i fàcil que en els models *in vivo*. Ara bé, donades les potencials característiques artefactuals d'aquests models, com la selecció del tipus de neurones que són viables en cultius, la manca de valoració del paper que poden jugar la resta de components del SNP, com les cèl·lules glials i les endotelials, així com l'ús de medis artificials, fan necessària que les troballes *in vitro* es validin en models establerts *in vivo* abans de generar abordatges terapèutics a nivell clínic (Leclere et al. 2007; Tucker et al. 2008). A més, en base als mecanismes i dianes identificats *in vitro* i *in vivo*, podem dissenyar diferents estratègies neuroprotectores i testar diferents fàrmacs per pal·liar la simptomatologia de la neuropatia o frenar la seva evolució, tot validant així les troballes mecanístiques prèvies i reforçant l'interès de la teràpia testada per que passi a assaig humà.

En tercer i darrer lloc, una neuropatia ben caracteritzada en models animals, permet testar a nivell preclínic la utilitat i les potencials interaccions entre els fàrmacs neuroprotectors i l'eficàcia del tractament antineoplàsic si s'utilitzen alhora en models amb transplantament de línies neoplàsiques. De la mateixa manera, també permet analitzar diferents paradigmes clínics, com per exemple els efectes de l'edat, la susceptibilitat que representa tenir una neuropatia de base abans d'iniciar un tractament

neurotòxic o l'efecte dels metabòlits o diferents enantiomers dels fàrmacs avaluats (Screnci et al. 1997), evitant la presència dels efectes confusors que genera una població de pacients heterogènia respecte aquestes característiques.

Tipus de models murins

Històricament s'han utilitzat majoritàriament els models murins, rata i ratolí. No hi ha una preferència de soques, ja que no s'ha identificat cap espècie amb especial susceptibilitat per a desenvolupar aquest tipus de neuropaties tòxiques, al contrari del que passa en altres malalties, com les endocrinològiques o algunes neurodegeneratives. Aquesta preferència pel model murí respecte altres tipus de mamífers probablement ve justificada pel seu menor cost, la facilitat d'estabulació i la còmode manipulació en grans quantitats. En rates s'han caracteritzat models de neuropaties en les soques Sprague Dawley, Wistar, Fisher i Swiss i en ratolins en les soques CD1, ICR, C57BL/6 i Swiss (Carozzi 2011). Malgrat que històricament el major volum d'estudis s'ha realitzat en models que usen rates, possiblement perquè la seva mida facilita la manipulació i extracció de mostres sense requerir especial entrenament, el ratolí presenta una sèrie d' avantatges sobre aquestes. Principalment, el seu manteniment té un cost menor i es poden estabular fàcilment en grans quantitats. A més a més, els estudis sobre dosis letals i màxima dosis tolerada dels fàrmacs utilitzen ratolins i la majoria de línies cel·lulars neoplàsiques han estat desenvolupades sobre teixits de ratolins (http://www.harlan.com/research_models_and_services/research_models_by_research_use/oncology/cell_line_references/cell_line_references.hl). Per tant, en els ratolins es poden fer estudis combinats que valorin l'eficàcia dels fàrmacs antineoplàsics sobre les cèl·lules tumorals, la neurotoxicitat que generen i la possible interacció amb d'altres fàrmacs administrats com a potencials neuroprotectors,

En general, s'han establert dos tipus diferenciats de models per a l'estudi de les neuropaties induïdes per citostàtics, definits per la posologia del fàrmac. Per una banda tenim els models on s'administren baixes dosis del fàrmac durant intervals curts de temps i per l'altre, models que administren concentracions més elevades durant períodes més llargs de tractament. El primer tipus de model, més extensament estudiat, està enfocat a reproduir i estudiar els símptomes relacionats amb el dolor i els mecanismes moleculars implicats de forma aguda en la neuropatia causada per l'antineoplàsic. En canvi, el segon tipus de model, més escàs en la literatura, es centra en intentar reproduir les troballes característiques dels pacients amb NIQ. En aquests segons, la forma establerta i més

crònica de la neuropatia permet també estudiar els efectes dels tòxics sobre l'esfera sensitiva.

Contribucions del models murins en les NIQ

Els diferents models existents de NIQ han servit, en el cas dels compostos del platí, per a confirmar a nivell morfològic que les alteracions produïdes es donen sobre el soma neuronal dels GRP (Cavaletti et al. 2001) i alhora bastir la principal hipòtesis etiopatogènica a nivell molecular, encara que s'ha de ressaltar que no ha estat validada per altres estudis (Gill et al. 1998; McDonald et al. 2002). Aquests models presenten electrofisiològicament característiques similars a la dels pacients (Verdú et al. 1999; Ceresa et al. 2011) i, per tant, són útils per monitoritzar l'efecte que poden exercir els fàrmacs neuroprotectors. L'exemple paradigmàtic, si bé per desgràcia encara únic, el trobem en el treball que mostra que l'administració de suplements de vitamina E té un efecte neuroprotector en un model de neuropatia induïda per cisplatí en ratolins (Leonetti et al. 1993). Aquesta troballa s'ha verificat posteriorment en assajos en pacients, i actualment la vitamina E és l'únic agent amb potencial neuroprotector acceptat clínicament amb un nivell d'evidència II (Pace et al. 2003; Pace et al. 2010) per aquest tipus de neuropatia. Malauradament, altres fàrmacs que s'han testat en models murins de NIQ per platins o altres citostàtics amb resultats prometedors, no han tingut la mateixa traducció a nivell clínic o encara no han superat la fase experimental (Cavaletti et al. 2008). Altres models murins de neuropatia han aportat coneixements similars respecte a la neurofisiologia i a l'estructura diana danyada pel citostàtic. Així, el citoesquelet de l'axó és l'estructura més afectada per vincristina i les epotilones, mentre que els taxans afecten tant del citoesquelet axònic com somàtic, amb una potencial implicació d'altres components cel·lulars com macròfags i dany col·lateral de les cèl·lules de Schwann (Schlaepfer 1971; Cavaletti et al. 1995; Tanner et al 1998; Topp et al. 2000; Peters et al. 2007; Chiorazzi et al. 2009).

Respecte als estudis sobre dolor agut administrant citostàtics a models murins, les troballes són difícils de correlacionar adequadament amb el problema clínic dels pacients amb NIQ. Això és degut, principalment al fet que els diferents símptomes positius no són avaluats de forma sistemàtica en la gran majoria d'estudis en pacients i per tant desconeixem, en part, les seves característiques i moment d'aparició durant l'evolució de

la neuropatia. A més, la identificació en aquest models d'alteracions algèsiques de forma precoç i a baixes dosis en relació a l'equivalent alomètric en pacients, es contraintuïtiu a l'experiència clínica observada. Una excepció a aquests models de dolor, seria el model de neuropatia induïda per oxaliplatí. En aquest cas, les característiques electrofisiològiques i histològiques són compatibles amb la neuropatia crònica clàssica dels platins, i s'indueix, al llarg de l'administració del fàrmac, una hiperalgèsia induïda per fred similar a l'observada en pacients (Renn et al. 2011).

OBJECTIUS

L'objectiu general d'aquest treball consisteix en desenvolupar i caracteritzar un model animal de neuropatia induïda per bortezomib que permeti conèixer exhaustivament els efectes d'aquest fàrmac sobre el sistema nerviós perifèric i sigui, alhora, útil tant per testar potencials agents neuroprotectors com per investigar mecanismes fisiopatològics i, així, dilucidar controvèrsies i paradigmes d'utilitat clínica que es plantegen al tractar els pacients amb bortezomib.

Per aquest propòsit, s'han plantejat els següents objectius específics:

1) **Desenvolupar un model experimental per a l'avaluació detallada i quantitativa dels efectes del bortezomib sobre el sistema nerviós perifèric:**
   a. Establir la posologia adequada de bortezomib en ratolins Swiss OF1 que permeti el desenvolupament de neuropatia sense causar altres majors toxicitats.
   b. Caracterització funcional, electrofisiològica, histològica i immuno-histoquímica de la neuropatia.

2) **Estudiar l'evolució natural de la neuropatia per bortezomib un cop instaurada:**
   a. Valoració del grau de recuperació de la neuropatia establerta per bortezomib després de finalitzar el tractament mitjançant tècniques electrofisiològiques, morfològiques i funcionals.

3) **Avaluar si la presència de neuropatia de base prèvia, constitueix un factor de risc per desenvolupar neuropatia a l'administrar bortezomib:**
   a. Establir i caracteritzar a nivell funcional, electrofisiològic i histològic un model de neuropatia crònica induïda per vincristina en ratolins Swiss OF1.
   b. Valorar, utilitzant una pauta seqüencial d'administració de vincristina i bortezomib en dos fases separades en el temps, la severitat de la neuropatia resultant comparada a la de ratolins que únicament han rebut bortezomib.

4) **Avaluar els efectes sobre el sistema nerviós perifèric del retractament amb bortezomib:**
   a. Valorar quantitativament mitjançant tècniques electrofisiològiques, morfològiques, d'immunohistoquímica i funcionals, diferències entre ratolins tractats amb bortezomib en dues fases separades en el temps i ratolins que només reben un esquema de tractament.

5) **Avaluar si l'afectació de fibres nervioses petites pot ser un marcador precoç d'utilitat en predir la instauració d'una neuropatia severa per bortezomib:**
   a. Valorar quantitativament mitjançant tècniques d'immunohistoquímica, l'afectació sequencial de fibres petites i grans al llarg del tractament amb bortezomib i correlacionar les troballes amb el desenvolupament de neuropatia severa definida electrofisiològicament.

DISSENY EXPERIMENTAL

Model animal

Per tal d'estudiar els objectius abans exposats s'han utilitzat ratolins femella de 2,5 mesos d'edat de la soca Swiss OF1. Els fàrmacs emprats en els diferents experiments han estat el bortezomib (Velcade®, suministrat per Millenium Pharmaceuticals Inc, i Johnson & Johnson Pharmaceutical Research & Development, L.L.C.) i la vincristina (Vincrisul®; Lilly, France).

Alhora d'escollir la dosis del fàrmac a administrar per tal de caracteritzar tant la neuropatia per bortezomib com la de vincristina, s'ha procedit de la següent forma. En primer lloc, es tria un rang de dosis. En el cas del bortezomib, on no hi ha referències prèvies, aquesta es calcula seguint les recomanacions de la FDA (Reagan-Shaw et al. 2008), per tal que la dosis total acumulada que rebrà l'animal durant un tractament complert de duració no superior als 2 mesos sigui similar a la dosis total acumulada que reben els pacients. Posteriorment, es realitza un estudi pilot amb grups de 5 animals cadascun, on es testen aquestes dosis a nivell electrofisiològic cada setmana. La posologia final escollida és aquella que aconsegueix desenvolupar una neuropatia rellevant i al mateix temps presenti una tolerància adequada. La tolerància es mesura mitjançant les guies habituals de bona praxis de manipulació d'animals d'experimentació (http://www.iclas.org; Real decreto1201/2005) i pel percentatge de mortalitat durant l'experiment pilot. En el cas del bortezomib, es van avaluar dosis de 0,8 i 1 mg/kg, administrades dues vegades per setmana i una dosis de 1 mg/kg tres cops a la setmana durant 6 setmanes. La primera posologia no causava cap efecte mesurable sobre la funció del nervi perifèric, mentre que la dosis més alta tenia una ratio d'elevada mortalitat. En el cas de la vincristina, existia un referent previ publicat usant 1,7 mg/kg dues vegades per setmana, en una altra soca de ratolins, si bé aquesta dosis provocava una alta taxa de mortalitat (superior a la LD50) i una mala tolerància física (Contreras et al. 1997). Per aquest motiu, el rang de dosis testat a nivell pilot va ser de 1, 1,25, 1,5 i 1,7 mg/kg. Totes les dosis avaluades van provocar, a nivell electrofisiològic, una neuropatia evident a les 4 setmanes de tractament, si bé la dosis més elevada provocava un índex de mortalitat i de mala tolerància excessius. Per tal d'induir neuropaties de diferent grau de severitat, es van escollir les dosis de 1 i 1,5 mg/kg.

Les vies d'administració van ser escollides en funció de la informació disponible i la seva comoditat de dispensació. El bortezomib es va administrar per via subcutània, ja que el laboratori propietari de la patent va comunicar que la biodisponibilitat entre aquesta via d'administració i la endovenosa eren similars, igual com s'ha demostrat recentment en pacients (Moreau et al. 2011). La vincristina es va administrar via intraperitoneal, ja que era la reportada en estudis previs en ratolins i rates (Contreras et al. 1997; Ja'afer et al. 2006).

Resum dels mètodes experimentals

1. Estudis Funcionals

Conducció nerviosa

El registre dels PAMC i PANCS es va realitzar mitjançant elèctrodes d'agulla posicionats en els músculs tibial anterior i el tercer interossi (múscul plantar) i a prop del nervi digital del quart dit respectivament, prèvia estimulació del nervi ciàtic a nivell de la fenedura ciàtica i del nervi tibial, branca del ciàtic, a nivell del turmell. Els estímuls consistien en polsos elèctrics rectangulars (estimulador Grass S88), de 0,01 ms de duració, amb un voltatge un 25% superior al necessari per obtenir una resposta màxima. D'aquesta forma, es van poder mesurar les latències, les amplituds dels potencials i les velocitats de conducció motores i sensitives a nivell proximal i distal (Navarro et al. 1994; Navarro et al. 2009). Per als estudis electrofisiològics, els animals es van anestesiar amb pentobarbital (40 mg/kg intraperitonealment) i es van col·locar sobre una superfície a temperatura constant, controlada per mitjà d'un circuit d'aigua calenta circulant a 36ºC per tal de mantenir la temperatura corporal del animal estable.

Avaluació de la funció autonòmica

La funció simpàtica sudomotora es va avaluar mitjançant la tècnica del motllo de silicona. Per tal d'estimular la sudoració del animal, se'ls hi administra una dosis de pilocarpina (5 mg/kg) subcutàniament i 10 minuts després s'aplica sobre la superfície plantar de la pota del darrere un material de silicona (Silasoft Normal, Detax GmbH & Co.). La impressió de les gotes de suor sobre la matriu de silicona, que correspon a cadascuna de les gotes secretades per cada ducte de les glàndules innervades, es va contabilitzar mitjançant un microscopi de dissecció (Vilches et al. 2002).

La variabilitat del R-R cardíac es va analitzar, en animals anestesiats, utilitzant un registre electrocardiogràfic de 3 minuts de duració, obtingut tot clavant electrodes de registre als palmells de les potes davanteres i a la cua, per obtenir les derivacions

d'Einthoven. Els registres electrocardiogràfics van ser adquirits per mitjà del sistema PowerLab (ADInstruments) i registrats mitjançant el programa Chart. L'anàlisi de la variabilitat registrada es va valorar amb el Coeficient de Variació de Pearson.

Avaluació de la sensibilitat dolorosa

Per valorar la funció de les fibres nociceptives C es va utilitzar la tècnica de l'algesimetria tèrmica. El ratolí es col·loca en un cubicle de plàstic situat sobre un terra de vidre i s'aplica un feix de llum directe sobre la planta del peu de l'animal (Plantar Algesimeter, Ugo Basile). Gràcies a un cronòmetre acoblat a un detector d'infrarojos, s'obté el temps que triga l'animal a aixecar la pota escalfada. El valor de cada animal testat és la mitja de 3 tests consecutius, separats cadascun per 10 minuts de repòs (Hargreaves et al. 1988).

Funció sensitivo-motora

Per tal de d'avaluar de manera integrada la funcionalitat del sistema nerviós sensitiu (tàctil i propioceptiu) i del motor, es va utilitzar un aparell de rotarod (LIAP). Els ratolins es col·loquen sobre una barra giratòria, a una velocitat de 8 revolucions per minut durant 120 segons i es mesura el temps que l'animal roman sobre aquesta abans de caure. El valor que se li dona a cada animal és la mitja de l'obtingut en tres proves consecutives deixant intervals de 10 minuts entre elles. Abans de cada tractament els animals es van entrenar durant 5 dies consecutius. El valor considerat com a normal és de 120 segons, punt on s'atura l'experiment si l'animal l'assoleix.

2. Estudis histològics

Per a l'obtenció de les mostres, els animals es van sacrificar a diferents temps en funció del grup experimental al qual pertanyien, mitjançant perfusió cardíaca de paraformaldehid (4% en PBS 0,1M, pH 7,4) prèvia anestèsia profunda. Posteriorment, es van extreure els GRP de la quarta arrel lumbar, el tronc comú del nervi ciàtic i el nervi tibial. Les mostres es post-fixen en una solució de glutaraldehid-paraformaldehid (3%:3%), es renten amb PBS (0,1M, pH 7,4) i es fixen amb un 2% de tetroxid d'osmi durant 4h a 4ºC. Les mostres es deshidraten mitjançant concentracions graduals d'etanol i finalment són embegudes en resina epon. Es realitzen talls semifins, de 0,5 µm en un ultramicròtom, de la totalitat del GRP i del nervi ciàtic i tibial a nivell mig de la cuixa i la pota respectivament. Les mostres es tenyeixen amb blau de toluïdina i s'analitzen mitjançant microscòpia òptica. S'estima el nombre d'axons mielínics de cada nervi, mitjançant el càlcul de la densitat d'axons sobre camps seleccionats sistemàticament a

1000x augments, cobrint una part representativa de la secció del àrea del nervi (almenys el 15% del total), i l'àrea de la secció transversal total del nervi. Mitjançant el programa *Object Image* (NIH) es mesura també el perímetre i el diàmetre de 500 axons mielínics per cada nervi, en diferents camps escollits a l'atzar. Amb aquestes dades es calculen el gruix de la mielina, el diàmetre de l'axó i la raó g (Gómez et al. 1996). En funció de les troballes al microscopi òptic, es van obtenir també seccions ultrafines de mostres seleccionades, tant de nervi com de GRP, tenyides amb citrat d'uranil i examinades sota microscopia electrònica (Hitachi 7000).

3. Estudis immunohistoquímics

Innervació de la pell

Els coixinets plantars dels animals sacrificats s'extreuen i es post-fixen durant tota la nit amb solució de Zamboni i, posteriorment es mantenen amb PBS i sacarosa per crioprotegir-los. Es van obtenir seccions de 40 ó 70 μm de gruix mitjançant un criostat (Leica CM1950). La tinció immunohistoquímica de les mostres es va realitzar mitjançant la tècnica de *free-floating*. Les mostres es van incubar en PBS, 0.3% Triton-X100 i en sèrum normal de cabra al 1% durant 1h, després es van incubar tota la nit amb una dilució 1:800 d'anticòs primari de conill contra el *protein gene product* 9.5 (PGP, ABSserotec o Ultraclone) i 1:1000 d'anticòs primari contra el *calcitonin gene-related peptide* (CGRP, Chemicon). Després de tres rentats amb PBS, les seccions es van incubar en una solució amb immunoglobulina G conjugada amb cianina 3 (Cy3) durant dues hores. Finalment, les mostres es van analitzar sota microscòpia d'epifluorescència amb els filtres adequats. Es van utilitzar cinc seccions de cada mostra per quantificar la innervació dels coixinets, comptabilitzant el número i la densitat de les fibres nervioses epidèrmiques (Navarro et al. 1995).

En les mateixes seccions, en la intersecció entre la dermis i l'epidermis, es van identificar i quantificar el número de receptors de Meissner innervats. Mitjançant el software *ImageJ* (NIH) es va mesurar la longitud de la interfase dermis-epidermis del total de la secció per poder calcular la densitat de receptors innervats (número de receptors innervats per μm).

Innervació de les glàndules sudorípares

Per a quantificar el cabdell de fibres que innerven a les glàndules sudorípares es van utilitzar almenys 4 imatges captades a 20 augments, de les seccions dels coixinets emprades per a la valoració de la innervació de la pell. La mesura usada va ser la

immunoreactivitat al PGP 9.5 present en el contorn de les glàndules ubicades a la dermis del coixinet. Per a realitzar-ho es van utilitzar ROIs (*regions of interest*) col·locats sobre aquestes, i es van obtindre els paràmetres de densitat integrada d'immunoreactivitat i percentatge entre el número de píxels marcats i no marcats per PGP 9.5 en el ROI.

Quantificació de cèl·lules de Langerhans

La mateixa tècnica immunohistoquímica utilitzada per a la valoració de la innervació de la pell, afegint-hi com a comarcador, l'anticòs primari de cabra contra la Langerina (1:100, Santa Cruz) va ser l'utilitza't per a la quantificació de les cèl·lules de Langerhans presents a l'epidermis. Les cèl·lules de Langerhans marcades van ser contades a 20 augments i el seu valor expressat com a número total de cèl·lules en 0,05 mm^2 (500x100 µm) d'epidermis, en un mínim de 4 seccions de coixinet per cada animal.

Ganglis raquidis posteriors

Els GRPs extrets es van fixar en paraformaldehid al 4% durant 4h i després es van guardar en una solució de PBS al 30% de sucrosa. Mitjançant un crisotat (Leica CM1950), es van obtenir talls de 8 µm de gruix sobre porta. La tinció immunohistoquímica es a realitzar sobre porta i després de bloquejar les mostres en PBS, 0.3% Triton-X100 i en sèrum normal de cabra al 1% durant 1h, posteriorment es van incubar durant tota la nit amb els anticossos primaris anti-PGP, anti-CGRP i anti-Lectina I Isolectina B4 (IB4). El PGP és un panmarcador neural, el CGRP marca les neurones peptidèrgiques de grandària mitja i petita i la isolectina les no peptidèrgiques de petita grandària (Tucker et al. 2008). Després de 3 rentats en PBS, les seccions es van incubar durant 2h amb immunoglobulina G conjugada amb Cy3. Les neurones tenyides es van analitzar en fotografies preses a 200 augments i es va obtenir el percentatge relatiu de neurones tenyides per cadascun dels anticossos. Només es van comptabilitzar les neurones on es distingia clarament el nucli tenyit, per tal d'evitar la redundància en el còmput. L'anàlisi morfomètrica dels somes neuronals es va realitzar sobre les neurones tenyides amb PGP, en camps seleccionats sistemàticament al atzar, per tal de valorar canvis en la grandària de la neurona. Els somes d'un mínim de 300 neurones tenyides per gangli, van ser resseguits manualment i les seves àrees determinades, usant el programa *Object Image* (Lago et al. 2007).

Esquema dels grups experimentals (Figura 2)

A. Grups control

Animals als quals únicament se'ls injectava sèrum fisiològic subcutàniament i eren seguits en els mateixos temps que els animals tractats amb que se'ls aparellava.

B. Grups tractats amb bortezomib

Animals als que s'administrava 1 mg/kg de bortezomib subcutàniament, dues vegades per setmana, durant 6 setmanes i eren sacrificats 4 dies després de la darrera dosis. Els testatges de proves funcionals es realitzaven a nivell basal pretractament i cada 2 setmanes durant el tractament. En la valoració de l'afectació de fibra petita com a predictor precoç de neuropatia severa, l'extracció de coixinets s'efectuava a nivell basal i cada 2 setmanes durant el tractament.

C. Grup de recuperació postractament amb bortezomib

Animals tractats amb bortezomib i seguits igual que en el grup anterior, però als quals després d'administrar la darrera dosis del fàrmac, a la sisena setmana de tractament, se'ls deixava en repòs durant 4 setmanes més, i se'ls realitzava aleshores un darrer estudi funcional pre-sacrifici del grup.

D. Grup de retractament amb bortezomib

Animals tractats amb bortezomib amb la mateixa posologia que els del grup B. Al finalitzar la sisena setmana de tractament, se'ls deixava en repòs durant 4 setmanes més i després es reiniciava de nou una pauta de bortezomib a 1 mg/kg, dues vegades per setmana, durant 6 setmanes més. Per tant, aquest grup era seguit 16 setmanes, i es realitzaven avaluacions funcionals a nivell basal i cada 2 setmanes durant les primeres 6 setmanes de tractament. Passades les 4 setmanes de repòs, es reiniciava el seguiment funcional amb el mateix esquema que durant el primer tractament. A la finalització d'aquest segon tractament amb bortezomib, eren sacrificats 4 dies després d'haver rebut la darrera dosis.

E. Grups tractats amb vincristina a dosis alta i baixa

Animals als quals s'administrava vincristina a 1 mg/kg (dosis baixa) i a 1,5 mg/kg (dosis alta) intraperitonealment, dues vegades per setmana, durant 4 setmanes i que eren seguits

posteriorment durant 8 setmanes més abans de ser sacrificats. Els seguiments funcionals es realitzaven a nivell basal, pretractament, i cada 2 setmanes fins al final.

F. Grups amb neuropatia instaurada tractats amb bortezomib

Animals tractats amb vincristina a dosis de 1 i 1,5 mg/kg amb la mateixa pauta que en el grup anterior, per tal d'induir-los-hi una neuropatia de base amb diferents graus de severitat. Després de 2 setmanes de repòs sense tractament, se'ls iniciava un tractament amb bortezomib a 1 mg/kg, dues vegades per setmana durant 6 setmanes més, i eren sacrificats 4 dies després de la darrera dosis de bortezomib. En total foren seguits durant 12 setmanes. Els seguiments funcionals també es realitzaven a nivell basal i cada 2 setmanes fins a finalitzar la darrera dosis del tractament amb bortezomib.

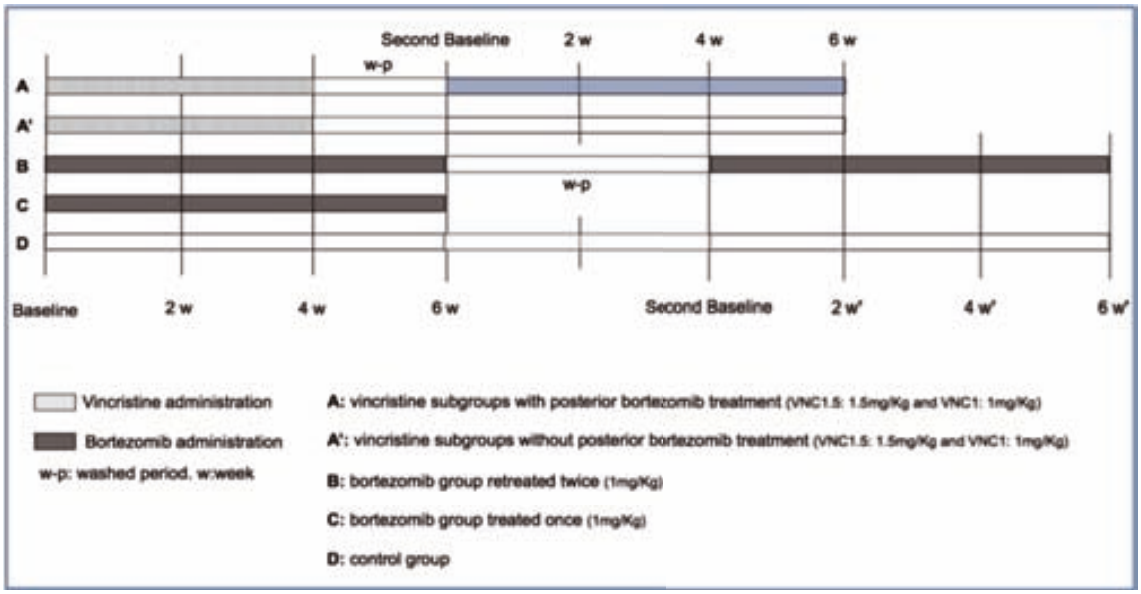

Figura 2. Esquema dels tractaments i pauta de seguiment dels diferents grups experimentals.

TREBALLS

1. Neurophysiological, histological and immunohistochemical characterization of bortezomib-induced neuropathy in mice. Bruna J, Udina E, Alé A, Vilches JJ, Vynkier A, Monbaliu J. Silverman L, Navarro X. *Experimental Neurology 2010; 223: 599-608*.

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Neurophysiological, histological and immunohistochemical characterization of bortezomib-induced neuropathy in mice

Jordi Bruna [a,b,c], Esther Udina [a,b], Albert Alé [a], Jorge J. Vilches [a], Ann Vynckier [d], Johan Monbaliu [d], Lee Silverman [e], Xavier Navarro [a,b,*]

[a] Group of Neuroplasticity and Regeneration, Institute of Neurosciences and Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Bellaterra, Spain
[b] Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Spain
[c] Unit of Neuro-Oncology, Department of Neurology, Hospital Universitari de Bellvitge, Hospitalet de Llobregat, Spain
[d] Johnson & Johnson, GPCD, Beerse, Belgium
[e] Millennium, Takeda Oncology Company, Cambridge, MA, USA

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ABSTRACT

Bortezomib, a proteasome inhibitor, is an antineoplastic drug to treat multiple myeloma and mantle cell lymphoma. Its most clinically significant adverse event is peripheral sensory neuropathy. Our objective was to characterize the neuropathy induced by bortezomib in a mouse model. Two groups were used; one group received vehicle solution and another bortezomib (1 mg/kg/twice/week) for 6 weeks (total dose as human schedule). Tests were performed during treatment and for 4 weeks post dosing to evaluate electrophysiological, autonomic, pain sensibility and sensory-motor function changes. At the end of treatment and after washout, sciatic and tibial nerves, dorsal ganglia and intraepidermal innervation were analyzed. Bortezomib induced progressive significant decrease of sensory action potential amplitude, mild reduction of sensory velocities without effect in motor conductions. Moreover, it significantly increased pain threshold and sensory-motor impairment at 6 weeks. According to these data, histopathological findings shown a mild reduction of myelinated (−10%; $p=0.001$) and unmyelinated fibers (−27%; $p=0.04$), mostly involving large and C fibers, with abnormal vesicular inclusion body in unmyelinated axons. Neurons were also involved as shown by immunohistochemical phenotypic switch. After washout, partial recovery was observed in functional, electrophysiological and histological analyses. These results suggest that axon and myelin changes might be secondary to an initial dysfunctional neuronopathy.

Introduction

Bortezomib (BTZ) is the first of a new class of anticancer agents known as proteasome inhibitors. BTZ is a reversible inhibitor of 20S ubiquitin-dependent proteasome complex, the major extralysosomal pathway responsible for nuclear and cytoplasmic protein degradation (Nandi et al., 2006). Proteasome inhibition is an interesting approach to cancer treatment because it acts by disruption of various critical cell signaling pathways, such as cell cycle regulation, cell adhesion and gene transcription. Thereby it leads cancerous cells to cell cycle arrest, inhibits angiogenesis and induces apoptosis (Voorhees et al., 2003; Jackson et al., 2005; Ludwig et al., 2005). BTZ is effective in the treatment of recurrent and newly diagnosed multiple myeloma (Richardson et al., 2003; San Miguel et al., 2008), and of recurrent mantle cell lymphoma (Kane et al., 2007). It is also under investigation as treatment for many common solid and hematological neoplasms, alone or in combination with other chemotherapeutic drugs (Awada et al., 2008; Caponigro et al., 2009; Davies et al., 2009).

However, one of most clinically significant adverse events and dose-limiting toxicity in BTZ therapy is the peripheral neuropathy, mainly characterized by hypoesthesia and sometimes painful paraesthesia (Richardson et al., 2006). The incidence of neuropathy reported in several phase II and III clinical trials using BTZ both in combination and as a single agent ranged from 31% to 55% (Richardson et al., 2003; Jagannath et al., 2004; Richardson et al., 2005; Harousseau et al., 2006; Bang et al., 2006; Badros et al., 2007; Min et al., 2007; Mateos et al., 2008). The neuropathy becomes severe, to grades 3 and 4 of the National Cancer Institute Common Toxicity Criteria (NCI-CTC) score (Trotti et al., 2003) in 13–17% and 1–7% of the patients respectively (Richardson et al., 2003; Jagannath et al., 2004; Richardson et al., 2005; Mateos et al., 2008). Moreover, neuropathy leads to a dose reduction in 12% and discontinuation of treatment in 5% of patients (Richardson et al., 2006) with the corresponding repercussion on patients' quality of life and survival (Jagannath et al., 2008). The

* Corresponding author. Unitat Fisiologia Mèdica, Facultat de Medicina, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Spain. Fax: +34 935812986.
E-mail address: xavier.navarro@uab.cat (X. Navarro).

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overall incidence of neuropathy is similar between patients without or with baseline neuropathy prior to the start BTZ therapy, a feature not unusual in myeloma patients (Richardson et al., 2006; Richardson et al., 2009). However, severe BTZ induced neuropathy is more frequent in the patients with baseline neuropathy (Richardson et al., 2006).

Current knowledge on the mechanisms underlying the BTZ induced peripheral neuropathy (BIPN) is scarce and contradictory. Only one group has described experimental studies of BIPN in a rat model (Cavaletti et al., 2007; Meregalli et al., 2009), in the first work, the electrophysiological findings were not in accordance with the main neurographic abnormalities found in BIPN patients. The study suggested that BTZ induces damage to satellite cells of dorsal root ganglia (DRG) (Cavaletti et al., 2007), whereas their second and most recent work proposes that BTZ induces painful unmyelinated axonopathy (Meregalli et al., 2009). On the other hand, in vitro studies identified the cell bodies of DRG neurons as the primary target for proteasome inhibitor peripheral neuropathy (Silverman et al., 2006). A recent in vitro study also supports the view that BTZ provokes dysfunction in sensory neurons by interfering with transcription and mRNA processing (Casafont et al., 2010). Thus, it is important to have an in vivo model available to further elucidate the mechanisms involved in BIPN, and to help in improving the clinical management of patients with BIPN. Moreover, it may be a useful model to evaluate neuroprotective agents designed to ameliorate or restore BIPN. The aim of our study was to develop and characterize an adequate model of BIPN in mice, assessed by electrophysiology, functional tests, morphology and immunohistochemistry.

Materials and methods

Animals, treatment and dose schedule

In a preliminary pilot study 20 Swiss OF1 female mice aged 2.5 months were used to assess different doses of BTZ and verify the safety and the development of neuropathic findings in this mouse strain. BTZ (provided by Millennium Pharmaceuticals Inc., and Johnson & Johnson Pharmaceutical Research & Development, L.L.C.) was dissolved in sterile saline solution and administered at doses of 0.8 mg/kg twice per week ($n=5$), 1 mg/kg twice per week (2pw) ($n=5$) or 1 mg/kg three times per week (3pw) ($n=5$). A fourth group ($n=5$) received only vehicle solution (control group). Treatment was administered subcutaneously during 6 weeks.

After determining the adequate dose to achieve a clinically relevant BIPN, a larger group of Swiss OF1 female mice aged 2.5 months ($n=20$) received BTZ treatment at the dose selected (e.g. 1 mg/kg) in the pilot study during 6 weeks, on days 1-4-8-11-15-18-22-25-29-32-36-39. At the end of the treatment period, half of the mice were sacrificed while the remaining animals were left untreated and followed-up for an additional 4-week period. In parallel, a second group of 10 mice receiving vehicle solution on the same days was used as control (CTROL).

The animals were housed under standard conditions in cages with soft bedding. Artificial lighting was provided on a fixed 12-h light–dark cycle with food and water available ad libitum. The general condition of the animals was assessed daily and body weight was recorded before each BTZ administration. The experimental procedures were approved by the Ethical Committee of our institution, and followed the rules of the European Communities Council Directive 86/609/EEC.

Functional tests

Functional evaluation was performed at baseline, before starting BTZ administration, and then every 2 weeks during treatment, and 4 weeks after finalization of treatment. Tests performed were aimed at quantitatively evaluating motor and sensory nerve conduction, autonomic sudomotor and heart function, pain sensibility, and integration of sensory-motor function.

Nerve conduction studies

For nerve conduction studies, the sciatic nerve was stimulated percutaneously through a pair of needle electrodes placed at the sciatic notch (proximal site) and at the ankle (distal site). Rectangular electrical pulses (Grass S88 stimulator) of 0.01 ms duration were applied up to 25% above the voltage that gave a maximal response (Navarro et al., 1994; Verdu et al., 1999). The compound muscle action potentials (CMAPs) were recorded from the tibialis anterior and the third interosseus muscle with microneedle electrodes. Similarly, the sensory compound nerve action potential (SNAP) was recorded by electrodes placed at the fourth toe near the digital nerves. Latencies and amplitudes of the action potentials were measured and nerve conduction velocities of motor and sensory nerve fibers were estimated. During electrophysiological tests, the animals were anesthetized (pentobarbital 40 mg/kg i.p.) and placed over a warm flat steamer controlled by a hot water circulating pump to maintain the body temperature constant.

Autonomic function tests

Sympathetic sudomotor function was evaluated by the silastic imprint technique. Sweating was stimulated by subcutaneous injection of pilocarpine (5 mg/kg), and 10 min later a silicone material (Silasoft Normal, Detax GmbH & Co., Ettlingen, Germany) was spread over the plantar surface of the hindpaw. Sweat droplet impressions made in the silicone mold were counted under a dissecting microscope (Navarro et al., 1994; Vilches and Navarro, 2002).

Heart rate variability was analyzed using an electrocardiographic recording for 3 min, acquired through a PowerLab system (ADInstruments) and stored by Chart software. Heart R-R periods were evaluated using Pearson Variation Coefficient.

Pain sensibility tests

The algesimetry technique was used to evaluate the functional status of nociceptive C fibers (Hargreaves et al., 1988). Mice were placed into a plastic box with an elevated glass floor (Plantar Algesimeter, Ugo Basile). The light of a projection lamp was focused directly onto the plantar surface of one hindpaw and the time to elevation of the heated paw was obtained from a time-meter coupled with infrared detectors. The value for a test was the mean of three trials separated by 10 min resting periods.

Sensory-motor function

A rotarod apparatus for small rodents (LIAP) was used. Mice were placed on the rod, turning at 8 rpm, and the time that each animal remained on it before falling was measured. The value for a test was the mean of three trials separated by 10 min resting intervals. Before treatment, mice were trained for 5 days. The ability to remain on the rotarod for 120 s was taken as an index of normal sensory-motor function (Navarro et al., 1993; Verdu et al., 1999).

Histological methods

At the end of the sixth week treatment period, half of the animals belonging to BTZ treated and control groups were anaesthetized and perfused with paraformaldehyde (4% in PBS 0.1 M, pH 7.4). The other half of animals was perfused after 4 weeks of washout period. A segment of the sciatic nerve at mid-thigh and a segment of the tibial nerve at the ankle level were removed. The nerve samples were fixed in glutaraldehyde–paraformaldehyde (3%:3%), washed in cacodylate buffer (0.1 M, pH 7.4), then post-fixed overnight with acetate uranile in 70% alcohol and 2% osmium tetroxide during 2 h at 4 °C, dehydrated in graded concentrations of ethanol and embedded in epon. Light

microscopy observations were performed on 0.5 μm semithin sections stained with toluidine blue. A morphological evaluation, including cross-sectional area, axonal counts, myelinated axon and fiber perimeters and diameters were made from systematic randomly selected fields at 1000× final magnification, covering representative part of the cross-sectional nerve profile (at least 15% of the total). The density of myelinated nerve fibers, myelin thickness and *g* ratio were then derived according to previously reported methods. Myelinated axons analyzed were at least 500 for each animal, only fibers whose contour was completely within each picture were counted and their perimeters outlined (Gómez et al., 1996). Morphometrical evaluation was performed at the sciatic level and axonal counts were made at both proximal (sciatic) and distal (tibial) sites manually using Object image software from NIH. Based on the light microscopic findings, ultrathin sections were prepared from selected blocks, counterstained with lead citrate and examined under a Hitachi 7000 electron microscope.

Immunohistochemistry

Skin innervation

Plantar pads removed from mice euthanized after the 6 weeks of treatment to obtain nerve samples were stored in Zamboni's fixative and thereafter cryoprotected. Cryotome pad sections 40 μm thick were washed free-floating in PBS with 0.3% Triton-X100 and 1% normal goat serum for 1 h, then incubated in primary rabbit antisera against protein gene product 9.5 (PGP, 1:800; ABSserotec) and calcitonin gene-related peptide (CGRP, 1:1000, Chemicon). After washes, sections were incubated in secondary antiserum and processed as described previously (Navarro et al., 1995). Samples were viewed under an epifluorescence microscope using appropriate filters. Five sections from each sample were used to quantify skin pad innervation by analyzing number and density of epidermal nerve fibers (Verdu et al., 1999).

Dorsal root ganglia

Dissected L4 dorsal root ganglia from the same mice were post-fixed in 4% paraformaldehyde, and stored in 30% sucrose in PBS solution. Cryostat sections were done at 8 μm thickness. Sections were stained using primary antibodies anti-PGP, anti-CGRP and anti-Lectin I Isolectin B4 (IB4). After three washes, sections were incubated with Cy3 conjugated immunoglobulin G (Lago and Navarro, 2007). Photographs to analyze the number of stained neurons were taken at 200× magnification. Neuronal cell counts were performed in order to estimate the proportion of labeled cells of each type. Only neurons with distinguished nucleus and diffuse nuclear staining were counted to avoid redundancy of neuronal counts. Morphometrical analyses of neuron bodies were performed in randomly selected PGP stained sections to evaluate changes in neuron size. The somas of at least 300 labeled neurons were outlined manually, and their sizes determined using Image software (Lago and Navarro, 2007).

Statistical analysis

Comparisons between control and experimental group values in functional tests were made using repeated ANOVA tests, and Student's *t*-test was used to assess differences between groups in histological and immunohistochemical evaluations. Results are expressed as mean and standard error of the mean. For normalization, the results of functional tests during the whole study period are expressed as the percentage with respect to baseline values for each mouse. All calculations were performed using the SPSS software package version 12.0 (SPSS Inc.) and graphics using software package GraphPad Prism version 4 (GraphPad Software, Inc.).

Results

Pilot study

BTZ administration at doses of 0.8 mg/kg 2pw was well tolerated; mice did not show distress and neither prostration nor reduced weight gain were observed. However, this schedule and dose induced only very mild electrophysiological changes without functional and histological abnormalities. Doses of 1 mg/kg 2pw were also well tolerated and induced mild to moderate sensory neuropathy with abnormalities in functional and histological evaluations. Doses of 1 mg/kg 3pw caused more severe changes in neurophysiologic tests, including motor conduction tests, and a more marked loss of axons (data not shown), but the high dose was also associated with an increased mortality in the first weeks (60% by 3 weeks). Since our aim was to obtain a mild neuropathy model with similar features to the human BINP, we chose the schedule of 1 mg/kg 2pw to fully characterize its induced neuropathy.

Characterization of BTZ induced neuropathy

Tolerance and safety of the 1 mg/kg 2pw bortezomib dose regime tested was confirmed in the larger animal group used for neuropathy characterization, and only one mouse died during the second week of treatment after the third dose of BTZ.

Nerve conductions tests

In motor nerve conduction tests, the amplitude of CMAP of both the tibialis anterior and plantar muscles and the motor nerve conduction velocity were similar in BTZ and control groups (Fig. 1A). Only one animal suffered a pathological reduction (two standard deviations below the normal mean) of the plantar CMAP amplitude. Latencies of H waves did not show differences between groups. In contrast, when sensory nerve conduction was evaluated, the SNAP amplitude of digital nerves in the BTZ group showed a significant progressive decrease with regard to the control group from 2 weeks of follow up; at the sixth week of treatment the SNAP amplitude averaged about 60% of baseline values ($p = 0.0001$) (Fig. 1B). Sensory nerve conduction velocity was also significantly decreased at 4 week of follow-up, slightly more at distal ($p = 0.014$) than at proximal segments of the sciatic nerve ($p = 0.035$). The control group showed the expected increase of conduction velocities due to nerve maturation during follow-up (Fig. 1C and D). After the washed period, sensory amplitude and conduction velocity values recovered to near normal values.

Autonomic evaluation

BTZ treated and control groups did not show significant differences in sudomotor function and in the coefficient of heart rate variability (data not shown). However, three of the treated mice (15%) showed a 30% decrease in the number of reactive sweat glands at 6 week of follow-up, indicating sudomotor fiber involvement.

Pain sensibility

Pain sensibility, evaluated by thermal algesimetry, showed a progressive increase of withdrawal threshold in BTZ treated mice, achieving significantly higher values at the maximal accumulated dose when compared to the control group ($p = 0.045$). The values (mean ± SD) in CTROL and BTZ groups at basal evaluation were 13.46 ± 2.02 and 12.92 ± 2.87 s respectively. After 6 weeks treatment, the withdrawal latency averaged 13.85 ± 3.32 s in the CTROL group and 17.79 ± 5.59 s in the BTZ group. After the washout period, the pain threshold of BTZ treated mice recovered and achieved values even lower than basal values (Fig. 2A).

Sensory-motor function

In the rotarod test BTZ treated mice showed a significant decline in the time of sustained walking in the rod only at the end of 6 weeks of

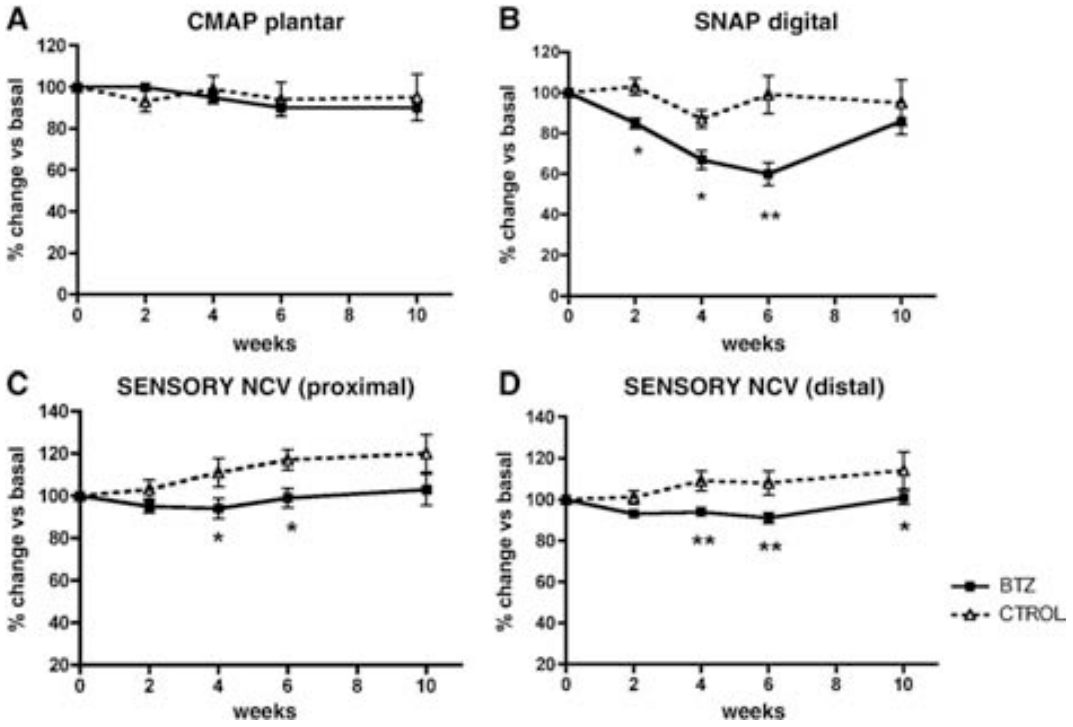


Fig. 1. Mean amplitude of the compound muscle action potential (CMAP) of plantar muscle (A) and the sensory compound nerve action potential (SNAP) of digital nerve (B) stimulating at the sciatic notch. Proximal (from sciatic notch to ankle) and distal (from ankle to toe) sensory nerve conduction velocities (C and D). BTZ group was treated during 6 weeks and followed-up during four more weeks of wash out period. Values expressed as percentages with respect to baseline. Error bars: SEM. $^{*}p<0.05$, $^{**}p<0.001$.

treatment ($p=0.026$). The values (mean ± SD) in CTROL and BTZ groups at baseline were 177.17 ± 6.38 and 175.75 ± 5.75 s, respectively, whereas after 6 weeks of treatment, CTROL group values were 178.17 ± 5.29 s and BTZ treated group were 123.85 ± 38.34 s. After the washout period BTZ treated mice returned to normal values (Fig. 2B).

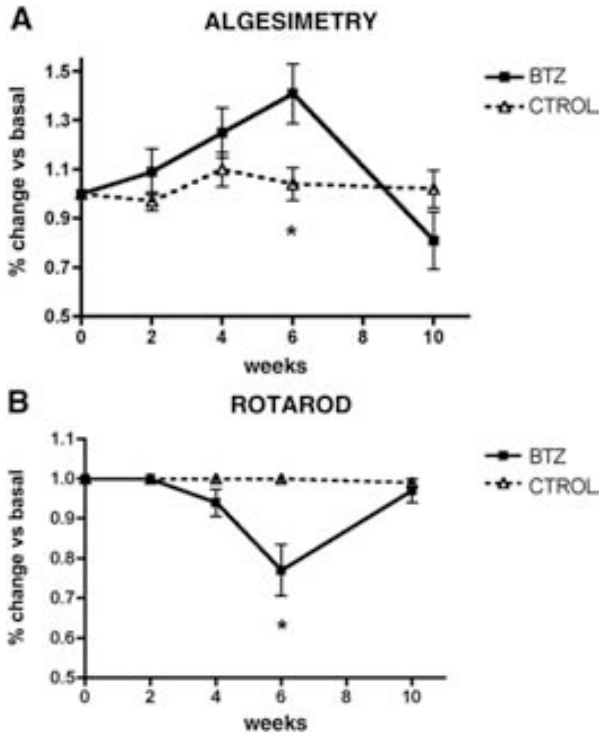


Fig. 2. (A) Algesimetry test results, expressed as time to withdrawal to hot pain stimulation. (B) Rotarod test results, expressed as time of maintenance in the rotating rod relative to baseline. BTZ group was treated during 6 weeks and followed-up during 4 weeks of wash out period. Values expressed as percentages with respect to baseline. Error bars: SEM. $^{*}p<0.05$.

Histopathological examination

Sciatic nerve histology after 6 weeks of BTZ treatment. The sciatic nerves showed a lower density of myelinated large fibers and more fibers with irregular forms in BTZ treated mice compared to control mice. There were no obvious signs of degeneration and macrophage infiltration (Fig. 3). The estimated count of myelinated fibers was significantly lower in the BTZ treated than in the control nerves (Table 1). The morphometrical analysis showed a significant reduction of the mean diameter of myelinated fibers in BTZ group (7.2 ± 0.04 μm vs. 8.0 ± 0.08 in controls). The decrease of fiber diameter was due to a reduction in myelin thickness (1.3 ± 0.01 μm vs. 1.5 ± 0.02 in controls), and also to axonal atrophy and some axonal loss (Fig. 4, table). The relative distribution of myelinated fibers according to diameter showed a marked decrease in the population of large fibers with a consequent increase in the relative percentage of small fibers, being the distribution shifted to the left compared to the control distribution (Fig. 4).

Tibial nerve histology after 6 weeks of BTZ treatment. Similar to the more proximal sciatic nerve sections, tibial nerve sections showed a well preserved nerve architecture, without signs of Wallerian degeneration (Fig. 3F). Analysis of tibial nerves showed a reduction of about 10% in the estimated count of myelinated fibers compared to the control group, although this difference was not statistically significant ($p=0.09$) (Table 1). There was a correlation between the electrophysiological and the histological findings, since BTZ treated animals with more than 50% reduction in amplitude of digital nerve SNAP from basal values ($n=5$) showed a significant ($p=0.04$) loss of myelinated axons in the tibial nerve (mean 1027 ± 69.2) compared to controls (1270 ± 59, $n=9$), whereas in the subgroup ($n=4$) of treated animals with milder electrophysiological involvement (less

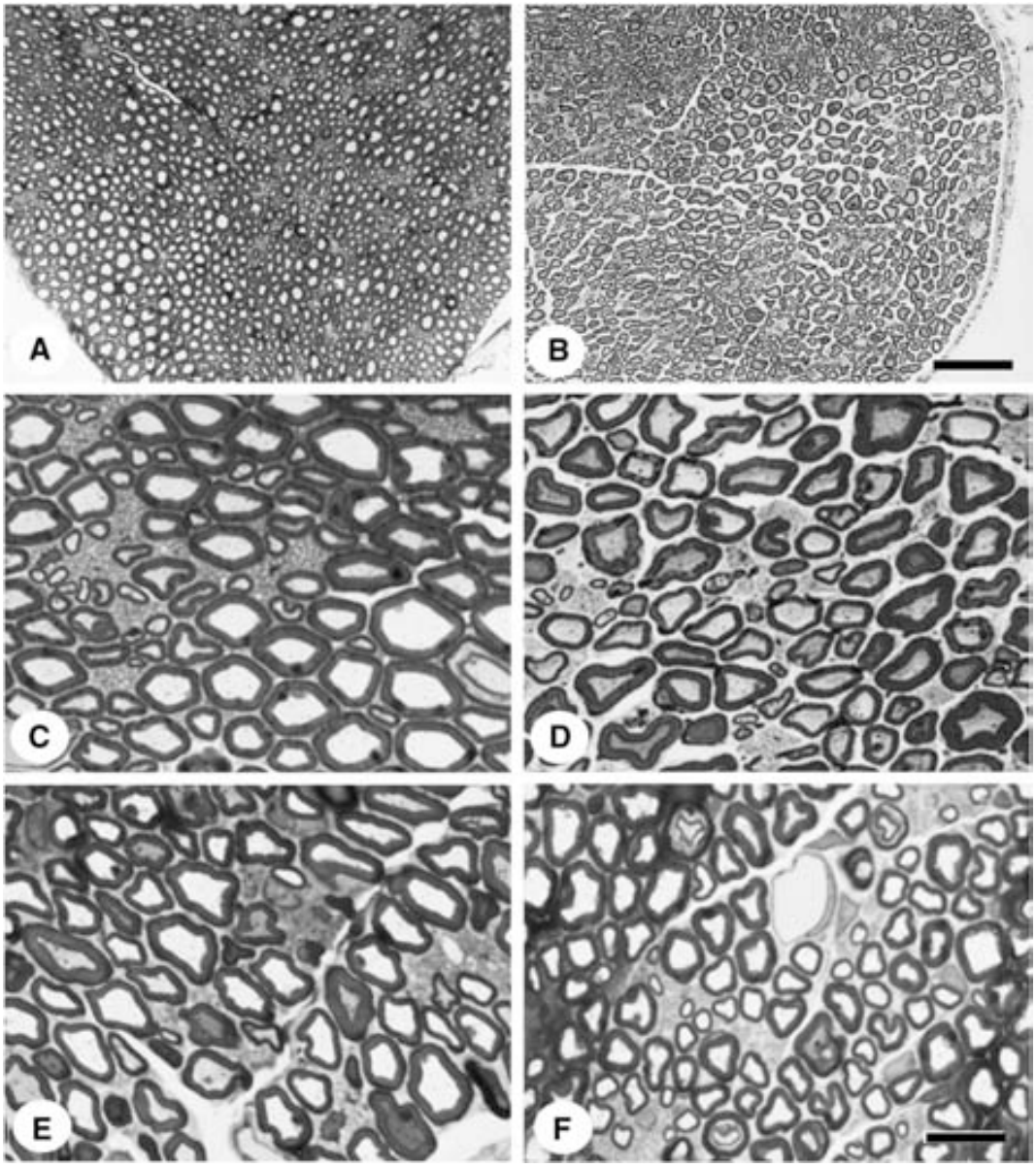


Fig. 3. Representative micrographs of semithin sections of sciatic nerves at the mid thigh (A–D) and of tibial nerves at the ankle (E–F) stained with toluidine blue. (A, C) Sciatic nerve from a control animal. Treatment with BTZ reduced the density of myelinated fibers in the sciatic nerve, their size and myelin thickness (B, D). (E) Tibial nerve at the ankle level of a control animal. After BTZ treatment, the number of myelinated axons was slightly reduced (F). Bar = 200 μm for A–B and 10 μm for C–F.

than 50% reduction in SNAP amplitude) the numbers where similar to the control group (1260 ± 34).

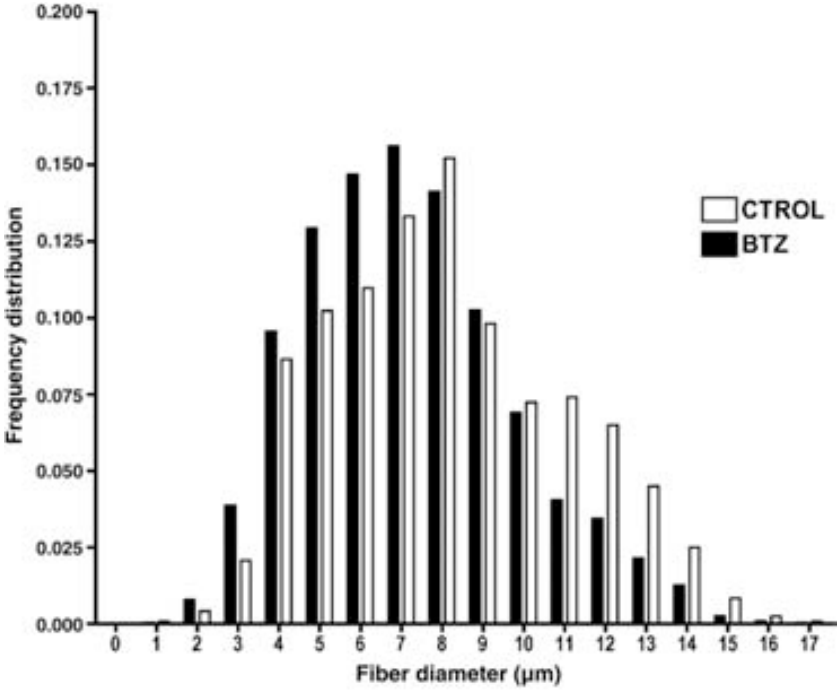
Nerve histology after the washout period

In contrast to the sciatic nerves of the animals studied at 6 weeks of BTZ administration, in the mice followed during the four additional weeks washout period, the morphological appearance of most nerves was similar to controls, with a normal density of myelinated fibers in sciatic and tibial nerves. Counts of myelinated fibers did not show significant differences between control and BTZ washout groups (Table 1). Only two animals in this group showed a reduced number of myelinated fibers in sciatic and tibial nerves, with thinner myelin sheaths, and a few degenerating axonal profiles. The morphometrical analysis showed a significant recovery of the fiber diameter (7.5 ± 0.05 μm) compared to the 6 weeks BTZ treated group (7.2 ± 0.04 μm). The increase in fiber diameter was mainly due to a significant recovery of myelin thickness (1.4 ± 0.01 μm vs. 1.3 ± 0.01 in BTZ 6w); thus, the *g* ratio was normalized (0.61 ± 0.08). Nevertheless, values of fiber

Table 1
Morphological results. Counts of myelinated fibers (MF) in sciatic and distal tibial nerves after 6 weeks (6w) of bortezomib (BTZ) treatment and following the washout period (10w).

	Sciatic nerve (mid thigh)				Tibial nerve (ankle)	
	Total MF	Total area	Tibial MF	Tibial area	Tibial MF	Tibial area
CTROL ($n = 10$)	4021 ± 95	0.16 ± 0.007	2362 ± 86	0.096 ± 0.004	1270 ± 63	0.038 ± 0.001
BTZ 6w ($n = 10$)	3624 ± 72*	0.15 ± 0.007	2185 ± 55	0.090 ± 0.003	1130 ± 57	0.038 ± 0.004
BTZ 10w ($n = 9$)	3966 ± 89	0.15 ± 0.004	2385 ± 79	0.10 ± 0.004	1286 ± 30	0.036 ± 0.003

Results expressed as mean ± SEM. *$p < 0.01$ BTZ vs. Control group.



GROUP	Fiber Perimeter	Axon perimeter	G Ratio	Fiber Diameter	Myelin thickness
CTROL	25.25 ± 0.26	15.56 ± 0.18	0.61 ± 0.08	8.04 ± 0.08	1.54 ± 0.02
BTZ 6w	22.75 ± 0.14*	14.79 ± 0.1*	0.65 ± 0.11*	7.24 ± 0.04*	1.27 ± 0.01*
BTZ 10w	23.42 ± 0.18*,#	14.49 ± 0.12*	0.61 ± 0.08#	7.45 ± 0.05*,#	1.42 ± 0.01*,#

Fig. 4. Histogram of the distribution of myelinated fibers of the sciatic nerve according to diameter in the control group and the BTZ-treated group. Table: Morphometric results of sciatic nerve myelinated fibers in the control group, and in BTZ-treated for 6w (BTZ 6w) and after 4w washout period (BTZ 10w) groups. Perimeter, diameter and myelin thickness in μm. Values expressed as mean ± SEM. *$p<0.001$ vs. CTROL; #$p<0.016$ vs. BTZ 6w.

perimeter and myelin thickness were still significantly lower than in control mice, suggesting a partial or ongoing recovery (Fig. 4, table).

Electron microscopy

In two mice of the BTZ group there were some abnormal clusters of unmyelinated axons of unusually large size. To further study the unmyelinated axons, we performed electron microscopy of the affected nerves, and found that in the axoplasm of some unmyelinated fibers, there were accumulations of large size vacuoles with degradation products (see Fig. 5B and C vs. A control). A few myelinated fibers in BTZ treated animals had signs of Wallerian degeneration (Fig. 5D), whereas in the soma of some Schwann cells the endosomal membrane system was markedly dilated (Fig. 5E, treated vs. F, control). These abnormalities were more marked in nerves sampled from mice that received BTZ 1 mg/kg 3pw in the pilot study.

Evaluation of skin innervation

Plantar pads of treated animals did not show obvious signs of skin atrophy. The number and the density of the intraepidermal axonal profiles, labeled by PGP, in the BTZ treated group was significantly lower than in the control group ($p=0.046$) (Fig. 6), but the subpopulation of CGRP positive profiles was not affected by BTZ (Table 2).

DRG examination

The mean area of DRG neurons labeled against PGP (a pan-neuronal marker) was $398 \pm 282\ \mu m^2$ in the BTZ treated group, similar to control values ($415 \pm 132\ \mu m^2$), thus indicating that BTZ did not induce marked atrophy of sensory neurons. When the neurons were distributed by size groups, there were no changes in the distribution of large DRG neurons (>$800\ \mu m^2$) but a slight not significant decrease in the population of medium neurons (400–$800\ \mu m^2$) with a consequent increase in the relative percentage of small neurons (<$400\ \mu m^2$) in treated animals (Fig. 7A). The two main subpopulations of small neurons were further analyzed by means of CGRP (peptidergic neurons) or IB4 (non-peptidergic small neurons) immunolabeling. In the treated animals it was a slight but significant increase in the number of DRG neurons expressing CGRP ($p=0.013$) and IB4 ($p=0.005$) with respect to the control group (Fig. 7B). The distribution by size groups of CGRP labeled neurons showed an increase in the relative percentage of large neurons (>$1200\ \mu m^2$) in treated animals ($p=0.0003$). The slight increase of neurons labeled by CGRP corresponding to large neurons, without significant changes in size of the total DRG neurons labeled by PGP suggests a phenotypic switch of the population of large neurons in BTZ treated animals (Fig. 7C). Details of immunohistochemical CGRP labeled DRG are shown in Fig. 8.

Discussion

The aim of our study was to characterize the neuropathic involvement induced by BTZ in mice, and find if this experimental model had similar features to the BIPN described in humans. Peripheral neurotoxicity is one of the main limiting adverse events for the therapeutic use of BTZ, and reduces the options to investigate

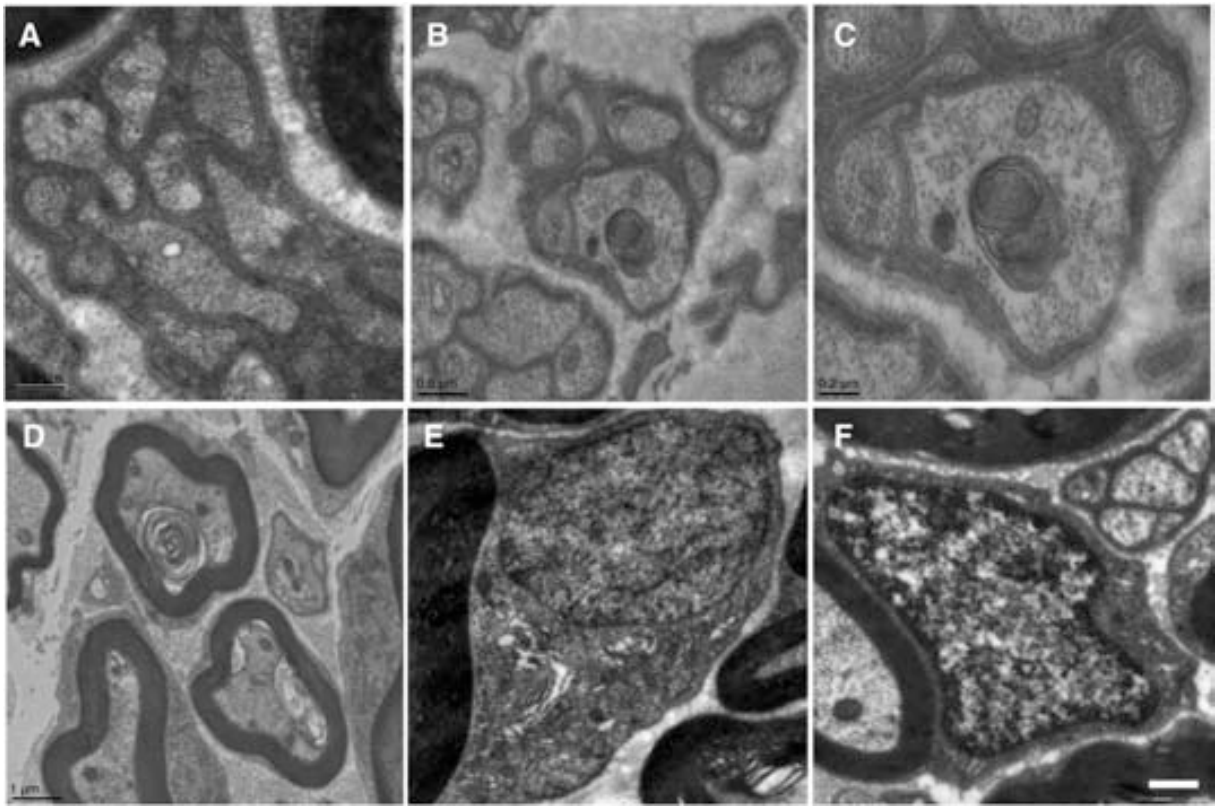


Fig. 5. Electron microscope micrographs of unmyelinated axons of a control animal (A). After 6 weeks of treatment with 1 mg/kg of BTZ two or three times per week, some unmyelinated axons show pathological features (B) (bar = 0.5 μm). At higher magnification, details of the vesicles in unmyelinated axons, with accumulation of degenerative products (C), suggestive of autophagosomes (bar = 0.2 μm). Details of degenerative signs in a myelinated axon in a mouse treated with 1 mg/kg three times per week (D, bar = 1 μm). Magnification of the soma of a Schwann cell in an animal treated with BTZ two times per week (E), where a marked dilatation of the endosomal membrane system can be observed when compared to a Schwann cell of a control animal (F) (bar = 1 μm).

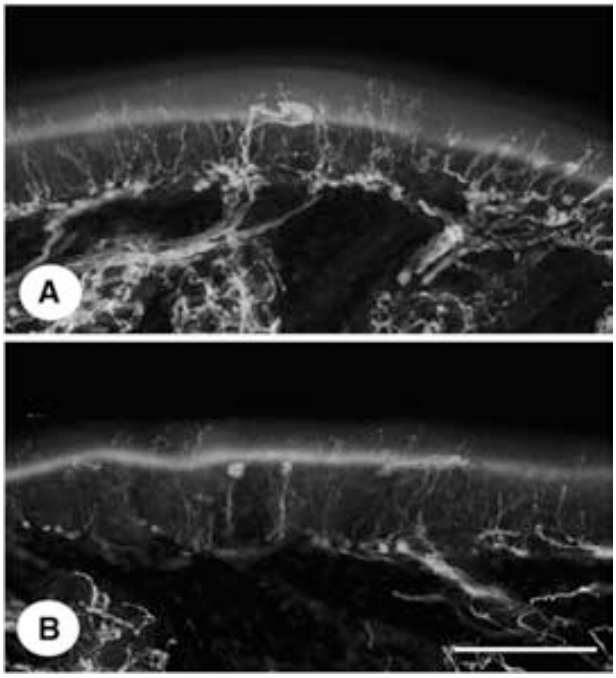


Fig. 6. Immunohistochemical images of protein gene product 9.5 (PGP) immunoreactivity in footpads representative of a control animal (A) and an animal treated with BTZ for 6 weeks (B). Bar = 150 μm. The density of immunoreactive profiles is considerably reduced in the epidermis of treated mice.

more active dose schedules and to combine BTZ treatment with other effective chemotherapeutic agents with known neurotoxic profile. Our present results demonstrate that BTZ given at a dose of 1 mg/kg twice per week during 6 weeks, induced a mild to moderate sensory neuropathy in Swiss OF1 mice, evidenced by neurophysiologic and functional tests and with corresponding histopathological findings.

Although the BTZ schedule used differs from that used in humans with respect to duration of administration and dosage, according to FDA recommendations (Reagan-Shaw et al., 2008) to convert doses between animals and humans, the total BTZ dose received at the end of the study by the mice given 1 mg/kg 2pw was 42.8 mg/m^2, similar to the standard dose used in patients with a schedule of 1.3 mg/m^2 four times each 21 days during eight cycles (41.6 mg/m^2).

When using the dose of 1 mg/kg 2pw in our mouse model, the resulting neuropathy shares common features with the one described in the clinic (see below). Although difficult and subjective, we can translate the severity of the neuropathy in mice to human neurotoxicity scales, by considering a decrease of more than 50% in amplitude of the SNAPs together with an increase above 50% of pain withdrawal threshold to a grade 2 neurotoxicity in the NCI-CTC scale, and an

Table 2
Analysis of the skin innervation after 6 weeks of bortezomib (BTZ) treatment. Number and density of intraepidermal nerve fibers (IENF) in the plantar skin, immunolabeled against protein gene product 9.5 (PGP) and calcitonin gene-related peptide (CGRP).

	PGP + IENF		CGRP + IENF	
	Number	Density	Number	Density
CTROL ($n = 10$)	39.4 ± 4.1	788.6 ± 81.6	18.9 ± 1.4	377.3 ± 28.8
BTZ ($n = 10$)	28.6 ± 3.0*	572.9 ± 60.8*	21.3 ± 1.5	426.3 ± 30.3

Values are the mean ± SEM. *$p < 0.05$ BTZ vs. Control group.

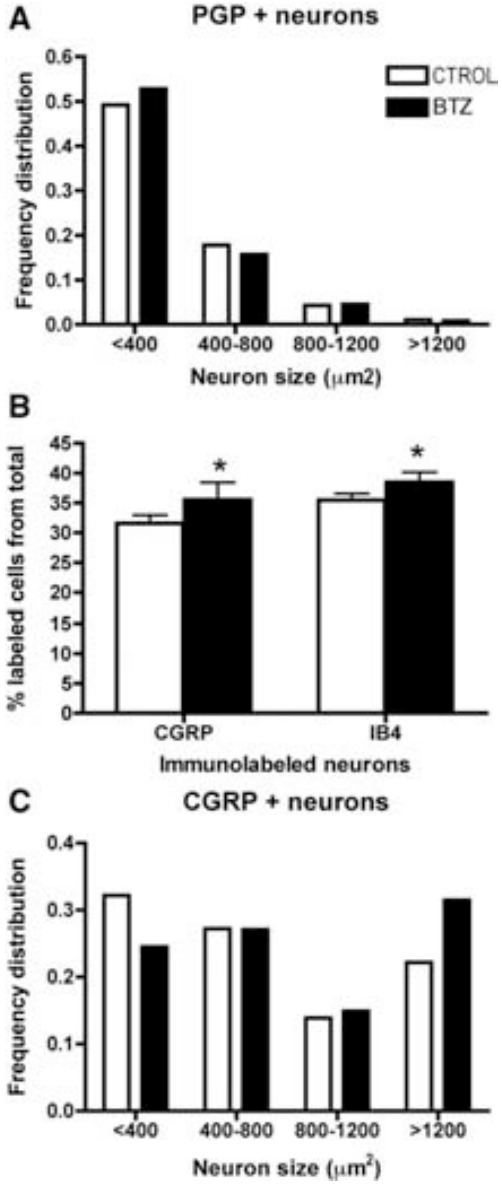


Fig. 7. (A) Histograms of the distribution of protein gene product 9.5 (PGP) labeled neurons in dorsal root ganglia (DRG) according to size in the CTROL and the BTZ treated group. (B) Relative percentage of neurons positive for CGRP and IB4 among all PGP labeled neurons of the DRG. (C) Size distribution of CGRP positive neurons in controls and treated animals. Error bars: SEM. *$p<0.01$ BTZ vs. CTROL group.

additional reduction above 50% in the scores on rotarod (a test that assesses the integration of sensory-motor function and evaluates general functional involvement) to an equivalent grade 3 NCI-CTC toxicity, in which daily activities of patients are impaired. According to this criteria, 47% (9/19) of our mice would have a grade 2 and 15% (3/19) a grade 3 of neurotoxicity, these percentages being similar to those observed in humans under standard schedule of BTZ (Badros et al., 2007; Richardson et al., 2003; Richardson et al., 2005).

When analyzing the features of the neuropathy in the mouse model, we found a predominantly sensory neuropathy, with a significant decrease in the amplitude and velocity of sensory nerve fibers but not of motor fibers. Motor nerve involvement was only observed in the pilot study at the highest dose used (1 mg/kg three times per week). The neurophysiological findings, similar to the ones recorded in patients treated with BTZ (Argyriou et al., 2008; Chaudhry et al., 2008; Richardson et al., 2009) suggest that the neuropathy is axonal or neuronopathic whereas demyelinating changes are probably secondary. The predominant sensory involvement may be explained by the lack of an efficient blood–nerve barrier in DRG ganglia, thus allowing toxic agents easier access to sensory than to motor neurons. BIPN has been previously described in the rat by Cavaletti et al. (2007) but their electrophysiological findings, suggesting demyelinating changes, with morphological alterations in Schwann and satellite cells do not correlate with the main neurographic findings compatible with axonal damage usually described in patients with BIPN (Argyriou et al., 2008; Chaudhry et al., 2008; Richardson et al., 2003). These divergences can be attributed to differences in duration of BTZ treatment and species used, thus, pointing out to the usefulness of studying different animal models to evaluate and understand the neurotoxicity induced by chemotherapeutic agents.

The decrease in the amplitude of the SNAP indicates an alteration of the large myelinated sensory fibers. The reduction in the number of myelinated fibers, together with the decrease of fiber diameters and slight decrease of myelin thickness in all size fibers in our BTZ treated mouse nerves are in accordance with the electrophysiological decrease of the sensory action potentials and conduction velocities. Earlier significant changes in SNAP amplitude than in conduction velocity likely reflect a predominant effect of BTZ on the neuron rather than on the Schwann cell. However, the increased *g* ratio and the decrease in the myelin thickness of the myelinated fibers of treated animals indicate that Schwann cells were also affected later on the course of BTZ treatment. The degree of fiber myelination recovered faster than axonal atrophy after 4 weeks of washout period, reinforcing the idea that BTZ primarily affects neurons and alterations related with Schwann cells might be secondary. On the other hand, the comparative analysis of proximal (sciatic nerve) and distal (tibial nerve) myelinated fiber counts points against a retrograde dying-back type of neuropathy. In fact, myelinated fibers in the tibial nerve of animals treated with BTZ showed only a trend to decrease with

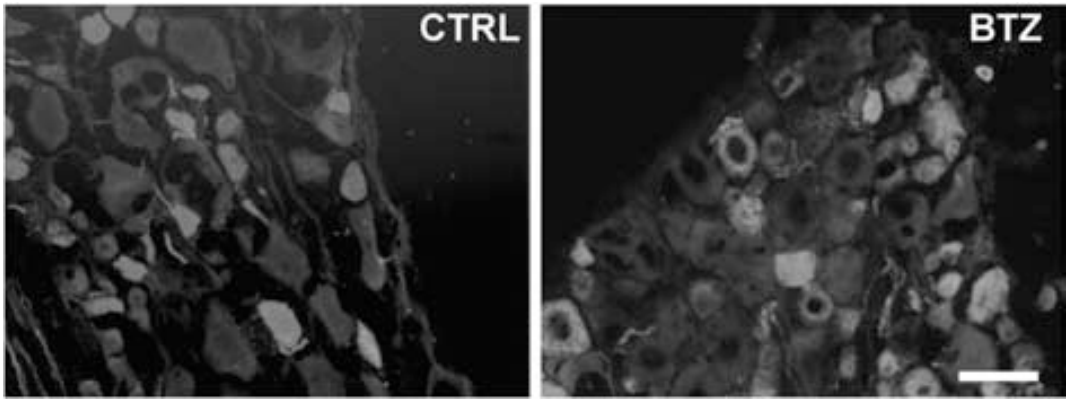


Fig. 8. Details of DRG sections labeled with anti-CGRP from representative animals of the control group (left) and the group treated with BTZ for 6 weeks (right). Bar = 50 μm.

respect to the control group, in contrast to the significant decrease at the sciatic level. However, when analyzing treated animals with marked reduction of SNAPs, the reduction of myelinated fibers at the distal tibial nerve was statistically significant. In correspondence with the neurophysiological recovery, the number of myelinated fibers returned to normal values after 4 weeks of washout. Injured axons could have regenerated, although histological samples did not show overt typical signs of regenerating fibers, with clusters of small regenerating axons.

The histopathological findings observed in BTZ treated mice are mild, with some axonal degenerative figures and Schwann cell endosomal membrane dilatation, as reported previously in rats (Cavaletti et al., 2007). We also observed pathological features in the unmyelinated axons of the most affected mice. These axons contained vesicles with degrading products inside, suggestive of autophagosomes. It is known that autophagy, a process responsible for the degradation and recycling of proteins and damaged organelles (Shimizu et al., 2004), can act as a compensatory degradation system when unfolded protein response is impaired (Shao et al., 2004; Shimizu et al., 2004; Shintani and Klionsky, 2004), as caused by proteasome inhibition by BTZ, and helps survival of neurons.

The involvement of unmyelinated fibers is in accordance with our neurophysiological and immunohistochemical assessments that demonstrate functional alteration of small fibers, as indicated by the increase in pain threshold. Although autonomic dysfunction was not significant, three animals (15%) showed abnormal sudomotor function, a similar proportion to the dysautonomia reported in humans (Richardson et al., 2003). On the other hand, BTZ treatment caused a significant loss of intraepidermal nerve profiles in the plantar skin when immunostained for PGP, a sensitive marker for cutaneous nerve fibers (Navarro et al., 1995) that is reduced in small-fiber neuropathies when patients have pain and deficits in cutaneous pain sensibility (Holland et al., 1998; Pan et al., 2001). Thus, the reduction of PGP positive profiles in our model, together with the hypoalgesia found in the algesimetry test indicates that BTZ also affects small nociceptive fibers. However, we failed to demonstrate hyperalgesic phenomena, in contrast to what was reported in some patients during BTZ treatment (Cata et al., 2007). A nonsignificant hyperalgesia was only observed after the washout period, similar to what has been described in situations of nerve regeneration and reinnervation (Casals-Diaz et al., 2009). Interestingly, when we analyzed the subpopulation of CGRP afferents of the skin, we did not find a decrease, suggesting that this subtype of nociceptive fibers is not affected by BTZ. To further elucidate the degree of alteration of different subpopulations of nociceptive neurons, we also studied CGRP (peptidergic) and IB4 (non-peptidergic) containing neurons in the DRG. The increased number of neurons of the DRG expressing CGRP that we observed in our model has been related to pain (Nishigami et al., 2009; Ohtori et al., 2007; Xu et al., 2005). When analyzing the distribution of neurons expressing CGRP after BTZ treatment, we detected an increased proportion of medium- to large-sized neurons. The up-regulation of CGRP in the medium to large subpopulation of neurons has also been reported after injury (Chao et al., 2008; Li et al., 2004; Miki et al., 1998; Zheng et al., 2008), as a phenotypic change probably linked to dynamic adaptive responses of injured neurons.

The preservation of the subpopulation of IB4 positive neurons, which are particularly dependent on trophic support of glial-derived neurotrophic factor (Averill et al., 2004; Molliver et al., 1997; Zhao et al., 2004), suggests a preservation of axonal transport and function of the Schwann cells. Thus, although we observed morphological changes in the endosomal membrane system of some Schwann cells, these alterations might not have functional translation. In fact, our results suggest that BIPN, at least at the initial stage, is mainly due to dysfunction at the neuronal level, and changes in axons and myelin might be secondary to this initial event. A possible activation of autophagy as compensatory pathway for neuron survival, preserved axonal transport (as suggested by the maintenance of CGRP skin profiles and of IB4 positive neurons), absence of motor nerve conduction deficit, lack of retrograde axonal dying-back, together with a marked impairment of sensory nerve conduction, and the reversibility of these neurophysiological changes after a resting period of 4 weeks are findings in agreement with functional neuronopathic impairment as the likely cause of BIPN. However, further studies have to be done in order to elucidate which mechanisms are mainly responsible for the neurotoxicity of BTZ treatment. A recent in vitro study, using DRG neuron cultures treated with BTZ, reported that neuronal dysfunction is the result of nuclear accumulation of ubiquitylated proteins and poly(A)RNAs in nuclear granules and a reduction of transcription that leads to a deficit in mRNA translation (Casafont et al., 2010).

In conclusion, the neuropathy induced by BTZ administration to mice, showing similar features to those described in patients with BIPN, can be a useful model to elucidate potential mechanisms underlying the BTZ neurotoxicity, and to test neuroprotective treatments or safeness of new BTZ administration schedules.

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RESEARCH REPORT

Evaluation of pre-existing neuropathy and bortezomib retreatment as risk factors to develop severe neuropathy in a mouse model

Jordi Bruna[1,2,3], Albert Alé[1,2], Roser Velasco[1,2,3], Jessica Jaramillo[1,2], Xavier Navarro[1,2], and Esther Udina[1,2]

[1] *Group of Neuroplasticity and Regeneration, Institute of Neurosciences and Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, Bellaterra, Spain;* [2] *Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Barcelona, Spain; and* [3] *Unit of Neuro-Oncology, Department of Neurology, Hospital Universitari de Bellvitge, Hospitalet de Llobregat, Barcelona, Spain*

Abstract Pre-existing neuropathy, a not uncommon feature in oncologic patients, is a potential but non-confirmed risk factor to develop early or severe chemotherapy-induced neuropathy. The main goal of this study is to evaluate the role of pre-existing neuropathy induced by vincristine (VNC) or bortezomib (BTZ) as a risk factor to develop more severe BTZ-induced neuropathy in a mouse model. VNC, at doses of 1 and 1.5 mg/kg given twice per week for 4 weeks, induced a moderate and severe sensory-motor neuropathy, primarily axonal, with predominant involvement of myelinated sensory axons. The neuropathy induced by BTZ at dose of 1 mg/kg given twice per week for 6 weeks was a mild axonal sensory neuropathy involving myelinated and unmyelinated fibers. The neuropathy in mice previously treated and retreated with the same schedule of BTZ after 4 weeks of washout period was similar in profile and severity to the one observed after the first treatment. When basal neuropathy was classified as moderate (most of BTZ-treated animals) or severe (all VNC-treated animals and two BTZ-treated animals), there was a more marked decline in sensory nerve function during BTZ retreatment in the group with basal severe neuropathy (−86%) than in the groups with basal mild (−57%) or without neuropathy (−52%; $p < 0.001$). Histopathological findings supported the functional results. Therefore, this study shows that the presence of a severe neuropathy previous to treatment with an antitumoral agent, such as BTZ, results in a more marked involvement of peripheral nerves.

Key words: bortezomib, mice, pre-existing neuropathy, vincristine

Introduction

Peripheral neuropathy (PN) has become the principal dose-limiting side effect of many employed chemotherapeutic agents with the advent of hematopoietic growth factors that ameliorate the hematologic toxicity also associated with chemotherapy *(Windebank and Grisold, 2008; Velasco and Bruna, 2010)*. It has been suggested that the pre-existing neuropathy can be a risk factor to develop more severe chemotherapy-induced PN (CIPN) based on clinical experience and few studies *(Chaudhry et al., 2003; Windebank and Grisold, 2008)*. However, in most reported cases, patients had an unsuspected underlying inherited or inflammatory neuropathy *(Graf et al., 1996; Kalfakis et al., 2002; Trobaugh-Lotrario*

Address correspondence to: Dr. Esther Udina, Unitat Fisiologia Mèdica, Facultat de Medicina, Universitat Autònoma de Barcelona, E-08193 Bellaterra, Spain. Tel: +(34)-935811966; Fax: +(34)-935812986; E-mail: esther.udina@uab.cat

et al., 2003). Moreover, indirect evidence usually employed to justify this hypothesis, like prior exposure to neurotoxic agents, presence of diabetes, and alcohol intake, is also scarce or absent *(Thant et al., 1982; Mollman et al., 1988; Van der Hoop et al., 1990; Ramanathan et al., 2010)*. In contrast, it has also been described that patients with pre-existing neuropathy may undergo chemotherapy with neurotoxic agents without experiencing serious exacerbations *(Van den Bent et al., 2002; Goetz et al., 2003)*.

Bortezomib (BTZ) is a proteasome inhibitor used in the treatment of multiple myeloma *(Richardson et al., 2003; San Miguel et al., 2008)*. The relationship of BTZ-induced PN with the presence of a prior neuropathy has been extensively evaluated during the last years, but clinical studies have reported contradictory results *(Richardson et al., 2006; Stubblefield et al., 2006; Badros et al., 2007; Argyriou et al., 2008; El-Cheikh et al., 2008; Lanzani et al., 2008; Corso et al., 2010; Velasco et al., 2010; Dimopoulos et al., 2011)*. Several reasons have contributed to this controversy; first, multiple myeloma itself can cause PN in approximately 11%–54% of patients *(Plasmati et al., 2007; Chaudhry et al., 2003; Richardson et al., 2009)*; second, therapies commonly employed in these patients in the past have neurotoxic profile [thalidomide, vincristine (VNC)]; third, multiple myeloma is usually a disease affecting old patients *(Mohty et al., 2010)*; and last, retreatment with BTZ can be offered to the same patient when favorable responses are observed. The main question in this regard is if patients with pre-existing neuropathy, and thus worst neurologic and neurophysiologic baseline status, will have more severe neurological dysfunction when treated with neurotoxic agents than patients without pre-existing neuropathy. The answer to this question will contribute to optimize the therapies that can be offered to oncologic patients, mainly in the setting of advanced or recurrently treated patients. Furthermore, this knowledge may be useful to extend the findings of clinical trials to the general population.

The first goal of this study is to evaluate the role of pre-existing neuropathy, as a risk factor to develop more severe BTZ-induced PN in mice, using a recently described model *(Bruna et al., 2010)*. Neuropathic involvement of different severity was induced by treating the animals previously with the same schedule of BTZ or with VNC. The study further describes the neurophysiological, histological, and immunohistochemical features of VNC-induced neuropathy in the mouse model.

Materials and Methods

Animal groups, treatment, and dose schedule

Five groups of Swiss OF1 female mice aged 2.5 months were used to assess the different paradigms (Fig. 1). To induce a basal neuropathy, the chemotherapy drug VNC (Vincrisul; Lilly France, Fegersheim, France) was used. Preliminary pilot studies were performed to verify the safety of VNC administration and the development of neuropathic signs in this mouse strain. These preliminary data determined a lethal dose 50 of 1.7 mg/kg intraperitoneally given twice per week (2 pw) for 4 weeks (n = 10). Therefore, we treated two groups of mice with doses of 1 (VNC1, n = 20) and 1.5 mg/kg (VNC1.5, n = 26) 2 pw during 4 weeks to induce a moderate/severe neuropathy, and one-half of the

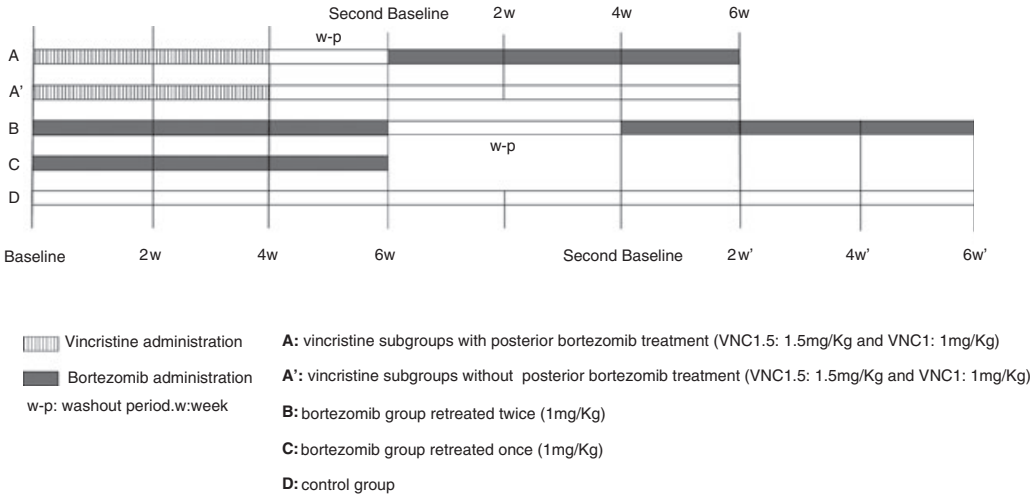

Figure 1. Schematic diagram of treatment and dose schedules that the different experimental groups received. Striped bars represent the period of vincristine treatment, gray bars the period of bortezomib treatment, and white bars are the periods without treatment.

animals were left untreated during two additional weeks before retreatment with BTZ (1 mg/kg 2 pw for 6 weeks, provided by Janssen Pharmaceutical Companies of Johnson & Johnson Pharmaceutical R&D, Beerse, Belgium), whereas the other half was followed with no further treatment until the 16th week.

Another group of animals was treated with BTZ at a dose of 1 mg/kg 2 pw subcutaneously during 6 weeks and left untreated for 4 more weeks before retreatment (BTZ-R, n = 10). Other animals were treated with the same dose of BTZ for only 6 weeks (BTZ, n = 20). In parallel, an untreated group of mice was followed and served as control (CTROL, n = 15).

During the study, animals were housed under standard conditions in cages with soft bedding. The general condition of the animals was assessed daily and body weight was recorded before each drug administration. The experimental procedures were approved by the Ethical Committee of our institution, and followed the rules of the European Communities Council Directive 86/609/EEC.

Functional tests

Functional evaluation was performed at baseline (before the first treatment regimen and the day before the onset of the second treatment regimen) and then every 2 weeks during the periods of treatment (Fig. 1). The tests performed were aimed to quantitatively evaluate motor and sensory nerve conduction, pain sensibility, and integration of sensory-motor function.

Nerve conduction studies

For nerve conduction studies, the sciatic nerve was stimulated percutaneously through a pair of needle electrodes placed at the sciatic notch (proximal site) and at the ankle (distal site). Rectangular electrical pulses (Grass S88 stimulator, Quincy, MA, USA) of 0.01 ms duration were applied up to 25% above the voltage that gave a maximal response *(Navarro et al., 1994; Verdú et al., 1999; Bruna et al., 2010)*. The compound muscle action potentials (CMAPs) were recorded from the tibialis anterior and the third interosseus muscle with microneedle electrodes. Similarly, the sensory compound nerve action potential (SNAP) was recorded by electrodes placed at the fourth toe near the digital nerves. The onset latency and the maximal amplitude of the action potentials were measured and nerve conduction velocity (NCV) of motor and sensory nerve fibers was estimated. During electrophysiological tests, the animals were under anesthesia (pentobarbital 40 mg/kg i.p.) and placed over a warm flat steamer controlled by a hot water circulating pump to maintain the body temperature constant.

To classify the grade of neuropathy induced by the first drug treatment, mice were arbitrarily grouped before the second treatment based on the amplitude reduction of the digital SNAP with respect to the first baseline recording. An amplitude reduction >50% was considered as a criteria of severe neuropathy, whereas a reduction between 40% and 50% was classified as moderate neuropathy.

Pain sensibility tests

The plantar algesimetry technique was used to evaluate the functional status of nociceptive C fibers *(Hargreaves et al., 1988)*. Mice were placed into a plastic box with an elevated glass floor (Plantar Algesimeter, Ugo Basile, Comerio, Italy) and the light of a projection lamp was focused directly onto the plantar surface of one hindpaw. The time to withdrawal of the heated paw was obtained from a time meter coupled with infrared detectors. The value for a test was the mean of three trials separated by 10-min resting periods.

Sensory-motor function

A rotarod apparatus for small rodents (Letica, Panlab, Barcelona, Spain) was used. Mice were placed on the rod, turning at 8 rpm, and the time that each animal remained on it before falling was measured. The value for a test was the mean of three trials separated by 10-min resting intervals. Before treatment, mice were trained for 5 days. The ability to remain on the rotarod for 120 s was taken as an index of normal sensory-motor function *(Verdú et al., 1999)*.

Histological methods

At the end of the studies, all animals were anesthetized and intracardially perfused with paraformaldehyde [4% in phosphate-buffered saline (PBS)]. A segment of the sciatic nerve at mid thigh and of the tibial nerve at the ankle level was removed in each animal. Dorsal root ganglia (DRG) from the fourth lumbar root were also harvested. The samples were fixed in glutaraldehyde–paraformaldehyde (3% : 3%), washed in cacodylate buffer, then post-fixed with 2% osmium tetroxide, dehydrated in graded concentrations of ethanol, and embedded in epon. Light microscopy observations were performed on 0.5 m semithin sections stained with toluidine blue. Myelinated fiber counts were made from systematically selected fields at ×1,000 final magnification, covering a representative part of the cross-sectional nerve profile (at least 15% of the total). The density of myelinated nerve fibers was then derived according to a previously reported method *(Gómez et al., 1996)*. The fiber counts were made at both proximal (sciatic) and distal (tibial) sites using Image J software (NIH, Bethesda, MA, USA). A morphometrical evaluation, including cross-sectional area, axonal counts, myelinated axon and fiber perimeters and diameters, and calculated myelin thickness

was made from systematically selected fields from the sciatic nerve sections using Object Image software (NIH, Bethesda, MA, USA).

On the basis of the light microscopy findings, ultrathin sections were prepared from nerve and DRG selected blocks, counterstained with lead citrate, and examined under a Hitachi 7000 electron microscope (Hitachi, Japan).

Immunohistochemistry

Plantar pads removed from perfused mice after the end of treatments were stored in Zamboni's fixative overnight and thereafter cryoprotected. Cryotome pad sections 40- m-thick were washed free-floating in PBS with 0.3% Triton-X100 and 1% normal goat serum for 1 h, then incubated in primary rabbit antiserum against protein gene product 9.5 (PGP, 1 : 800; AbD Serotec, Dusseldorf, Germany). After washes, sections were incubated in secondary antiserum and processed as described previously *(Navarro et al., 1995)*. Samples were viewed under an epifluorescence microscope using appropriate filters. Five sections from each sample were used to quantify skin pad innervation by analyzing the number and density of epidermal nerve fibers *(Verdú et al., 1999)*.

Statistical analysis

Comparisons among experimental group values in functional tests were made using repeated measures analysis of variance (ANOVA) test, whereas one-way ANOVA was used to assess differences between groups in histological and immunohistochemical results. The Bonferroni test was used as the *post hoc* test in each case. Results are expressed as mean and standard error of the mean. For normalization, the results of functional tests during the study period are expressed as the percentage with respect to baseline values for each mouse. All calculations were performed using the SPSS software package version 15.0 (SPSS Inc.) and graphics using software package GraphPad Prism version 4 (GraphPad Software Inc., La Jolla, CA, USA).

Results

VNC-induced PN

Assessment of general toxicity

VNC treatments showed a moderate toxicity profile. In the VNC1.5 group, body weight was significantly decreased (mean loss of 13%) compared to CTROLs ($p < 0.001$), whereas in the VNC1 group, the mean body weight was stable but did not follow the normal gain of CTROL mice of about 12% ($p = 0.003$). Despite body weight changes, mice did not show prostration or behavioral abnormalities. With the high dose of VNC three mice died (11.5%), whereas with the lower dose only one died (5%) during the 4 weeks of treatment. After 2 weeks of washout period both groups recovered body weight compared to the CTROL group, although another three and two mice died in groups VNC1.5 and VNC1, respectively.

Neurophysiological assessment

The results of motor nerve conduction tests showed that the CMAP amplitude of both tibialis anterior and plantar muscles and the motor NCV were similar in VNC1 and CTROL groups. In contrast, VNC1.5 group suffered a significant decrease in CMAP amplitude in both muscles compared to CTROL (tibialis anterior: $p = 0.033$, plantar: $p = 0.014$) and VNC1 groups (tibialis anterior: $p = 0.001$, plantar: $p < 0.001$; Fig. 2A). The reduction of CMAP amplitudes was more pronounced in the plantar (−37%) than in the tibialis anterior muscle (−16%), indicating a distal to proximal gradient. Motor NCV was also significantly decreased at the end of treatment in the VNC1.5 group compared to the CTROL group ($p = 0.037$; Fig. 2B).

In the sensory nerve conduction tests, the digital SNAP amplitude showed a significant decrease in both VNC-treated groups. At the end of treatment, the SNAP amplitude averaged about 16% ($p < 0.001$) and 21% ($p < 0.001$) of baseline values in the VNC1.5 and VNC1 groups, respectively (Fig. 2C). Sensory NCV was also significantly decreased in distal and proximal segments of the sciatic nerve in VNC1.5 ($p < 0.001$) and VNC1 ($p < 0.001$) groups compared to the CTROL group (Fig. 2D). Likewise, increased latencies of the H waves were also found in treated groups (VNC1.5: $p = 0.002$, VNC1: $p = 0.013$) compared to CTROLs.

During the washout period, there was a recovery of the amplitude of CMAPs of the VNC1.5 group that became closer to normal values after 4 weeks of treatment withdrawal, whereas motor NCV normalized at 2 weeks. In contrast, the SNAP amplitude of the digital nerves in both the VNC-treated groups remained significantly reduced after 10 weeks of follow-up. The sensory NCV recovered to normal values at 8 weeks after treatment in the VNC1 group, but remained lower than normal in the VNC1.5 group (Fig. 2D).

Pain sensibility

Pain sensibility, evaluated by hot thermal algesimetry, was unaffected with VNC treatment during all the follow-up, although there was a non-significant slight increase of the mean withdrawal threshold in the VNC1.5 group at the highest accumulated dose (Fig. 3A).

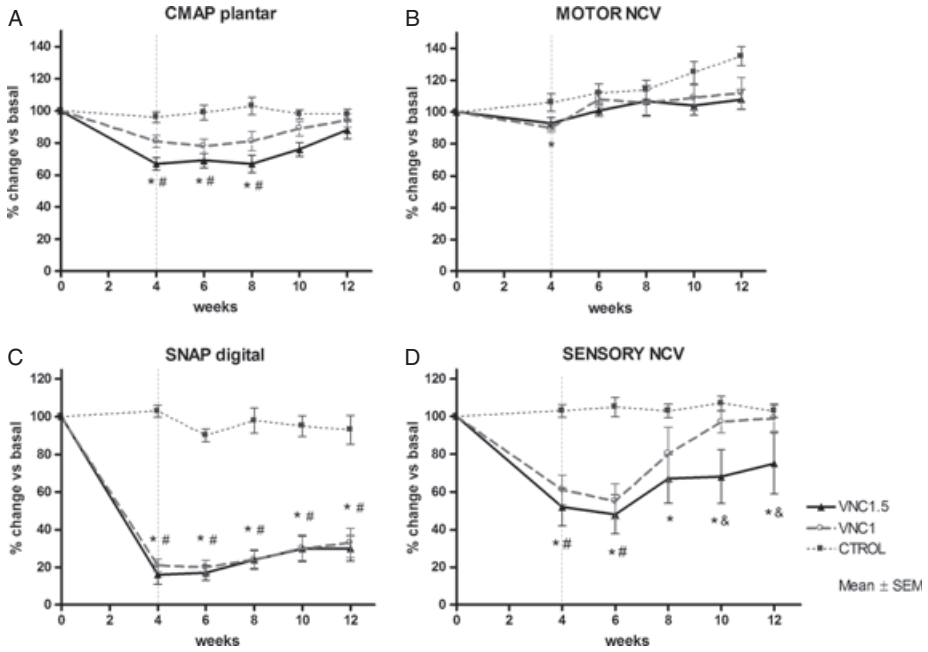

Figure 2. Percentage of amplitude changes vs. baseline of (A) the compound muscle action potential of plantar muscles, (B) motor nerve conduction velocity, (C) sensory compound nerve action potential of digital nerves, and (D) sensory nerve conduction velocity during follow-up of the experimental groups treated with VNC. Animals were treated during 4 weeks with 1 (VNC1) and 1.5 mg/kg (VNC1.5) of VNC and followed-up during 8 more weeks (washout). Values are expressed as percentages with respect to baseline. Error bars: SEM. $^{\#}p < 0.05$ in VNC1 vs. CTROL; $^{*}p < 0.05$ in VNC1.5 vs. CTROL; $^{\&}p < 0.05$ in VNC1.5 vs. VNC1. CTROL, control; VNC, vincristine.

Sensory-motor function

In the rotarod test, VNC-treated mice showed a significant decline in the time of maintenance in the rotating rod at the end of treatment. Animals in the VNC1.5 group were almost unable to maintain the position on the rod ($p < 0.001$) whereas animals in the VNC1 group were less affected ($p < 0.001$), their time of maintenance on the rod about 60% of baseline. After the washout period, the VNC1.5 and VNC1 groups slightly recovered sensory-motor function (+12% and +13%, respectively) but without reaching normal values (Fig. 3B).

Histopathological examination

The sciatic and tibial nerves of VNC-treated mice showed a lower density of myelinated large fibers, with evident signs of Wallerian degeneration and macrophage infiltration in both animal groups, although these signs were more obvious in the VNC1.5 than in the VNC1 group (compare Figs. 4C and 4B, respectively). The estimated counts of myelinated fibers were significantly lower in sciatic nerves of VNC1.5-treated animals when compared to CTROLs ($p = 0.027$; Table 1).

Evaluation of skin innervation

Plantar pads of treated animals did not show obvious signs of skin atrophy. The number and the density of the intraepidermal axonal profiles, labeled by PGP, showed only a significant decrease in the VNC1.5 group ($p = 0.006$) with respect to the CTROL group (Table 1).

PN induced by BTZ and BTZ retreatment

Mice treated with BTZ at 1 mg/kg 2 pw for 6 weeks showed a sensory neuropathy, with a significant decrease in the amplitude of SNAP (−52%, $p = 0.013$) and the conduction velocity of sensory nerve fibers (−17%, $p = 0.049$) without changes in motor conduction tests. Mice retreated with BTZ after 4 weeks of washout presented a similar neurotoxic pattern (reduction of 34% in the amplitude of SNAP: $p = 0.014$ and of 12% in the NCV: $p = 0.03$); thus, there were no differences in the percentage of decline with respect to the corresponding baseline values between first and second treatments (Figs. 5A and 5B). Surprisingly, the impairment of SNAP amplitudes was more marked during first treatment than second treatment, although the trend was not significant ($p = 0.072$).

Pain sensibility showed a progressive increase of the withdrawal threshold during the first treatment followed by recovery during the washout period, and a similar behavior during the second BTZ treatment, although the changes in pain threshold were not significant with respect to CTROLs (Fig. 5C).

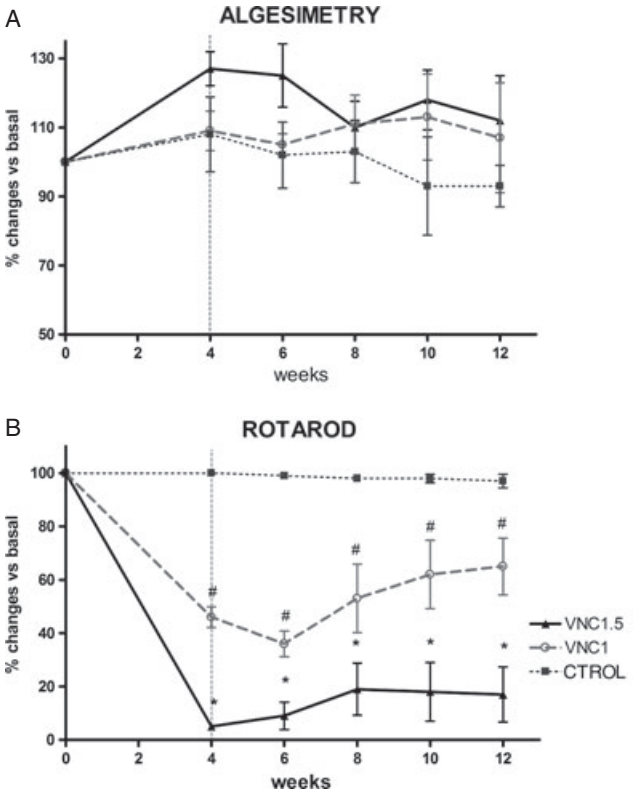

Figure 3. Percentage of changes vs. baseline of (A) algesimetry test results, expressed as time to withdrawal from hot pain stimulation and (B) rotarod test results, expressed as time of maintenance in a rotating rod. Animals were treated during 4 weeks with 1 (VNC1) and 1.5 mg/kg (VNC1.5) of VNC and followed-up during 8 more weeks (washout). Values are expressed as percentages with respect to baseline. Error bars: SEM. *$p < 0.001$ VNC groups vs. CTROL. CTROL, control; VNC, vincristine.

A significant decline in the time of rotarod test was also observed during the first ($p = 0.027$) and the second BTZ treatments ($p = 0.006$), although there were no differences between the two treatment administrations (Fig. 5D).

When analyzing the skin of the paws by immunohistochemistry, we observed a significant loss of intraepidermal fibers in both the animals treated and retreated than in CTROLs ($p < 0.001$; Table 1). Sciatic and distal tibial nerve cross sections showed a preserved architecture in all BTZ-treated animals, with no clear signs of degeneration but a lower density of myelinated fibers than in CTROLs. The reduction in the number of myelinated fibers was of about 12% and 11% at mid thigh (sciatic) and ankle (tibial) levels, respectively, although the decrease only reached significance at the sciatic level (Table 1). The counts of myelinated fibers in animals retreated with BTZ were similar to the ones that received single treatment. Morphometrical analysis showed that the mean area of the axons was significantly

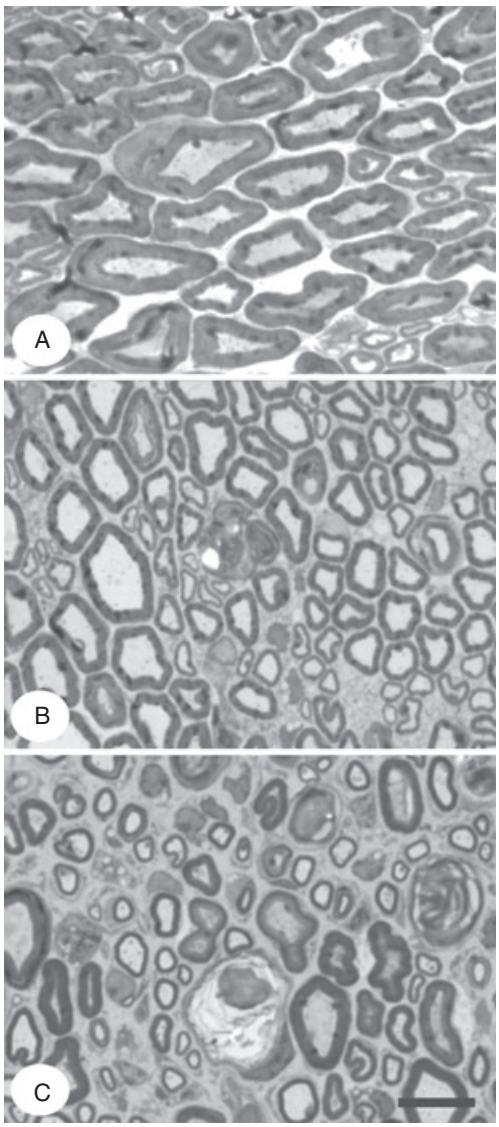

Figure 4. Semithin cross sections of representative sciatic nerves of a control animal (A), an animal treated with 1 mg/kg VNC (B), and one treated with 1.5 mg/kg VNC (C) during 4 weeks. There is a reduction in the density of myelinated fibers, with signs of degeneration after VNC treatment, more marked in the animals that received higher doses. Small axons with thin myelin sheaths, compatible with regenerative clusters, can be observed in treated animals. Bar = 10 m. VNC, vincristine.

reduced in both BTZ (7.6 ± 0.2 m^2) and BTZ-R animals (7.1 ± 0.3 m^2) compared to CTROLs (8.0 ± 0.9 m^2; Table 2). Both treated groups showed a shift to the right in the fiber diameter distribution graph, although more marked in the BTZ-R animals (Fig. 6).

Pre-existing neuropathy as a risk factor to develop BTZ-induced neuropathy

On the basis of pre-defined criteria of SNAP amplitude loss, we grouped the animals depending

Table 1. Counts of myelinated fibers of the sciatic nerve at mid thigh and of the tibial nerve at the ankle level, and analysis of intraepidermal profiles in the plantar skin, immunolabeled against the pan-neuronal marker protein gene product 9.5.

Group	CTROL	VNC1	VNC1.5	BTZ-R	BTZ
Sciatic nerve	4333 ± 58	4156 ± 110	3811 ± 177*	3727 ± 119*	3785 ± 90*
Tibial nerve	1290 ± 65	1291 ± 40	1182 ± 90	1160 ± 36	1130 ± 57
Intraepidermal fibers	23.97 ± 0.27	21.98 ± 0.65	20.25 ± 0.72*	15.83 ± 0.51*	14.76 ± 0.65*

Histological and immunohistochemical analyses were made after 8 weeks of washout period in VNC groups and immediately after completion of BTZ treatment in BTZ and BTZ-R groups. Values are expressed as mean ± SEM.
BTZ, bortezomib; BTZ-R, BTZ retreatment; CTROL, control; VNC, vincristine; VNC1, VNC 1 mg/kg; VNC1.5, VNC 1.5 mg/kg.
*$p < 0.05$ vs. CTROL.

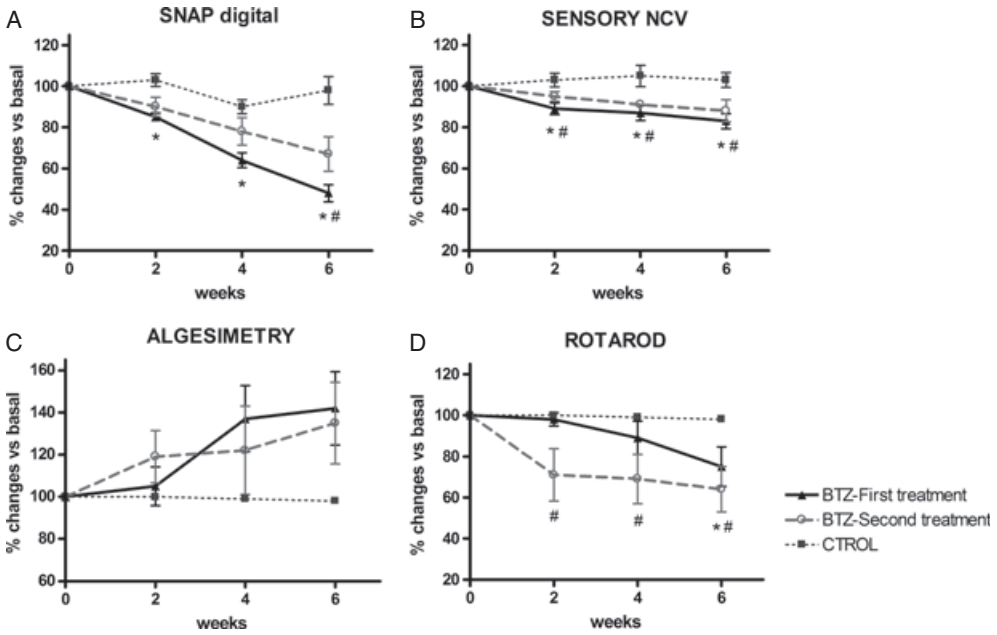

Figure 5. Percentage of changes vs. baseline of (A) sensory compound nerve action potential of digital nerves, (B) sensory nerve conduction velocity, (C) algesimetry test results, and (D) rotarod test results during follow-up in groups of mice treated with BTZ. Animals were treated for 6 weeks with BTZ (BTZ-first treatment group) and retreated during 6 more weeks after a 4-week washout period (BTZ-second treatment group). Values are expressed as percentages with respect to baseline. Error bars: SEM. *$p < 0.05$ BTZ-first treatment vs. CTROL; #$p < 0.05$ BTZ-second treatment vs. CTROL. BTZ, bortezomib; CTROL, control.

Table 2. Morphometrical analysis of myelinated fibers of sciatic nerves from CTROL animals, animals treated with BTZ for 6 weeks, animals retreated with BTZ (BTZ-R), and animals with basal severe neuropathy induced by VNC that received further BTZ treatment (VNC + BTZ).

Group	Fiber area (mm^2)	Axon area (mm^2)	Fiber diameter (mm)	Myelin thickness (mm)
CTROL	29.3 ± 1.5	8.0 ± 0.9	8.4 ± 0.1	1.6 ± 0.03
BTZ	23.0 ± 0.7	7.6 ± 0.2	7.4 ± 0.1	1.3 ± 0.05
BTZ-R	23.3 ± 2.0	7.1 ± 0.3	7.1 ± 0.5	1.3 ± 0.09
VNC + BTZ	21.1 ± 0.9	5.9 ± 0.3	7.1 ± 0.1	1.34 ± 0.02

Values are expressed as mean ± SEM.
BTZ, bortezomib; CTROL, control; VNC, vincristine.

on the severity of their neuropathy at the end of the first treatment period with VNC or BTZ, and we further treated them with BTZ to evaluate the effects of a pre-existing neuropathy as a risk factor to develop BTZ-induced neuropathy. All the animals that had been treated with VNC, both at 1 and 1.5 mg/kg schedules, developed a severe sensory neuropathy (SNAP loss >50%, n = 14), whereas most of the animals treated with BTZ developed a mild neuropathy (SNAP loss between 40% and 50%, n = 7). However, two of the

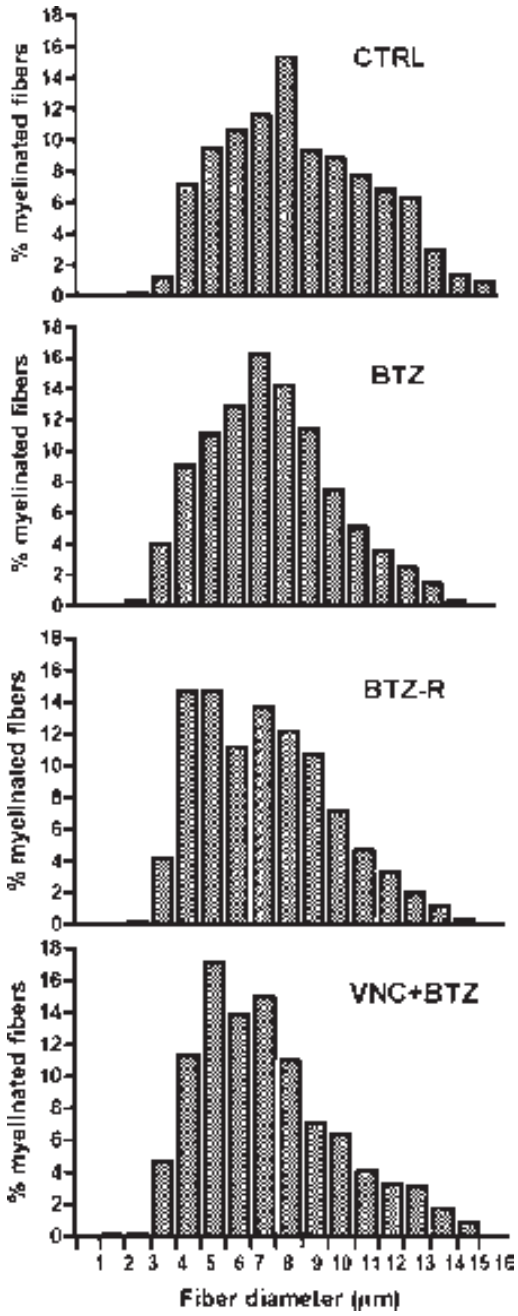

Figure 6. Frequency distribution (percentage) of the diameters of myelinated fibers from sciatic nerves of control animals (CTROL), animals treated with bortezomib (BTZ) for 6 weeks, animals retreated with BTZ (BTZ-R), and animals with basal severe neuropathy-induced by vincristine (VNC) that received further BTZ treatment (VNC + BTZ).

animals treated with BTZ showed a marked decrease in the SNAP amplitude reaching criteria of severe neuropathy. These two animals were thus included in the subgroup with severe neuropathy (final $n = 16$). The group of mice treated once with BTZ ($n = 20$) was used as a group of mice without previous basal neuropathy.

After BTZ treatment, the SNAP amplitude of digital nerves showed a significant decrease in all groups compared to the CTROL group. However, the decline of the SNAP amplitude was significantly more marked in the group with severe neuropathy (86% decline) than in the groups with mild neuropathy (57%) and without basal neuropathy (52% decline, $p < 0.001$). The sensory NCV was also significantly reduced in BTZ-treated animals with basal severe neuropathy when compared to CTROLs and BTZ-treated animals without basal neuropathy ($p = 0.002$; Fig. 6). BTZ-treated mice with basal severe neuropathy also showed a more marked decrease in the average time of maintenance in the rotarod compared to treated animals without pre-existing neuropathy (−67% vs. −22%, $p < 0.001$; Fig. 7D). However, no differences were observed in the algesimetry test between groups. As expected, CMAP swere not affected by the second BTZ treatment because it induces a pure sensory neuropathy. In contrast, previous VNC treatment impaired plantar CMAP amplitude in most of the animals (see above), and after VNC withdrawal, the amplitudes progressively recovered even in animals that received further treatment with BTZ (Fig. 7C).

The histopathological analysis at the end of the second BTZ treatment revealed that animals with basal severe neuropathy had more affected nerves, with lower density of myelinated fibers, marked signs of degeneration, and small clusters of thin myelinated axons compatible with regenerative units (Fig. 8C) than nerves from the groups with basal mild or no neuropathy (Figs. 8A and 8B). The estimated count of sciatic myelinated fibers was lower (3626 ± 251) in the group with severe basal neuropathy than in groups with basal mild neuropathy (4033 ± 240, $p = 0.02$), no pre-existing neuropathy (3898 ± 321, $p = 0.02$), and CTROLs (4333 ± 153, $p = 0.024$, Table 3). The estimated counts of myelinated fibers at the ankle level showed a similar trend but differences were not significant.

Morphometrical analysis of the myelinated fibers showed a significant reduction in the area of fibers and axons in animals with basal neuropathy treated with BTZ compared to CTROLs (29.3 ± 1.5 and 8 ± 0.9 m^2, respectively), although the reduction was more marked in animals with basal severe neuropathy (21 ± 0.9 and 5.9 ± 0.29 m^2) than in animals with basal mild neuropathy (23 ± 2.0 and 7.8 ± 0.8 m^2) or no neuropathy (23 ± 0.7 and 7.6 ± 0.2 m^2; Table 2). Fiber diameter distribution plots showed a shift toward the left in both treated groups with basal neuropathy, with an increased proportion of smaller fibers than in animals without basal neuropathy and in the CTROL group (Fig. 6).

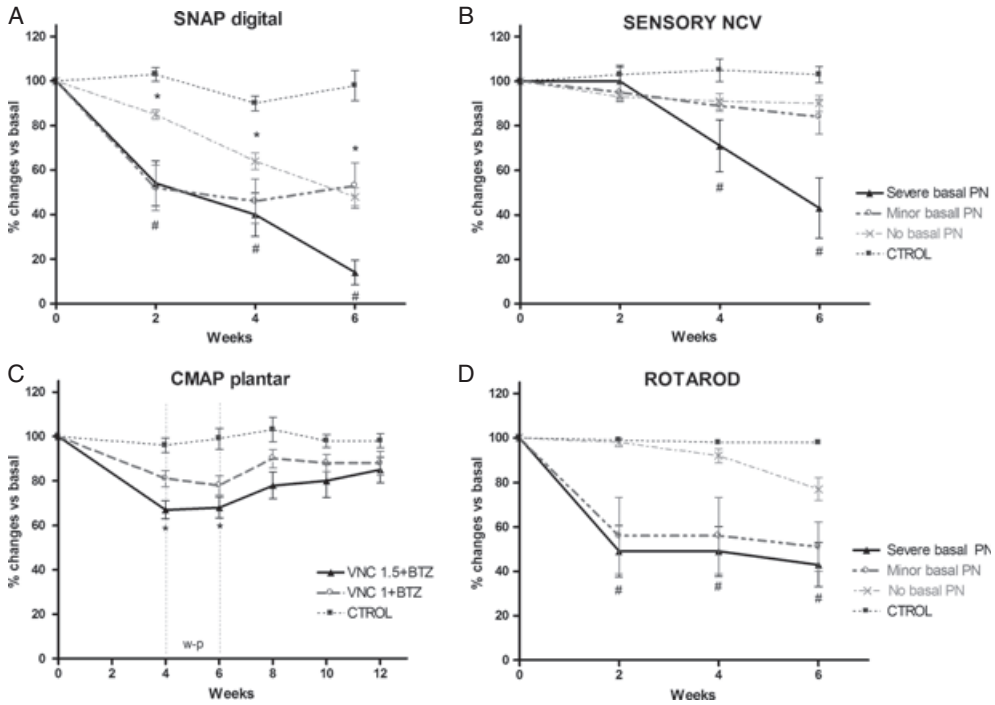

Figure 7. Percentage of changes vs. baseline of (A) SNAP of digital nerves, (B) sensory nerve conduction velocity, (C) compound motor action potential of plantar muscle, and (D) rotarod test results. After VNC or BTZ treatment, mice were classified as basal severe neuropathic (reduced SNAP amplitude >50%) or mild neuropathic (reduced SNAP amplitude between 40% and 50%). A third group was formed with animals without basal neuropathy. The three groups received BTZ treatment during 6 weeks and results are expressed vs. baseline values before the second treatment. BTZ, bortezomib; SNAP; sensory compound nerve action potential; VNC, vincristine.

Inspection of ultrathin sections of DRG under electron microscopy showed a preserved cytoarchitecture, with somas of neurons and satellite cells and clusters of myelinated and unmyelinated fibers regionally grouped, without noticeable differences between treated and CTROL mice (Figs. 9A&C and 9B&D). In BTZ-treated animals with severe neuropathy, the axoplasm of some unmyelinated fibers had vacuoles of large size with accumulation of degradation products (Fig. 9D). In some DRG neurons, large mitochondria with signs of swelling were observed (Figs. 9E vs 9F control), an abnormality that was not found in treated animals with basal mild neuropathy (Fig. 9G). The endosomal membrane system of neurons did not seem altered in any of the treated animals, in spite of their basal state (Fig. 9H), and showed no signs of dilatation or destructuration.

Discussion

In this study, we observed that the presence of a basal severe neuropathy previous to BTZ treatment further deteriorates sensory nerve function, evident by the decline in sensory conduction tests, when compared to treated animals without pre-existing neuropathy. Besides the marked sensory nerve function decline, histopathological findings provide evidence of the more marked toxicity of BTZ in neuropathic mice.

Although it is usually considered that pre-existing neuropathy is a risk factor to develop early or severe CIPN, the issue is currently under discussion. Clarification of this question may be useful to more appropriately select antitumoral drugs and schedules in patients under cancer treatment. Clinical studies addressed to answer this question have some limitations, as they are generally retrospective, based on case reports or on studies with a limited low number of patients *(Badros et al., 2007; El-Cheikh et al., 2008; Lanzani et al., 2008; Velasco et al., 2010)*. Moreover, phase III studies do not provide useful information because oncological trials usually do not use the adequate tools to assess neuropathy *(Cavaletti et al., 2010)*, and inclusion criteria often exclude patients with severe PN *(Richardson et al., 2006; Corso et al., 2010; Dimopoulos et al., 2011)*. For these reasons, we wanted to evaluate this paradigm in a mouse model using graded degrees of basal neuropathy previous to a BTZ treatment schedule that induces a mild sensory neuropathy *(Bruna et al., 2010)*. Moreover, here we confirm the involvement of large sensory

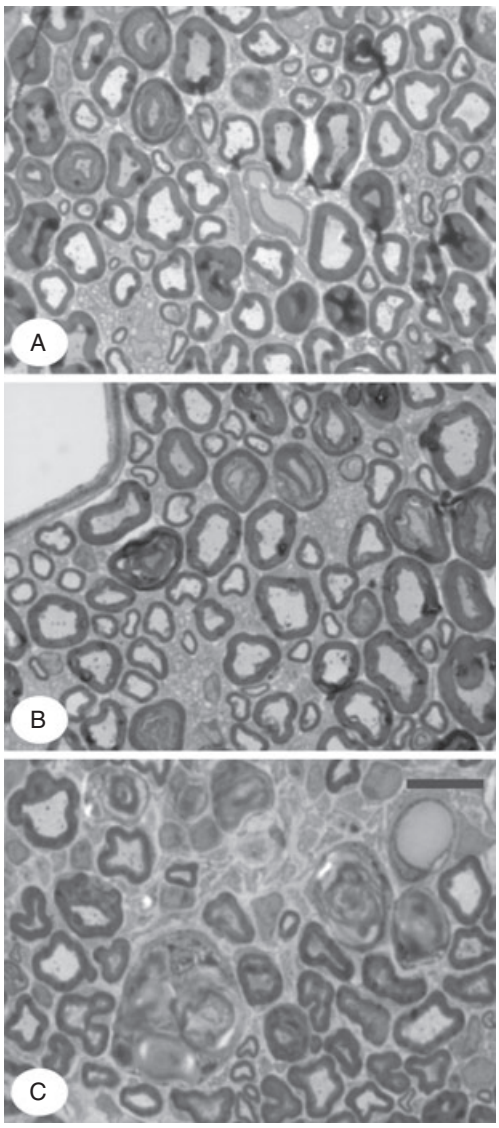

Figure 8. Semithin cross sections of representative sciatic nerves of BTZ-treated mice with no basal neuropathy (A), with mild basal neuropathy (B) and with severe basal neuropathy (C). Animals with severe basal neuropathy, when retreated, presented degenerate profiles and a marked loss of myelinated fibers, whereas nerves of animals with mild basal neuropathy were less affected. Bar = 10 m. BTZ, bortezomib.

fibers and also of small nerve fibers in BTZ-induced PN as described in other experimental studies *(Carozzi et al., 2010; Meregalli et al., 2010)*.

Experimental models of chemotherapy-induced neuropathy

To induce a basal neuropathy, we used two chemotherapy drugs: the classic VNC and the more recently developed BTZ. VNC, a drug used for many years *(Johnson et al., 1963)*, is still included in almost all frontline combined treatments of hematological and lymphatic neoplasms. An increased dose of VNC is associated with better therapeutic outcome *(Carde et al., 1983)*; unfortunately, neuropathy remains the main dose-limiting toxicity. Therefore, it is important to further characterize the neurotoxic effects of this drug and the potential additive neurotoxicity when other chemotherapies are used in combination.

Table 3. Counts of myelinated fibers of the sciatic nerve at mid thigh and of the tibial nerve at the ankle level, and analysis of intraepidermal profiles in the plantar skin, immunolabeled against the pan-neuronal marker protein gene product 9.5.

Group	Severe basal PN (n = 16)	Mild basal PN (n = 7)	Non-basal PN (n = 20)
Sciatic nerve	3626 ± 65	4033 ± 135*	3898 ± 91*
Tibial nerve	1172 ± 38	1092 ± 66	1133 ± 44
Intraepidermal fibers	19.5 ± 1.1	18.5 ± 1.0	19.2 ± 1.2

Mice were grouped according to the basal neurophysiological registers previous to BTZ treatment and were analyzed immediately after completion of BTZ treatment. Values are expressed as mean ± SEM.
BTZ, bortezomib; PN, peripheral neuropathy.
*$p < 0.05$ vs. severe basal PN.

Several animal studies evaluated the effects of VNC on the peripheral nervous system, but most of them focused on the development of neuropathic pain *(Authier et al., 2009)*. These experimental pain models use short treatment periods that usually do not cause the functional impairments characteristic of the chronic neuropathy reported in patients. There are only a few studies that reported electrophysiological, histological, and functional findings in animals treated with prolonged VNC schedules *(Contreras et al., 1997; Authier et al., 2003; Ja'afer et al., 2006; Callizot et al., 2008)*. Most of the animal studies on VNC-induced neuropathy have a limited or incomplete evaluation, both when using short periods and low doses of treatment *(Ogawa et al., 2001; Fukuizumi et al., 2003; Kiguchi et al., 2008; Gauchan et al., 2009)* or longer treatments *(Djaldetti et al., 1996; Contreras et al., 1997; Kamei et al., 2006)*.

In this study, we describe a detailed dose-dependent VNC-induced sensory-motor neuropathy model in mice that has close similarities with the neuropathy observed in human patients *(Casey et al., 1973)*. There was a more marked decrease in sensory than in motor nerve conduction tests suggesting a primarily axonal neuropathy with predominant involvement of sensory axons. Moreover, after an 8-week period of washout, sensory involvement was still present, whereas motor nerve function recovered. Histological examination showed a significant loss of myelinated fibers with signs of Wallerian degeneration.

In contrast to the lesion of large sensory fibers, small sensory fibers were preserved. Therefore, nociceptive functional responses were normal and the density of unmyelinated intraepidermal fibers was only slightly decreased with high doses of VNC (1.5 mg/kg). The involvement of the different types of fibers could be related to a different composition of tubulin isotypes between myelinated sensory, motor, and unmyelinated axons, which provide different susceptibility to the action of VNC. Alternatively, it is also possible that VNC carriers are mainly present in myelinated axons, with a higher distribution in sensory than motor ones that would facilitate VNC access to the axolemma and thus a selective toxicity.

We also used the more recently discovered chemotherapy drug BTZ to induce a basal neuropathy. The present results following a single course of BTZ are in agreement with those previously described in detail in the mouse model of BTZ-induced neuropathy *(Bruna et al., 2010)*. Retreatment with the same schedule of BTZ after 4 weeks of washout period led to a decline in sensory nerve conduction and functional tests similar to that observed when animals were treated for the first time with the drug. In fact, there was a non-significant better tolerance to the drug during the second treatment. Furthermore, the loss of myelinated fibers after the second treatment was similar to that observed in mice with only one BTZ schedule. Morphometrical results were also similar between BTZ and BTZ-R animals, although the group that received twice the drug showed a reduction in the percentage of large myelinated fibers, similar to the one observed in the VNC + BTZ group. When analyzing the ultrastructure of DRG neurons, animals with BTZ retreatment did not show the marked signs of toxicity observed in animals that received BTZ after a VNC treatment. Mitochondrial swelling, a sign described also after treatment with other chemotherapeutic drugs *(Flatters and Bennett, 2006)*, was common in VNC–BTZ-treated mice but not seen in BTZ-retreated animals (Fig. 8). Our observations indicate that retreatment with BTZ does not cause severe neuropathy in the mouse model. Thus, this study supports the findings of a few clinical reports in which retreatment of patients with relapsing or refractory multiple myeloma was explored *(Berenson et al., 2005; Wolf et al., 2008; Sood et al., 2009)*.

Pre-existing PN as a risk factor to develop BTZ-induced neuropathy

As the pathogenesis of BTZ-induced PN is largely unknown, it is difficult to establish the reasons that

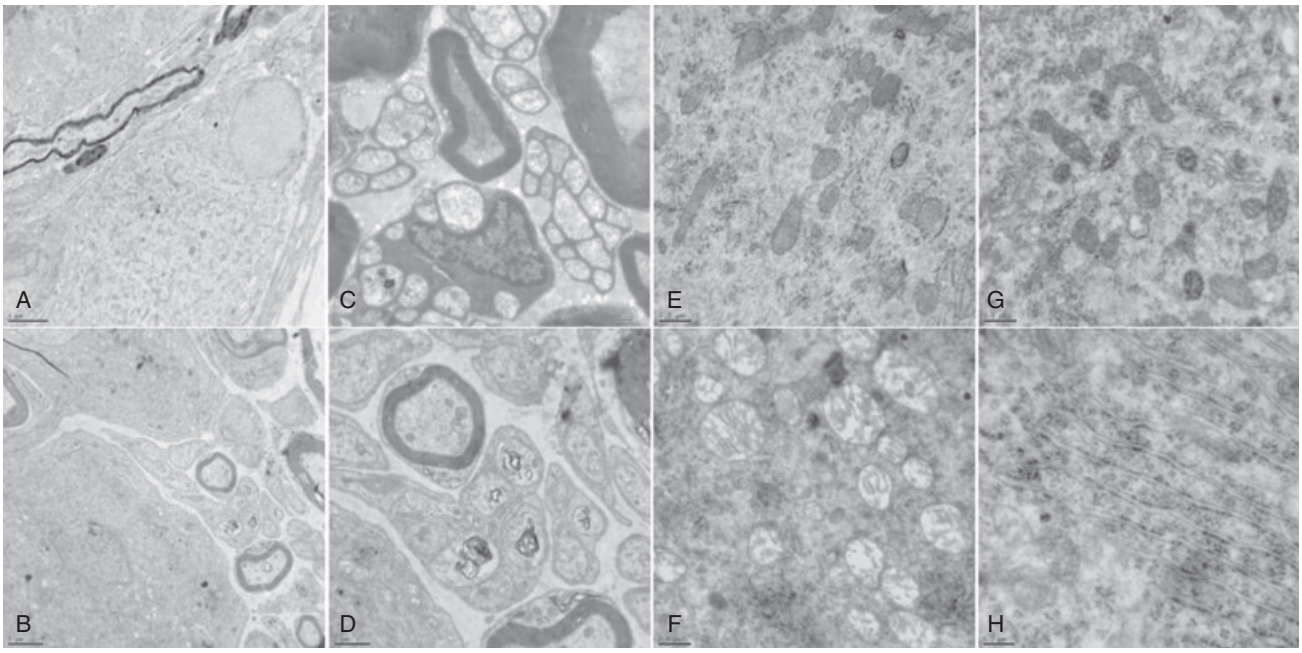

Figure 9. Ultrathin cross sections of DRGs from an intact animal (A, C, D), and animals treated with BTZ with basal mild neuropathy (BTZ retreatment, E) or severe neuropathy (VNC-BTZ treatment, B, D, F, G). Low magnification view (×3,000) of an intact DRG (A) and a DRG from a BTZ-treated animal with severe basal neuropathy (B). In the axoplasm of some unmyelinated fibers, there are vacuoles with accumulation of degradation products (×6,000 in D) not observed in intact unmyelinated fibers (C). Mitochondria were larger and swollen (×20,000 in F), compared to mitochondria of control animals (E) or BTZ-treated animals with basal minor neuropathy (G). The endosomal membrane system from neurons of BTZ-treated animals with basal neuropathy, such as the rough reticule, had a normal appearance without signs of dilatation (H, ×40,000). BTZ, bortezomib; DRG, dorsal root ganglia; VNC, vincristine.

can explain why a previous severe neuropathic state is a risk factor to develop more severe neurotoxicity. Nevertheless, it is worth to note that this increased neurotoxicity is specific for the type of neurons affected. Thus, we found that BTZ treatment, which specifically affected sensory fibers, did not interfere with recovery of motor nerve function, impaired by previous treatment with VNC. The characteristic pure sensory neuropathy induced by BTZ treatment *(Bruna et al., 2010)* suggests that the drug is mainly affecting DRG neurons, although other experimental studies reported both neuronal *(Casafont et al., 2009; Meregalli et al., 2010)* and glial *(Cavaletti et al., 2007; Watanabe et al., 2010)* affectation by BTZ. The neurotoxicity of BTZ has been linked to its interference with the pleiotropic effects of the nuclear factor kappa B (NF-kB) pathway *(Panwalkar et al., 2004)*, in the transcription and mRNA processing *(Casafont et al., 2009)* with the consequent decrease of neurotrophic factors, or in microtubule stabilization *(Poruchynsky et al., 2008)*. The decreased source of trophic factors or destabilization of microtubule dynamics might be more important for the survival of neurons that were under regeneration or metabolic stress than of neurons under physiological conditions, a fact that could explain why animals with basal previous neuropathy were particularly susceptible to develop severe BTZ-induced neuropathy.

In conclusion, pre-existing severe neuropathy is a risk factor to develop peripheral nerve dysfunction during BTZ treatment, whereas BTZ retreatment does not imply an added risk after a washout period to allow recovery of nerve function. These results reinforce the concept that patients with pre-existing PN should be closely monitored when subjected to chemotherapy.

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3

USEFULNESS OF IMMUNOHISTOCHEMICAL ANALYSIS OF SKIN BIOPSY FOR THE EARLY DIAGNOSIS OF BORTEZOMIB-INDUCED PERIPHERAL NEUROPATHY

J. Bruna[1,2], **A. Alé**[1], **E. Udina**[1], **X. Navarro**[1]

[1] Institute of Neurosciences and Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona, and Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), Bellaterra, Spain.

[2] Unit of Neuro-Oncology, Department of Neurology, Hospital Universitari de Bellvitge, Hospitalet de Llobregat, Spain.

Abstract

Peripheral neuropathy (PN) is a dose-limiting adverse event of bortezomib treatment. Conventional electrophysiological techniques identify the PN late, once it is established.

The aim of this pilot study is to evaluate the usefulness of the longitudinal assessment of skin biopsies to predict the development of bortezomib-induced PN and to extend the neuropathy characterization in a mouse model. During bortezomib administration, 1 mg/Kg/twice per week for 6 weeks, 20% and 60% of mice developed severe PN at 4 and 6 weeks of treatment respectively, according nerve conduction studies. In all treated mice, the intraepidermal nerve fibers (IENF) and the innervated Meissner corpuscles showed a decrease during the bortezomib administration, from second ($p<0.001$) and fourth ($p=0.001$) week respectively. Conversely, the decrease in the IENF was correlated with the increase in the number of Langerhans cells ($p=0.037$, $r=-0.9$). The sweat gland innervation did not show changes along the treatment with regard the control group. The decrease in the relative percentage of IENF during the second week was greater than the sensory nerve action potential (SNAP) amplitude decrease ($p=0.05$). No significant differences were observed between groups with regard to the neuropathy grade and the evaluated immunohistochemical skin parameters. Moreover, the reduction of SNAP amplitude during the treatment was not significantly correlated with the decrease of the IENF neither the other skin parameters. Therefore, small unmyelinated nerve fiber involvement is an early event in the bortezomib-induced neuropathy. However, this abnormality is not useful to predict the development of severe neuropathy of medium and large myelinated fibers.

Key words: bortezomib, immunohistochemistry, intraepidermal nerve fibers, neuropathy, mouse, Meissner, Langerhans cells

INTRODUCTION

Peripheral neuropathy (PN) is a dose-limiting adverse event of most chemotherapeutic drugs. The chemotherapy neurotoxic side effect may be permanent with possible negative impact on therapeutic outcome, thus compromising the survival and also negatively influencing the quality of life of cancer patients (Argyriou 12, Velasco 10, Cavaletti 10). Several factors have been identified as risk factors to develop chemotherapy-induced PN (CIPN) under different cytostatic therapy schedules, like subclinical neuropathy, combination regimens with several neurotoxic drugs, being the total accumulated dose the main one (Argyriou 12, Velasco 10, Windebank 08).

Nevertheless, physicians have not yet available a clinical routine tool to predict which patients under treatment are in high risk to develop symptomatic CIPN and consequently, adapt or modify the chemotherapy schedule. Careful clinical neurological monitoring was demonstrated useful for detection of early neuropathy (Velasco 10b), although it has not been evaluated in large patient series and may be subject to variable criteria between physicians. In addition, conventional electrophysiological techniques (i.e. nerve conduction tests) identify the CIPN relatively late, once it is well established. On the other hand, the evaluation of intraepidermal and dermal nerve fibers in skin biopsies provides an objective and sensitive method to diagnose early and established PN, especially when it affects small nerve fibers (England 09, Lauria 10, Lauria 11, Devigli 08). However, this technique has not been extensively used in patient cohorts under chemotherapy treatments, their predictive value is unknown and the involvement of small fiber in CIPN has only been described for a few chemotherapeutic drugs (Richardson 09, Burakgazi 11).

Bortezomib is the keystone for treatment of myeloma multiple and mantle lymphoma. However, it causes PN, that has been well characterized in the clinic as well as in experimental models (Richardson 09, Bruna 10, Bruna 11, Carozzi 10), and is the main dose-limiting adverse event (Cavaletti 10, Chari 10). CIPN animal models have demonstrated their usefulness to understand the pathophysiology, to fully characterize the type of involvement, and to test clinical paradigms related with these neuropathies (Bruna 11, Bruna 10, Cavaletti 08). The aim of this pilot study is to evaluate the usefulness of the longitudinal assessment of skin biopsies to predict the development of bortezomib-induced PN and to extend the characterization of the type of nerve fibers involved in the bortezomib-induced PN mouse model.

MATERIAL AND METHODS

Animals, treatment and follow-up

Two groups of Swiss OF1 female mice aged 2.5 months were used. The first group received bortezomib (provided by Millennium Pharmaceuticals Inc., and Johnson & Johnson Pharmaceutical Research & Development, L.L.C.), subcutaneously, at dose of 1 mg/Kg twice per week during 6 weeks to induce PN (BTZ group, n=10), as previously reported (Bruna 10). A second group, used as control, only received vehicle solution (n=5).

Electrophysiological studies were performed at baseline, before starting bortezomib administration, and then every two weeks during treatment. Skin biopsies were obtained after the electrophysiological assessment at the 2nd and 4rd week of treatment from distal plantar pads of right and left hindpaws, respectively.

The animals were housed under standard conditions in cages with soft bedding. The general condition of the animals was assessed daily and body weight was recorded before each bortezomib administration. The experimental procedures were approved by the Ethical Committee of our institution, and followed the rules of the European Communities Council Directive 86/609/EEC.

Nerve conduction studies

For nerve conduction studies, the sciatic nerve was stimulated percutaneously through a pair of needle electrodes placed at the sciatic notch. Rectangular electrical pulses of 0.01 ms duration were applied up to 25% above the voltage that gave a maximal response (Navarro 94, Verdu 99, Bruna 10). Sensory compound nerve action potentials (SNAP) were recorded by microelectrodes placed at the fourth toe near the digital nerves. Latencies and amplitudes of the action potentials were measured. During electrophysiological tests, the animals were anesthetized (pentobarbital 40 mg/kg i.p.) and placed over a warm flat steamer controlled by a hot water circulating pump to maintain the body temperature constant. A SNAP amplitude decrease $\geq$ 50% with respect to basal values during bortezomib treatment was the criterion used to identify mice with severe neuropathy.

Immunohistochemistry of skin samples

Plantar pads were stored in Zamboni's fixative overnight and thereafter cryoprotected. Cryotome pad sections of 70 μm thickness were washed free-floating in PBS with 0.3% Triton-X100 and 1% normal goat serum for 1 h, then incubated with rabbit antibody against protein gene product 9.5 (PGP, 1:1000; Ultraclone) and in goat antibody against Langerin (1:100, Santa Cruz). After washes, sections were incubated in secondary goat anti-rabbit Cy3 and Cy5 labeled IgG (1:200; Jackson Immunoresearch) overnight at 4°C, and processed as described previously (Navarro 95, Verdu 99). Samples were viewed under an epifluorescence microscope using

appropriate filters and analysis were made by an observer blind to the animal's group assignment and the extraction date.

Intraepidermal nerve fiber (IENF) quantification was performed in at least 4 sections from each sample by analyzing the number and the density nerve fibers present in the epidermis of the lateral side of the plantar pad (Verdu 99, Lauria 10).

To evaluate the degree of sweat gland innervation, a measurement of gland immunoreactivity (PGP 9.5) was done using a ROI (region of interest) placed on the sweet glands (Vilches 02). At least 4 images were taken at 20x magnification, and the integrated density and the percentage of labeled area (relation between the number of labeled and unlabeled pixels in a ROI) were quantified with ImageJ software.

The same sections used to quantify IENF were used to identify the number of innervated Meissner receptors in the intersection between epidermis and dermis, being most of them located in the dermal buds. The complete epidermal-dermal interface length at each pad section was measured using Image J software, and the Meissner density, total number of receptors with regard to length, was calculated.

The number of Langerhans cells (LCs) present in the same sections was counted at 20x magnification. LC counts were made in at least four sections from each pad, and expressed as the total number of cells per 0.05 mm^2 (500X100 µm) of epidermis.

Data analysis

Results are expressed as mean and standard deviation. To compare the changes between different variables, the results were normalized as the percentage with respect to baseline values per each animal. Comparisons between groups were made using one way ANOVA, using Bonferroni test as *post hoc* at each assessment time point of the follow-up. To assess the predictive value of the skin innervation evaluated parameters, these were compared using non-parametric U Mann Whitney test with regard to the severity of neuropathy categorization according to nerve conduction assessment. The correlation between neurophysiological results and immunohistochemical skin parameters was examined with the Spearman's rho correlation coefficient. All calculations were performed using the SPSS software package version 12.0 (SPSS Inc.) and graphics using GraphPad Prism version 4 (GraphPad Software, Inc.).

RESULTS

Table 1 and figures 1 and 2 summarize the evaluated skin parameters between mice groups and their neuropathy grade.

Table 1. Values of skin immunohistochemical parameters classified according to the severity of the SNAP amplitude decrease.

	2 weeks		4 weeks		Control
SNAP decrease	<50%	≥50%	<50%	≥50%	
IENF	14.8±1.3	17.8±5.9	11.8±3.9	13.5±3.9	21.1±4.0
Meissner	2.7±1.1	2.0±1.2	2.7±0.7	1.9±1.1	2.8±0.7
SG	30.2±8.6	33.8±3.2	27.1±15.7	28.5±6.7	25.9±2.7
Langerhan cells	48.9±10.1	49.2±9.2	37.6±6.6	33.0±18.7	48.3±5.6

Number of intraepidermal nerve fibers (IENF) per 1 mm length, density of innervated Meissner corpuscles (number per μm), percentage of sweat gland (SG) area immunoreactive against PGP9.5, and number of Langerhan cells (in 0.05mm^2) during the follow-up. Sensory nerve action potentials (SNAP) amplitude categorization corresponds to values recorded two weeks later with regard to skin sampling. Values expressed as mean ± standard deviation

Sensory nerve conduction

The SNAP amplitude of digital nerves in the BTZ group showed a progressive decrease compared to the control group from 2 weeks of follow-up. At the sixth week of treatment the SNAP amplitude averaged 44% of baseline values ($p<0.001$). During treatment, the number of mice that fulfilled the severe neuropathy criterion was none at 2 weeks, 2 (20%) at 4 weeks, and, 6 (60%) at the end of treatment.

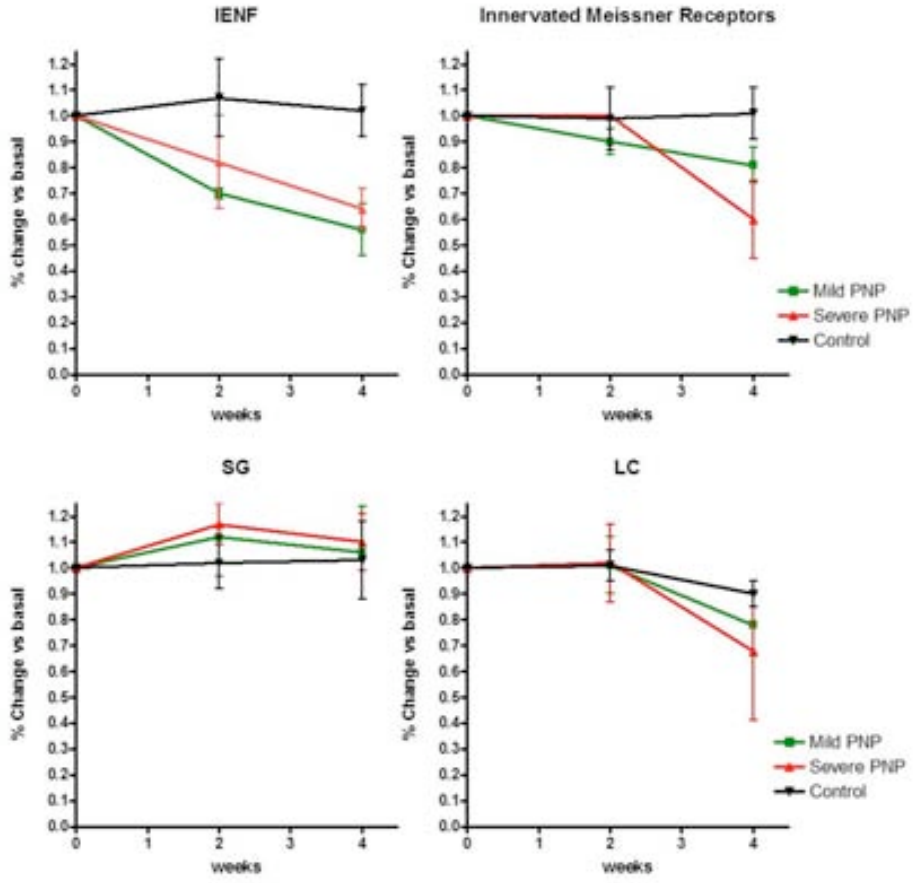
Intra and subepidermal innervation

The number and density of IENF also showed a progressive decrease during the bortezomib treatment with respect to control values ($p<0.001$) (Fig. 3). However, the decrease was more pronounced at the second week of follow-up than during the next two weeks of treatment, and the absolute normalized decrease was greater for the IENF than for the SNAP amplitude at the second week of treatment ($p=0.05$) (Fig. 1). Nevertheless, no significant differences were observed between groups with regard to the neuropathy grade (Table 1).

The density of Meissner corpuscles innervated showed a similar decline with regard the control group ($p=0.001$), although the decrease was observed from the fourth week of treatment (Figs. 1, 2). Despite a trend to show lower number of innervated corpuscles in the less severe

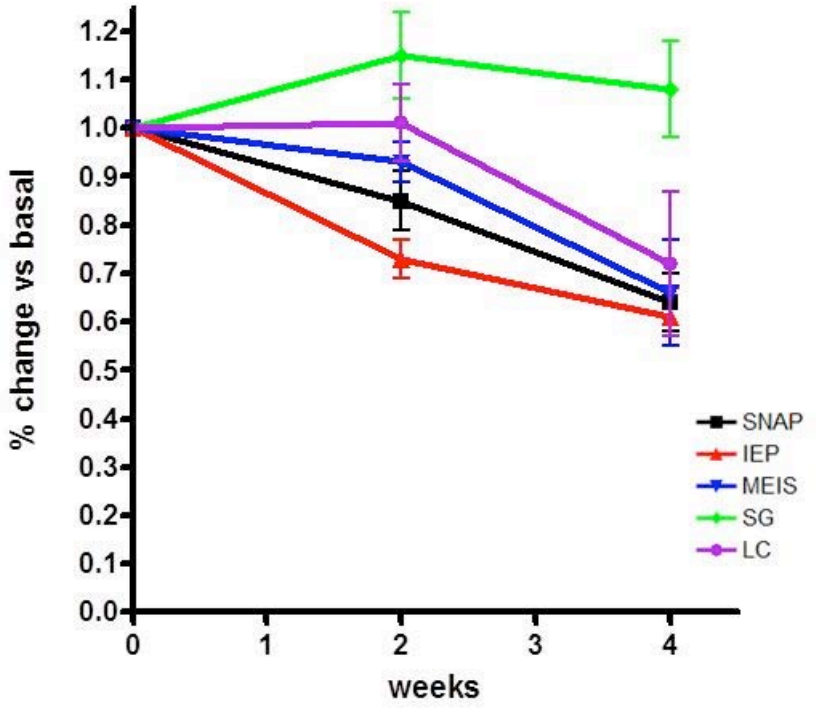
neuropathy subgroup, the differences were not significant (Table 1). Comparing the percentage of decrease between IENF and Meissner corpuscles innervation, the former was more accentuated during the first two weeks (p=0.037), but there were no differences between groups at the fourth week of treatment.

Figure 1. Immunohistochemical parameters during the bortezomib treatment in groups of mice divided according to the severity of the neuropathy developed at the end of the treatment.



IENF: intraepidermal nerve fibers. PNP: peripheral neuropathy. SG: sweat gland innervation. LC: Langerhans cells. Values expressed as percentages with respect to baseline (mean± standard deviation).

Figure 2. Evolution of immunohistochemical parameters in comparison with nerve conduction parameters during bortezomib treatment.



IENF: intraepidermal nerve fibers. SNAP: sensory nerve action potentials. SG: sweat gland innervation. LC: Langerhans cells.

Sweat gland innervation

The immunoreactivity intensity measured through the integrated density and the percentage of sweat gland surface labeled for PGP9.5 did not show significant differences during the follow-up between control and treated groups.

Langerhans cells

The LC density was similar between groups during the first two weeks. However, at four weeks all treated mice presented a decrease in the density of labeled LC without significant differences between the neuropathy grades and control group (Fig 4.).

Relationship between skin innervation parameters and nerve conduction results

When comparing the normalized decline of each parameter evaluated along the bortezomib treatment, the IENF number showed the most marked reduction, followed by the amplitude of the SNAP (Fig. 2), whereas the innervation of the sweat glands was the least affected. These observations point out to a marked involvement of sensory neurons, either of large or small size, with preservation of sympathetic neurons.

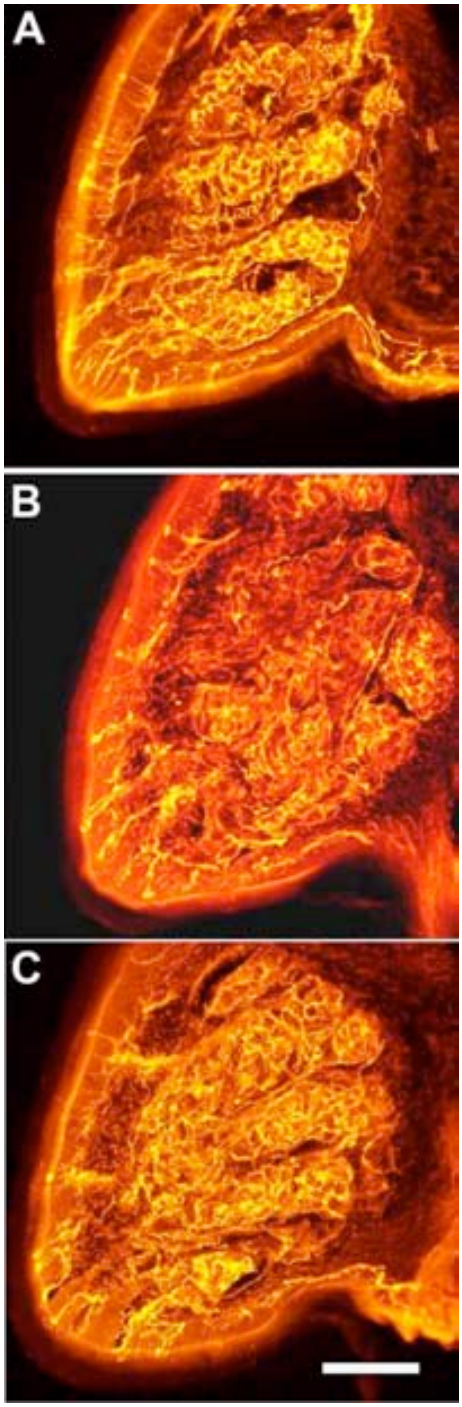


Fig. 3. Representative micrographs of immunohistochemical labeling against PGP of skin pad samples from a mice control (A), and a mice treated with bortezomib at 2 (B) and 4 (C) weeks. A progressive decrease in the IENF and Meissner corpuscles can be observed in treated animals. Bar = 200 µm

The decrease in the number of IENF was not significantly correlated with the reduction of SNAP amplitude during the treatment. The other immunohistochemical skin parameters were not correlated with nerve conduction neither at two nor at four weeks of bortezomib treatment. On the other hand, the decrease in the number of IENF was correlated with the Meissner corpuscles denervation ($p=0.004$, $r=0.85$) and with the increase in number of LC ($p=0.037$, $r=-0.9$).

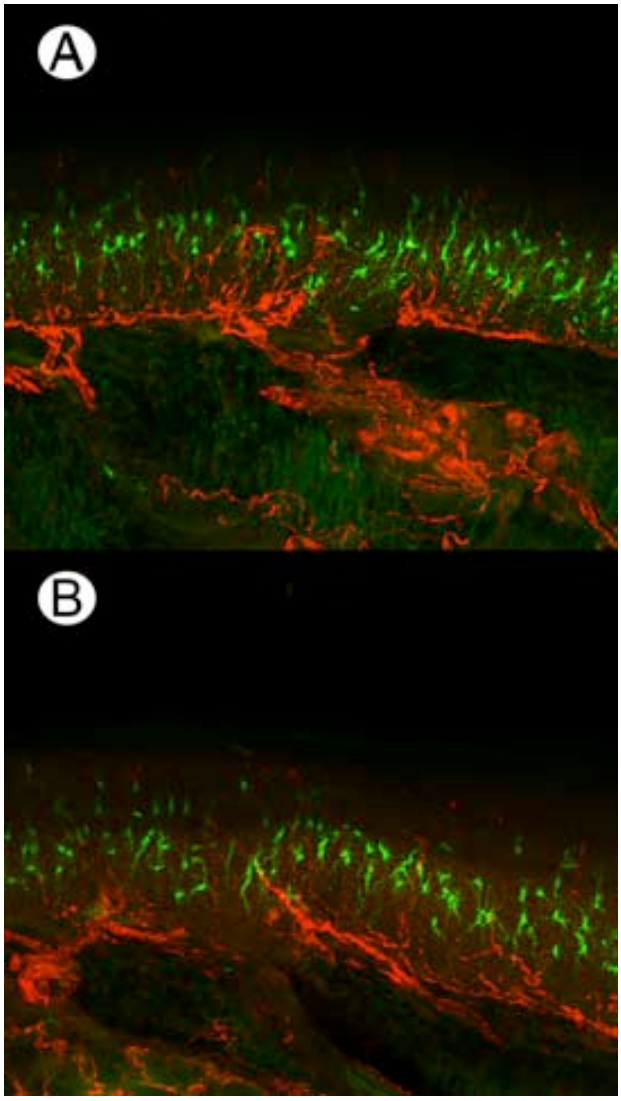


Figure 4. Confocal images of PGP9.5 (in red) and langerin (in green)-stained intraepidermal nerve fibers in the hind skin paw. Images from a representative control animal (A) and an animal treated with BTZ for 4 weeks (B). The number of immunoreactive langerin positive cells is cosiderably reduced in footpads of treated animals

DISCUSION

Early predictors of BTZ-induced neuropathy

This bortezomib-induced neuropathy mouse model is a well validated experimental model, in which an initial dysfunction at the sensory neuronal level is followed by secondary pathology affecting axons and myelin (Bruna 10). This exploratory study shows that the intraepidermal nerve fiber loss is an early feature during bortezomib treatment, showing a normalized IENF decrease greater than the reduction of SNAP amplitude observed after two weeks of treatment in the mouse. However, the loss of unmyelinated IENF neither predicted the severity of myelinated nerve fiber involvement assessed by nerve conduction tests nor it was correlated with the SNAP amplitude decline. This unexpected feature may indicate a different natural history of myelinated and unmyelinated fibers neuropathy induced by the same neurotoxic drug, suggesting a variable toxic tolerance between small and medium-large sensory

neurons (Perry 04). Therefore, although immunohistochemical analysis of skin biopsy is an easy and sensitive method to diagnose small fiber neuropathies, its use as an early diagnostic tool to predict the severity in toxic neuropathies is limited according to our results, at least in bortezomib treatment. On the other hand, the other immunohistochemical parameters evaluated in this study, Meissner corpuscles innervation, sweat gland innervation and LC counting, showed a later decrease in the follow-up than intraepidermal innervation. Therefore, they are not useful predictors of severity of CIPN.

However, it could be arguable the criterion of severe neuropathy used, although this criterion has been used in previous reports (Bruna 10, Bruna 11), since it directly reflects only the involvement of large sensory fibers. The small groups' size of this exploratory study may also limit the strength to evaluate the usefulness of early neuropathy predictors. However, the lack of correlation between SNAP values, independently of the neuropathy categorization grade, with the assessed immunohistochemical skin parameters, does not support the need of further extended studies that attempt to find early predictors.

Extended characterization of small myelinated and unmyelinated BTZ-induced neuropathy

The first event observed in bortezomib-induced neuropathy is the involvement of unmyelinated IENF that appeared before the loss of distal myelinated fibers, measured by SNAP amplitude of digital nerves and the counting of innervated Meissner corpuscles. Conversely, loss of IENF is corroborated by the significant correlation with the initial increase in the epidermal layers of LC, previously showed in other peripheral neurodegenerative conditions (Stankovic 99, Lauria 05) and in toxic neuropathies (Jin 08, Siau 06). This feature may indicate an involvement of LC in the axonal degeneration process. On the other hand, we did not observe autonomic nerve involvement by immunohistochemical analysis of SG innervation, corroborating our previously published findings (Bruna 10).

In conclusion, small unmyelinated nerve fiber involvement is an early event in the neuropathy induced by bortezomib administration. However, this abnormality is not useful to predict the development of severe neuropathy of medium and large myelinated fibers. These findings indicate the convenience of performing skin biopsy analyses in conjunction with nerve conduction tests regularly in patients receiving chemotherapy in order to detect the early development of PN.

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DISCUSIÓ GENERAL

El model animal descrit en aquest treball reprodueix les troballes neurofisiològiques observades en els pacients tractats amb bortezomib i és, per tant, una bona eina per a testar potencials tractaments neuroprotectors i per aprofundir en el coneixement etiopatogènic d'aquesta neuropatia. Per altra banda, també ha permés explorar diferents paradigmes clínics d'utilitat pràctica mèdica, així com analitzar comparativament la sensibilitat de diferents mètodes d'estudi de l'afectació de diferents tipus de fibres nervioses.

El model de NIB descrit presenta una gran versemblança amb la neuropatia observada en pacients. Malgrat que la distància filogènica que separa el ratolí de l'humà no permet posologies d'administració similars, s'ha aconseguit induir la neuropatia utilitzant dosis acumulades equivalents alomètricament, al contrari del que succeeix en alguns models publicats d'altres NIQ (Authier et al. 2003). La neuropatia induïda és purament sensitiva, afectant-se les amplituds dels PANCS, sense repercussió en les conduccions motores. Posteriorment, i amb menor intensitat, s'alteren les velocitats de conducció sensitives. Per altra banda, a nivell histològic s'observa una major pèrdua d'axons a nivell proximal (ciàtic) que no pas distal (tibial), fet que aniria en contra d'una degeneració tipus *dying-back*. Aquestes troballes, juntament amb la pèrdua de fibres amielíniques i la reducció de les fibres sensorials intraepidèrmiques, suggereixen que la NIB és, de fet, una ganglionopatia sensitiva pura. L'anàlisi immunohistoquímica demostra una manca de correlació entre l'afectació de les fibres petites amielíniques distals i la disfunció de les fibres sensorial gruixudes, reflexada en la reducció d'amplitud del PANCS. Aquesta troballa indica que existeix una afectació diferencial de les neurones sensorials grans i petites pel mateix agent neurotòxic, suggerint una variable tolerància entre elles, com també s'ha demostrat en altres neuropaties tòxiques (Perry et al. 2004).

Respecte al correlat amb la neuropatia descrita en pacients, restaria per determinar el grau d'afectació autonòmica i l'existència de dolor neuropàtic descrits en un nombre no menyspreable de pacients. En quan a la disautonomia, en el nostre model no s'observen alteracions en el grau de variabilitat en la despolarització del node sinusal cardíac dels ratolins, però cal tenir en compte que la tècnica utilitzada per a la seva valoració no és l'òptima i, per exemple, no va permetre induir respostes reflexes cardiovasculars amb maniobres de Valsalva. En alguns animals (15%) s'observa cert grau de denervació de les glàndules sudorípares, però no hi ha diferències significatives amb animals intactes si es

valora el conjunt d'animals tractats. Cal tenir en compte, però, la important capacitat de ramificació col·lateral, mantenint nivells d'innervació tissular quasi normals amb la denervació parcial, de les fibres simpàtiques sudomotores (Navarro et al. 1988; Verdú et al. 1999). Ara bé, els principals símptomes disautonòmics descrits en pacients són la hipotensió ortoestàtica i la constipació, difícils de valorar en els models animals. Per tant, l'absència d'alteracions significatives en les proves de valoració de la funció autonòmica realitzades en aquest model no permeten detectar l'existència d'una neuropatia autonòmica de predomini entèric o vasomotor. Respecte al dolor neuropàtic, malgrat la demostració d'afectació de fibres nociceptives amielíniques, no s'ha demostrat l'existència d'hiperalgèsia durant el tractament. Hipotètiques explicacions d'aquesta absència podrien estar lligades a les diferències en les dosis i a la via d'administració o al fet que les proves algesimètriques es realitzessin cada dues setmanes. No es pot descartar, de tota manera, que en els primers dies del tractament es puguin trobar respostes d'hiperalgèsia, que en fases posteriors quedin compensades per la pèrdua d'innervació cutània. Al mateix temps, manquen estudis clínics exhaustius sobre la història natural i el moment d'aparició del dolor neuropàtic en els pacients. Malauradament, aquest model no sembla útil per a l'exploració de teràpies analgèsiques per al tractament del dolor neuropàtic secundari a la NIB. No obstant aquestes petites diferències, les similituds entre el model murí desenvolupat i les troballes realitzades en una cohort de pacients tractats i seguits prospectivament, comunicades en el congrés de l'*American Society of Hematology* (Richardson, Bruna et al. 2010), són sòlides i confirmen la validesa d'aquest model.

Al mateix temps, els estudis histològics i immunohistoquímics de caracterització del model de NIB aporten certa informació que pot ser d'utilitat en la recerca futura de mecanismes etiopatogènics subjacents a aquesta neuropatia. A la dosis testada, l'administració de bortezomib produeix una repercussió funcional i electrofisiològica comparativament major que el dany estructural a nivell axònic i somàtic neuronal, suggerint que l'alteració induïda pel bortezomib sobre la neurona afectaria més primordialment a vies relacionades amb el metabolisme i l'homeòstasis cel·lular que no pas un dany estructural. La recuperació de la neuropatia al suspendre el tractament en aquest model, al igual que succeeix en el pacients tractats amb bortezomib, aniria a favor d'un tipus de neuropatia més 'disfuncional' que no pas 'estructural', al contrari del que s'ha observat amb altres neuropaties i ganglionopaties com les generades pels alcaloides de la vinca i els platins. Paral·lelament, l'observació en els axons de vesícules d'inclusió amb material electrodens en el seu interior, suggestives d'autofagosomes, podria indicar

la participació de la via de degradació autofàgica per compensar la inhibició de la via degradativa del proteasoma, que facilitaria la supervivència neuronal en un primer moment, però podria conduir a la neurona cap a una mort per autofàgia quan la compensació fos insuficient per permetre la seva viabilitat. Per altra banda, malgrat que estudis previs en models animals han fet èmfasis en l'acció del bortezomib sobre les cèl·lules de Schwann, les dilatacions del seu sistema reticular endoplasmàtic i l'alteració secundària del grau de mielinització dels axons observats en el present model, no presenten una traducció destacable a nivell funcional. Així, la velocitat de conducció s'altera de forma més tardana que el dany axonal i les cèl·lules de Schwann mantindrien intactes les seves funcions tròfiques sobre l'axó com es demostra indirectament pel manteniment de les neurones IB4 positives, dependents del *glial-derived neurotrophic factor* (Molliver et al. 1997; Averill et al. 2004).

Així doncs, aquest model constitueix una bona eina tant per la recerca de mecanismes etiopatogènics de la NIB, com en la recerca d'estratègies neuroprotectores pel bon correlat que presenta amb la neuropatia observada en pacients. Ara bé, l'obtenció d'un bon model animal, no només serveix per aprofundir en mecanismes casuístics o d'assaig preclínic de fàrmacs neuroprotectors, sinó que permet explorar diferents paradigmes clínics en els que existeix controvèrsia, moltes vegades difícils de solucionar amb un alt grau d'evidència, degut a les dificultats logístiques i de maneig dels pacients oncològics o bé, per manca d'interès de la indústria en posar en pràctica aquests estudis. En aquest sentit, s'han avaluat dos situacions d'especial rellevància clínica: el paper d'una neuropatia de base com a factor de risc per a desenvolupar una neuropatia més severa de l'esperada a l'administrar un citostàtic neurotòxic, i la seguretat a nivell de toxicitat sobre el SNP amb un nou retractament usant el mateix fàrmac.

Respecte al retractament, el bortezomib és un dels fàrmacs més eficaços que existeixen actualment contra el MM, encara que en la majoria de casos no s'aconsegueix la curació d'aquest. Seria d'interès conèixer si el perfil de seguretat d'aquest fàrmac permet reutilitzar-lo de nou, com a tractament de rescat en aquells pacients que han presentat una resposta parcial o complerta al primer tractament amb bortezomib i la seva malaltia torna a progressar. Com es demostra en un dels experiments efectuats, el grau de severitat de la neuropatia induïda per un segon tractament amb bortezomib, tant a nivell neurofisiològic, funcional, com histològic i ultraestructural, no difereix de la desenvolupada en animals amb un sol esquema de tractament. Per tant, segons les troballes realitzades en aquest model, no existeix un risc addicional de pitjor tolerància a

nivell del SNP en cas de requerir un rescat amb un segon tractament amb bortezomib. Aquest fet estaria en consonància amb les troballes obtingudes en petites cohorts de pacients on s'ha tornat a administrar aquest fàrmac per segona vegada (Berenson et al. 2005; Wolf et al. 2008; Sood et al. 2009; Taverna et al. 2012). Fins i tot s'observa una tendència, encara que no significativa, d'una menor afectació de les amplituds dels PANCS després d'un segon tractament, en línia amb les troballes clíniques descrites per Berenson et al. (Berenson et al. 2005). Aquesta fet, malgrat que no s'acompanya d'una menor afectació a nivell histològic, obriria les portes a especular sobre la possibilitat de l'existència de mecanismes adaptatius en la neurona que permetin superar la disfunció generada pel bortezomib i que, alhora, podrien ser una potencial diana per a plantejar teràpies neuroprotectores.

L'existència d'una neuropatia de base, com a factor de risc per a desenvolupar una neuropatia de major severitat de l'esperada quan s'administra un fàrmac citostàtic neurotòxic, és una situació llargament debatuda a nivell clínic però escassament comprovada. L'evidència a favor d'aquest factor de risc es basa en les experiències clíniques personals i en pocs estudis de casos aïllats (Chaudry et al. 2003; Windebank et al. 2008). Contràriament, existeixen altres estudis on no s'observen exacerbacions de la neuropatia de base després de l'administració d'agents neurotòxics (van den Bent et al. 2002). Aquest dilema adquireix especial rellevància en els pacients amb MM, donat que és una població amb risc de patir una neuropatia de base, ja que els pacients solen ser d'edat avançada i poden haver estat exposats a altres citostàtics neurotòxics, administrats prèviament a l'aprovació del bortezomib (Mothy et al. 2010). A més a més, el propi mieloma pot provocar neuropatia (entre un 11-54% dels pacients), com a efecte de la paraproteinèmia o d'una acció paraneoplàsica no determinada (Plasmati et al. 2007; Chaudry et al. 2008; Richardson et al. 2009). Els estudis realitzats per valorar aquest factor de risc en pacients tractats amb bortezomib, es troben amb un seguit de limitacions, com són el reduit nombre de pacients valorats i la natura retrospectiva d'aquests (Badros et al. 2007; El-Cheikh et al. 2008; Lanzani et al. 2008; Velasco et al. 2010b). Per a contribuir en l'avaluació d'aquest problema utilitzant una població controlada, com la que ofereixen el models animals, hem realitzat un experiment sobre una mostra de ratolins, als que s'ha induït prèviament una neuropatia mitjançant l'administració de vincristina a diferents dosis. Posteriorment, se'ls ha pautat el bortezomib amb la posologia descrita. Aquest treball permet constatar que la neuropatia de base constituïx un factor de risc per a presentar una alteració neurofisiològica i histològica més severa de l'esperada a

l'administrar el bortezomib, només en aquells subjectes que presentin una neuropatia prèvia severa, no observant diferencies entre els animals amb neuropaties de base lleu i els prèviament no tractats. A nivell ultrestructural s'observa una dilatació mitocondrial anormal en els somes de les neurones sensorials, que no es veu en els animals que reben exclusivament bortezomib. Aquesta troballa suggeriria un efecte sinèrgic mediat per un altre mecanisme patogènic com a explicació de l'existència d'aquest factor de risc, més que d'un efecte additiu provocat a l'administrar de nou un altre neurotòxic. Paral·lelament, en aquest experiment s'ha pogut observar un altre fet de potencial rellevància clínica. Durant l'administració del bortezomib, en els animals que presentaven la neuropatia sensitivo-motora induïda per la vincristina, i mentre les seves neurones sensitives estaven patint els efectes d'aquest, s'apreciava una regeneració de les fibres motores al igual que succeïa en aquells animals amb la mateixa neuropatia de base però que no rebien el tractament amb bortezomib. Aquesta troballa indicaria que l'especificitat de l'acció del bortezomib és exclusivament sobre les neurones sensitives perifèriques, no interferint en els processos de regeneració d'altres tipus de fibres del SNP. Aquesta especificitat o patoclisis del citostàtic, de ser extrapolable a altres fàrmacs neurotòxics, ens indicaria la seguretat d'administrar agents neurotòxics que actuen sobre les neurones sensitives en pacients amb neuropaties de base amb component motor, com per exemple les no infreqüents neuropaties per compressió del nervi ciàtic popliti extern, facilitades per la caquèxia dels pacients amb neoplàsies.

Finalment, el darrer paradigma testat en aquest model és l'avaluació de l'afectació de fibra petita amielínica, mitjançant l'estudi immunohistoquímic de biòpsies de pell, com a mètode de diagnòstic precoç i com a possible marcador predictiu de risc alhora de desenvolupar una neuropatia severa per bortezomib. L'absència de marcadors predictius de risc en pacients sota tractament citostàtic és una de les mancances actuals, a nivell terapèutic, en el seguiment i tractament d'aquests malalts, ja que l'electromiografia convencional identifica la NIQ massa tard, quan aquesta ja està instaurada. Encara que s'han descrit, de forma inconsistent, en petites cohorts de pacients i en estudis retrospectius alguns factors i marcadors de risc genètics, clínics i metabòlics, únicament la dosis total acumulada és acceptada com a tal (Windebank et al. 2008; Velasco et al. 2010, Bruna 2011a, Bruna 2011b, Argyriou et al. 2012). Donada la demostrada afectació de fibra petita amielínica en la NIB i el suggeriment, encara que amb nivell de recomanació C, de la *European Federation of Neurological Societies/Peripheral Nerve Society Guideline* de la utilitat de les biòpsies cutànies seriades en la detecció precoç de

neuropatia de fibra petita (Lauria et al. 2010) i, segons altres autors, de tot tipus de neuropaties (Lauria et al. 2007), es va procedir a la comprovació d'aquest fet en el nostre model. Malgrat que es va observar que l'afectació de fibra petita en la NIB és un esdeveniment precoç, malauradament, aquesta presenta una manca de correlació predictiva entre el seu grau o intensitat d'afectació i l'instauració d'una neuropatia severa de fibres mielíniques. Ara bé, l'experiment serveix per constatar la diferent susceptibilitat dels diferents tipus de neurones a un mateix agent citostàtic, com s'ha demostrat anteriorment amb altres agents neurotòxics (Perry et al. 2004). Així doncs, aprofundir en els mecanismes adaptatius de les diferents classes de neurones podria ser una altra estratègia útil, alhora de cercar potencials dianes en neuroprotecció d'aquesta classe de neuropaties. Per altra banda, aquest estudi corrobora el valor de l'ús de biòpsies de pell, complementàriament als estudis de conducció nerviosa i a l'exploració neurològica (Velasco et al. 2010b), per tipificar i identificar el desenvolupament precoç de la neuropatia perifèrica en pacients tractats amb citostàtics.

CONCLUSIONS

1. L'administració de bortezomib en ratolins Swiss OF1, a dosis d'1mg/kg dos cops per setmana durant 6 setmanes, indueix una neuropatia ben tolerada i reproduïble, de característiques similars a l'observada en humans.

1.1. La neuropatia induïda per bortezomib és una neuropatia sensitiva pura de gran espectre que afecta tan a les fibres mielíniques com a les amielíniques.

1.2. Les troballes electrofisiològiques i histològiques suggereixen que la neuropatia és una ganglionopatia i, per tant, secundària a l'efecte del bortezomib sobre els somes de les neurones. La pèrdua axonal seria producte d'aquesta alteració i els canvis més tardans observats en la mielina, secundaris a l'alteració axònica.

2. El pronòstic de recuperació espontània de la neuropatia induïda per bortezomib és bo, observant a les 4 setmanes d'haver suspès el fàrmac, una recuperació total dels paràmetres neurofisiològics i funcionals, juntament amb una recuperació parcial però significativa de les alteracions estructurals.

3. La presència de neuropatia severa prèvia al inici d'un tractament amb bortezomib constitueix un factor de risc per a desenvolupar una neuropatia de major severitat a nivell electrofisiològic i histològic respecte a no presentar-ne o a tenir una neuropatia lleu-moderada.

3.1. L'administració de vincristina en ratolins Swiss OF1 indueix una neuropatia de predomini sensitiu de fibra gruixuda a dosis de 1 mg/kg i una neuropatia sensitivo-motora a dosis de 1,5 mg/kg, dos cops per setmana, durant 4 setmanes.

3.2. La neuropatia per vincristina és de caràcter axonal amb fenòmens de desmielinització secundaris.

3.3. Dosis d'1 mg/kg o 1,5 mg/kg de vincristina, dos cops per setmana, durant 4 setmanes presenten una tolerabilitat acceptable i reprodueixen el gradient de troballes electrofisiològiques observada en humans.

3.4. Els esquemes de dosis testats són capaços d'induir una neuropatia sensitiva crònica amb una escassa recuperació electrofisiològica i funcional després de 8 setmanes de la suspensió del tractament.

3.5. El tractament amb bortezomib en animals que presenten una neuropatia sensitivo-motora crònica severa, provoca un decrement percentualment major tant a nivell dels potencials d'acció sensitius com del número d'axons, respecte als animals sense neuropatia o amb neuropatia lleu.

3.6. El bortezomib presenta una patoclisis específica sobre les neurones sensitives, permeten la recuperació o regeneració de les neurones motores perifèriques durant la seva administració.

4. El retractament amb el mateix esquema de bortezomib (1 mg/kg 2 cops per setmana, durant 6 setmanes) després d'un període de recuperació, és una opció terapèutica segura i, fins i tot, sembla presentar una millor tolerància a nivell electrofisiològic que una única pauta de tractament.

5. La neuropatia de fibra petita amielínica és un fenòmen que apareix precoçment, a partir de les dues setmanes del tractament amb bortezomib, amb la dosi de 1 mg/kg 2 cops per setmana durant 6 setmanes.

5.1. La neuropatia de fibra petita amielínica, malgrat que es un fenòmen precoç en la instauració de la neuropatia induïda per bortezomib, no es correlaciona ni prediu la severitat de la neuropatia de fibra mielínica gruixuda que acabarà desenvolupant-se.

ANNEXES

1. Chemotherapy-induced peripheral neurotoxicity (CIPN): An update. Argyriou AA, Bruna J, Marmiroli P, Cavaletti G. *Crit Rev Oncol Hematol 2012; 82:51-77.*

2. Platinum derivates-induced peripheral neuropathy. Bruna J. In: Effect of Chemotherapeutic Drugs on the Peripheral Nervous System of Human and Animal Experimental Models, 2011. ISBN: 978-81-308-0456-9. Editors: Arianna Scuteri and Giovanni Tredici.

3. Vinca alkaloids-induced peripheral neuropathy. Bruna J. In: Effect of Chemotherapeutic Drugs on the Peripheral Nervous System of Human and Animal Experimental Models, 2011. ISBN: 978-81-308-0456-9. Editors: Arianna Scuteri and Giovanni Tredici.

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Chemotherapy-induced peripheral neurotoxicity (CIPN): An update

Andreas A. Argyriou [a], Jordi Bruna [b,c], Paola Marmiroli [d], Guido Cavaletti [d,*]

[a] *Department of Neurology, "Saint Andrew's" General Hospital of Patras, Greece*
[b] *Unit of Neuro-Oncology, Department of Neurology, Bellvitge University Hospital, Hospitalet del Llobregat, Spain*
[c] *Department of Cell Biology, Physiology and Immunology, Universitat Autonoma of Barcelona, CIBERNED, Spain*
[d] *Department of Neuroscience and Biomedical Technologies, University of Milan-Bicocca, Monza, Italy*

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* Corresponding author.
E-mail address: guido.cavaletti@unimib.it (G. Cavaletti).

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Abstract

The peripheral nervous system can be vulnerable to the toxic action of several drugs since it is not protected as effectively as the central nervous system from noxious exogenous agents. Drug-induced neurotoxicity can affect the nerve fibers or the neuronal bodies (generally the dorsal root ganglia of the primary sensory neurons). Among the neurotoxic drugs antineoplastic agents represent a major clinical problem, given their widespread use and the potential severity of their toxicity. In fact, the peripheral neurotoxicity of antineoplastic agents frequently represents one of their dose-limiting side effects. Moreover, even when antineoplastic agents' peripheral neurotoxicity is not dose-limiting, its onset may severely affect the quality of life of cancer patients and cause chronic discomfort. Among the anticancer chemotherapy drugs, platinum derivates, antitubulins, thalidomide and bortezomib can induce the most severe effects on the peripheral nervous system of the treated patients. Therefore, we will review the features of chemotherapy-induced peripheral neurotoxicity (CIPN) resulting from the administration of these drugs with a focus on new classes of promising antineoplastic agents, such as epothilones and proteasome inhibitors.

Keywords: Chemotherapy; Peripheral neuropathy; Platinum drugs; Antitubulin drugs; Proteasome; Cancer

1. Introduction

The peripheral nervous system can be vulnerable to the toxic action of several drugs since it is not protected as effectively as the central nervous system from noxious exogenous agents. Drug-induced neurotoxicity can affect the nerve fibers or the neuronal bodies (generally the dorsal root ganglia (DRG) of the primary sensory neurons). The clinical features of such neurotoxicity are dependent on the type of agent involved and the site of action – ranging from motor, to sensory-motor or almost exclusively sensory neuropathies, with or without autonomic impairment. Among the neurotoxic drugs antineoplastic agents represent a major clinical problem, given their widespread use and the potential severity of their toxicity. In fact, the peripheral neurotoxicity (PN) of antineoplastic agents frequently represents one of their dose-limiting side effects. Moreover, even when antineoplastic agents' peripheral neurotoxicity is not dose-limiting, its onset may severely affect the quality of life of cancer patients and cause chronic discomfort.

The methods of assessment of the severity of CIPN in clinical trials are not homogeneous, but among the most commonly used scales the National Cancer Institute – Common Toxicity Criteria (NCI-CTC) and to the Total Neuropathy Score (TNS), a composite scale designed to assess the severity of distal polyneuropathies, deserve to be mentioned (Table 1).

We will review the features of chemotherapy-induced PN resulting from the administration of the most widely used and better investigated compounds, such as platinum drugs, taxanes, vinca alkaloids and thalidomide, with a focus on new classes of promising antineoplastic agents, such as epothilones and proteasome inhibitors (Fig. 1).

2. Platinum drugs

Since platinum compounds were identified as antineoplastic agents (cisplatin has been used since 1970), their use has been increasingly adopted in routine oncological clinical practice. While the toxicity profile differs among the different drugs, platinum-induced PN is a common feature.

2.1. Pathogenesis

DRG represent the main target of platinum compound-induced damage [1–5] and platinum levels in the DRG of treated patients correlate with the severity of their PN [3,6]. Although platinum compounds differ in several chemical properties, the primary mechanisms involved in platinum-induced PN are probably similar [5,7].

Two main mechanisms have been proposed to explain the physiopathology of platinum-induced PN. Firstly, the platinum compounds form intrastrand adducts and interstrand crosslinks which alter the tertiary structure of the DNA [8,9]. This effect on DNA promotes alterations in cell-cycle kinetics resulting in the upregulation of cyclin D1 expression and hyperphosphorylation of the retinoblastoma gene product, with an attempt of differentiated postmitotic DRG neurons to re-enter into the cell cycle

Table 1
Examples of two scales used to grade CIPN.

Adverse event	National Cancer Institute – Common Toxicity Criteria version 3.0 – grades					
	0	1	2	3	4	5
Neuropathy motor	Normal	Asymptomatic, weakness on exam testing only	Symptomatic weakness interfering with functioning but not interfering with ADL	Weakness interfering with ADL; bracing or assistence to walk (e.g. cane or walker) indicated	Life threatening disabling (e.g. paralysis)	Death
Neuropathy sensory	Normal	Asymptomatic loss of tendon reflex or paresthesia (including tingling) but not interfering with function	Sensory alterations or paresthesias interfering with function but not interfering with ADL	Sensory alterations or paresthesias interfering with ADL	Disabling	Death

Parameter	Total Neuropathy Score (TNS) – grades				
	0	1	2	3	4
Sensory symptoms	None	Symptoms limited to finger or toes	Symptoms extends to ankle or wrist	Symptoms extends to knee or elbow	Symptoms above knees or elbows, or functionally disabling
Motor symptoms	None	Slight difficulty	Moderate difficulty	Require help/assistance	Paralysis
Autonomics symptoms					
	0	1	2	3	4 or 5
Pin sensibility	Normal	Reduced in finger/toes	Reduced up to wrist/ankle	Reduced up to elbow/knee	Reduced above elbow/knee
Vibration sensibility	Normal	Reduced in finger/toes	Reduced up to wrist/ankle	Reduced up to elbow/knee	Reduced above elbow/knee
Strength	Normal	Mild weakness	Moderate weakness	Severe weakness	Paralysis
Tendon reflex	Normal	Ankle reflex reduced	Ankle reflex absent	Ankle reflex absent, others reduced	All reflexes absent
Vibration sensation (QST vibration)	Normal to 125% of ULN	126–150% of ULN	151–200% of ULN	201–300% of ULN	>300% of ULN
Sural amplitude	Normal/reduced to <5% of LLN	76–95% of LLN	51–75% of LLN	26–50% of LLN	0–25% of LLN
Peroneal amplitude	Normal/reduced to <5% of LLN	76–95% of LLN	51–75% of LLN	26–50% of LLN	0–25% of LLN

Adapted from the original version (available at http://www.cancer.gov).
Note: the first 7 items are used in the clinical TNS (TNSc).
QST: quantitative sensory test; ULN: upper limit of normal; LLN: lower limit of normal.

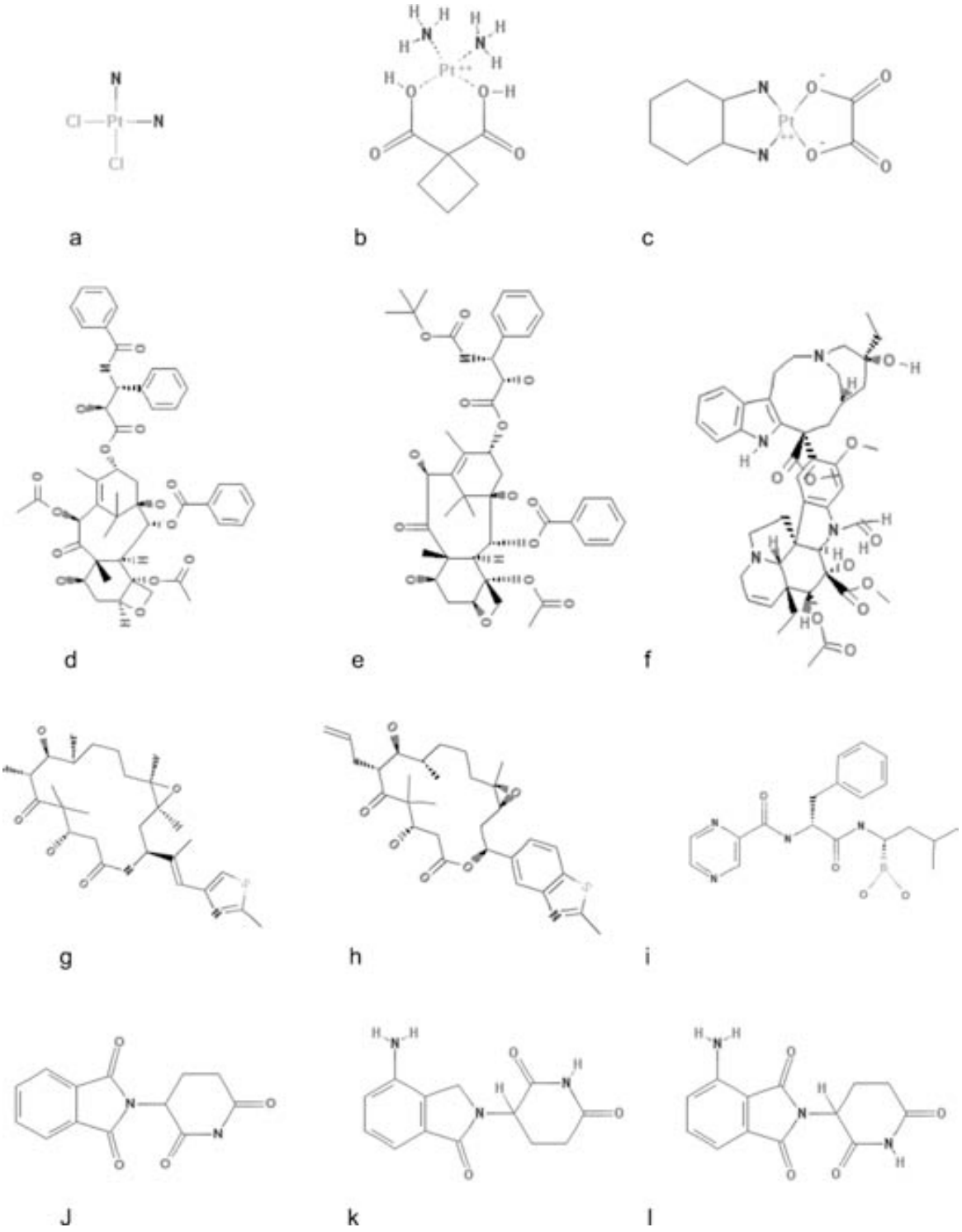


Fig. 1. Chemical structure of the most widely used neurotoxic antineoplastic drugs. a, cisplatin; b, carboplatin; c, oxaliplatin; d, paclitaxel; e, docetaxel; f, vincristine; g, ixabepilone; h, sagopilone; i, bortezomib; j, thalidomide; k, lenalidomide; l, pomalidomide.
Source: http://pubchem.ncbi.nlm.nih.gov/

resulting in an induction of apoptosis [10]. The second mechanism proposes an involvement of oxidative stress and mitochondrial dysfunction as the trigger of neuronal apoptosis [11]. PN could be modulated by a reduction in the activity of enzymes involved in DNA base excision, repair of oxidative damage and in redox regulation [12]. PN could also involve apoptosis, mediated by p53 increased activity and mitochondrial release of cytochrome-c pathway, independent of fas receptor activation [13]. Another recently described modulator of proteins involved

in platinum-induced apoptosis is the activation of p38 and ERK1/2 [14].

Acute oxaliplatin neurotoxicity (see below) is thought to be caused by a dysfunction of nodal axonal voltage-gated Na^+ channels [15], probably the Ca^{2+}-dependent channels. This effect is likely to be due to the oxalate chelating effect on both Ca^{2+} and Mg^{2+} which could interfere with channel kinetics [16] and reduce the overall Na^+ current [17].

2.2. Clinical and electrophysiological characteristics

The earliest signs of PN observed in platinum-treated patients are a decreased vibratory sensitivity in the toes and loss of ankle jerks, associated with numbness, tingling or paresthesias in finger and toes. Prolonged treatment may worsen symptoms and signs, with generalized loss of deep tendon reflexes (DTR) and more proximal vibratory sensitivity impairment. Pin and temperature sensation, joint position and light touch perception are less severely affected. In the worst cases the loss of propioception may result in an ataxic gait. Lhermitte's phenomenon secondary to DRG cell degeneration and spinal cord dorsal columns damage can be occasionally observed [18,19].

Oxaliplatin-induced PN presents as two distinct clinical syndromes: an acute, cold-induced and transient syndrome characterized by paresthesias in the distal extremities and perioral region that usually appears during or within hours after infusion, and a chronic cumulative sensory neuropathy with the typical features of platinum drug-induced PN. A smaller number of patients present with slurred speech, jaw pain during chewing and paresthesias in the extremities or calf cramps with walking that tend to persist for days to weeks [20,21].

Nerve conduction studies performed in patients treated with platinum drugs consistently demonstrated sensory axonal damage with reduced amplitude of the sensory nerve action potentials (SNAP) [1,15,22–25]. Motor nerve conduction velocities (NCV), compound muscle action potentials (CMAP) and F-wave latencies remain unchanged during treatment. Conflicting findings have been observed in autonomic testing [26,27]. Neurophysiological studies performed within 24–48 h after oxaliplatin infusion showed neuromyotonic discharges and repetitive compound muscle action potentials. All these abnormalities resolved within 3 weeks after oxaliplatin administration [28].

2.3. Outcome

PN usually develops during platinum drug treatment, but symptoms and signs may progress for 2–6 months after cessation of chemotherapy (the so-called "coasting" effect, occurring in up to 30% of cisplatin-treated patients) [29] and frequently recovery is incomplete. Recently, a cross-sectional study of testicular cancer patients re-evaluated between 23 and 33 years after finishing treatment, showed that PN remains detectable in up to 20% of patients, being symptomatic in 10% of them [30]. Similar results were found in another study that evaluated cisplatin-treated patients after a median follow-up of 15 years: 38% and 28% patients had asymptomatic and symptomatic neuropathy respectively, which was disabling in 6% [31]. The cumulative dose of cisplatin is the main risk factor associated with the persistence of neurotoxicity [30].

Chronic oxaliplatin PN is partially reversible in about 80% of patients and completely resolved in about 40% of them at 6–8 months after treatment discontinuation [32]. However, coasting may also occur with this drug. Two studies have reported persistence of neuropathy in almost 35% of patients 5–6 years after cessation of oxaliplatin treatment [33,34]. It has been reported that PN may be exacerbated by surgery just after finishing treatment with oxaliplatin. In these patients surgery-induced haemolysis is thought to release oxaliplatin that has accumulated in erythrocytes [35].

2.4. Incidence, severity and risk factors

Platinum-induced PN is related to the total cumulative dose [1,3,30,36] and to the dose-intensity of treatment [23,37]. When using cisplatin, the onset of PN is expected to occur after the administration of 250–350 mg/m^2 [1,30], while at a cumulative dose of 500–600 mg/m^2, almost all patients have objective evidence of neuropathy [38], being severely disabling in at least 10% of patients [36] (Table 2). Recently, reduced neurotoxicity with the use of a liposomal formulation of cisplatin (Lipoplatin) has been reported [39,40].

Carboplatin induced-PN is less frequent and severe at conventional doses than is in cisplatin-treated patients [36,41].

In combination schedules, the use of cisplatin or carboplatin with taxanes has been reported to induce higher incidence of PN, even at lower cumulative dose of each antineoplastic agent [42–46], but this result has not always been confirmed [47,48]. Retreatment with neurotoxic drugs is a feasible option in several patients [49]. Combinatory therapies with other neurotoxic agents and low magnesium serum levels have also been related to a higher incidence and severity of neuropathy [30,50]. Other risk factors including alcohol consumption, smoking, familial diabetes, serum creatinine levels, age, tumour characteristics or performance status are not considered as being related to the onset or the severity of PN [36,37]. Polymorphisms in genes involved in the platinum biotransformation [51] and in the mechanisms for repairing platinum-DNA adducts [6] could modulate and partially explain the individual differences in the severity of the PN induced by these drugs [52].

Oxaliplatin-induced acute symptoms are transiently present in the vast majority of patients. Sensory symptoms are usually reversible over hours to days. Conversely, chronic PN persists between cycles and severity increases with cumulative dose [25,53,54]. Severe PN has been observed with cumulated doses ranging from 510 to 765 mg/m^2 in up to 10% of patients, but it can affect approximately 50% of the

Table 2
Incidence of cisplatin-induced neuropathy. Selection criteria: phase III studies and prospective studies with specific neurologic assessment tools, excluding patients with other neurotoxic agents.

Author (year)	Patients (*n*)	Schedule	Dose-intensity planned (mg/m^2/week)	Neurological assessment	Neuropathy incidence
Phase III studies					
Crinò [195]	150	120 mg/m^2 every 21 days	40	WHO	PN in 3.3%
van der Hoop [36]	195	100 mg/m^2 every 36 days.	20	WHO	Grade 1: 20.5% Grade 2: 23.5%
	97	75 mg/m^2 every 21 days	25		Grade 3: 3.6% Grade 1: 25% Grade 2: 15% Grade 3: 5%
Kawahara [201]	80	80 mg/m^2 every 28 days	20	WHO	Grade 2: 2.5% Grade 3: 0%
Swenerton [194]	210	75 mg/m^2 every 28 days	25	ECOG criteria	Grade 1: 18.1% Grade 2: 7.3% Grade 3: 2.4%
Cartei [189]	52	75 mg/m^2 every 21 days	25	NCI-CTC	Grade 2: 11.5% Grade 3: 3.8%
Gandara [200]	103	50 mg/m^2 on days 1 and 8 every 28 days for 8 cycles	25	SWOG criteria	PN in 4%
	216	100 mg/m^2 on days 1 and 8 every 28 days for 4 cycles	50		PN in 2.8%
Kemp [197]	120	100 mg/m^2 every 21 days for 6 cycles	33.3	NCI-CTC	Grade 1: 25.8% Grade 2: 29.2% Grade 3: 12.5%
Sutton [202]	90	20 mg/m^2 during 5 every 21 days for 8 cycles	33.3	GOG criteria	Grade 2: 7.8% Grade 3: 12.2% Grade 4: 1.1%
Gatzemeier [203]	206	100 mg/m^2 every 21 days	33.3	NCI-CTC QLQ-C30	Grade 1–2: 11.6% Grade 3–4: 0.4%
Wachters [191]	119	80 mg/m^2 every 21 days for 5 cycles	26.7	NCI-CTC QLQ-C30	Grade 1: 14% Grade 2: 8% Grade 3–4: 1%
Fleming [190]	129	50 mg/m^2 every 21 days for 7 cycles	16.7	NCI-CTC FACT/GOG-Ntx	Grade 1–2: 3.1% Grade 3–4: 0.8%
Jehn [196]	21	100 mg/m^2 every 21 weeks for 6 cycles	33.3	NCI-CTC	Grade 1: 33.3% Grade 2: 33.3% Grade 3: 19% Grade 4: 0%
	25	100 mg/m^2 on days 1, 8, 15 every 21 days for 6 cycles	100 (liposomal cisplatin)		Grade 1: 27% Grade 2: 0% Grade 3: 0% Grade 4: 4%
Homesley [192]	261	50 mg/m^2 every 21 weeks for 6 cycles	16.7	NCI-CTC FACT/GOG-Ntx	Grade 1: 24.9% Grade 2: 3.1% Grade 3: 1.9% Grade 4: 0%

Wozniak [193]	200	100 mg/m^2 every 28 days for 4 cycles	25	SWOG criteria	Grade 1–2: NA Grade 3–4: 0.5%
Studies with specific neurological assessment tools					
van der Hoop [199]	13	75 mg/m^2 every 21 days for 6 cycles	25	Neurological Clinical scale	Symptoms Sum Score:1.75 ± 0.55
				VPT	Signs Sum Score: 7.42 ± 1.3
Thompson [1]	11	50 mg/m^2 every 28 days	12.5	Neurological clinical assessment NCS	PN in 91%
Roelofs [38]	24	60 mg/m^2 every 28 days	15	Neurological clinical assessment NCS Sural biopsy	PN in 92%
Daugaard [22]	8	40 mg/m^2 every 7 days for 3 cycles	40	NCS	PN in 100%
Mollman [37]	19	50 mg/m^2 every 28 days for 9 cycles	12.5	Neurological clinical assessment NCS	PN in 47%
		40 mg/m^2 every 28 days for 9 cycles	10		PN in 100%
Cavaletti [23]	20	75 mg/m^2 every 21 days for 6 cycles	25	NSS NDS NCS	PN: 38.6%
	40	50 mg/m^2 every 7 days for 9 cycles	50		PN: 16.6%
LoMonaco [204]	16	40 mg/m^2 during 5 days every 28 days for 3 cycles	50	Neurological clinical assessment NCS	PN in 89%
van den Bent [49]	33	50 mg/m^2 on days 1, 8, 15 and 29, 36, 43	42	Questionnaire for neurological symptoms VPT	NCI.CTC all group Grade 1: 55%
	47	70 mg/m^2 on days 1, 8, 15 and 29, 36, 43	58.8	NCI-CTC	Grade 2: 15% Grade 3: 3.8%
Pace [198]	14	Several	–	NSS WHO	PN in 85.7% NSS: 4.7 ± 2.9
Pace [70]	24	NA	NA	TNS NCS	TNS: 4.1 ± 4.5

WHO: World Health Organization; ECOG: Eastern Cooperative Oncology Group; NCI-CTC: National Cancer Institute-Common Toxicity Criteria; SWOG: South West Oncology Group; GOG: Gynecologic Oncology Group; PN: peripheral neuropathy; QLQ-C30: European Organization for the Treatment of Cancer quality of life questionnaire-30 items; FACT/GOG-Ntx: Functional Assessment of Cancer Therapy Scale/Gynecologic Oncology Group-Neurotoxicity scale; VPT: vibration perception threshold; NCS: nerve conduction studies; NSS: Neurological Symptom Score; NDS: Neurological Disability Score; TNS: Total Neuropathy Score; NA: not available.

Table 3
Incidence of oxaliplatin-induced neuropathy. Selection criteria: phase III studies and prospective studies with specific neurologic assessment tools.

Author (year)	Patients (*n*)	Chemotherapy schedule	Dose-Intensity planned (mg/m^2/week)	Neurological assessment	Neuropathy incidence
Phase III studies					
Land [207]	189	85 mg/m^2 at week 1, 3, 5 of a 8 weeks cycle for 3 cycles	31.875	FACT/GOG-Ntx NCI-CTC	At week 12:
				NCI-Sanofi grade	NCI-Sanofi. Grade 1–2: 68.3% Grade 3–4: 3% NCI-CTC Grade 1–2: 61.3% Grade 3–4: 2.6%
Giacchetti [213]	100	125 mg/m^2 every 21 days, as a continuous 6-hour intravenous infusion	41.67	Specific scale of peripheral neuropathy	Grade 1: 32% Grade 2: 13% Grades 3–4: 10%
Tournigand [214]	309	85 mg/m^2 every 14 days for 12 cycles	42.5	NCI-CTC	Grade 1: 34% Grade 2: 37% Grade 3: 18% Grade 4: 0%
	303	130 mg/m^2 every 21 days for 6 cycles, stop OXL 6 weeks and reintroduction other 6 OXL cycles	37.14		Grade 1: 36% Grade 2: 42% Grade 3: 13% Grade 4: 0%
de Gramont [55]	209	85 mg/m^2 every 14 days, until progression disease	42.5	NCI-CTC	Grade 1: 20.6% Grade 2: 29.2% Grade 3: 18.2% Grade 4: 0%
André [243]	1108	85 mg/m^2 every 14 days for 12 cycles	42.5	NCI-CTC	Grade 1: 46% Grade 2: 33.6% Grade 3: 12.4%
Rothenberg [244]	153	85 mg/m^2 every 14 days, median cycles 3 (1–18)	42.5	Specific scale (similar to NCI-CTC)	Acute: Grade 1–2: 51% Grade 3–4: 7% Chronic: Grade 1–2: 49% Grade 3–4: 2%
	150	85 mg/m^2 every 14 days, median cycles 3 (1–16)			Acute: Grade 1–2: 50% Grade 3–4: 3% Chronic: Grade 1–2: 48% Grade 3–4: 3%
Allegra [209]	1321	85 mg/m^2 every 14 days for 12 cycles	42.5	NCI-CTC	Grade 2: 29% Grade 3: 14%
	1326	85 mg/m^2 every 14 days for 12 cycles, plus bevacizumab			Grade 2: 33% Grade 3: 16%
Al-Batran [245]	112	85 mg/m^2 every 14 days, Until progression disease	42.5	NCI-CTC OXL-specific scale	Grade 1–2: 49.2% Grade 3–4: 14.3%
Wang [75]	44	85 mg/m^2 every 14 days	42.5	WHO NCS	Grade 1–2: 40.9% Grade 3–4: 31.8%
Ishibashi [79]	16	85 mg/m^2 every 14 days	42.5	NCI-CTC Debiopharm neurotoxicity scale	NCI-CTC Grade 1: 94% Grade 2: 6% Grade 3–4: 0%
Gibson [76]	324	85 mg/m^2 every 14 days	42.5	NCI-CTC	Grade 1: 38% Grade 2: 18.8% Grade 3–4: 16.7%
Cassidy [208]	655	130 mg/m^2 every 21 days, maximum 16 cycles	43.33	NCI-CTC	Equal in both groups Grade 1: 11%

Table 3 (*Continued*)

Author (year)	Patients (*n*)	Chemotherapy schedule	Dose-Intensity planned (mg/m^2/week)	Neurological assessment	Neuropathy incidence
	649	85 mg/m^2 every 14 days, maximum 24 cycles	42.5		Grade 2: 5% Grade 3: 4% Grade 4: 0%
Cunningham [211]	452	130 mg/m^2 every 21 days, maximum 8 cycles	43.33	NCI-CTC QOL-QC30	Grade 1–2: 75.2% Grade 3–4: 6.4%
Cathomas [210]	20	130 mg/m^2 every 21 days, maximum 6 cycles Heated at 37 °C	43.33	NCI-CTC Neurological symptoms questionnaire (NCI)	NCI-CTC Grade 1: 85% Grade 2: 15% Questionnaire: Dysestesias/paresthesias/acute Grade 1: 35%/40%/35% Grade 2: 10%/ 20%/5% Grade 3: 30%/20%/30% Grade 4: 5%/10%/20%
Qvortrup [212]	69	130 mg/m^2 every 21 days for 8 cycles	43.33	NIC-CTC	Grade 2: 42% Grade 3: 16%
	70	130 mg/m^2 every 21 days for 8 cycles, chronotherapy			Grade 2: 27% Grade 3: 19%
Poplin [206]	272	100 mg/m^2 every 14 days, until progression disease	50	NCI-CTC	Grade 3: 9.5%
Louvet [205]	157	100 mg/m^2 every 14 days, until progression disease	50	NCI-CTC	Grade 1–2: 76% Grade 3: 19.1%
Cascinu [74]	26	100 mg/m^2 every 14 days	50	NCI-CTC	After 8 cycles: Grade 1: 21.1% Grade 2: 31.6% Grade 3–4: 26.3%
Studies with specific neurological assessment tools					
Krishnan [15]	16	100 mg/m^2 every 14 days	50	NCI-CTC NSS Oxaliplatin-specific neurotoxicity score NCS	NCI.CTC: Grade 1–2: 12.5% Grade 3–4: 37.5% NSS: 1: 25% 2: 12.5% 3: 12.5%
Argyriou [77]	20	85 mg/m^2 every 14 days for 12 cycles	42.5	NSS NDS NCS	PN: 75% TNS: 11.2 ± 9.05 NDS:20 ± 23.1 NSS: 1.5 ± 1.3
Argyriou [45]	25	85 mg/m^2 every 14 days for 12 cycles	42.5	NSS NDS FGS NCS TNS WHO CIPN (1–3)	TNS: 1–11 (mild): 24% 12–23 (moderate): 32% ≥24 (severe): 8% Mean NDS: 21.1 ± 17.5 Mean NSS: 1.8 ± 0.8

FACT/GOG-Ntx: Functional Assessment of Cancer Therapy Scale/Gynecologic Oncology Group-Neurotoxicity scale; NCI-Sanofi: oxaliplatin Sanofi-developed specific questionnaire; NCI-CTC: National Cancer Institute-Common Toxicity Criteria; WHO: World Health Organization; QLQ-C30: European Organization for the Treatment of Cancer quality of life questionnaire-30 items PN: peripheral neuropathy; NSS: Neurological Symptom Score; NDS: Neurological Disability Score; FGS: Functional Grading Scale; NCS: nerve conduction studies; TNS: total neuropathy score. For a description of the scales see Cavaletti et al. [242].

patients receiving doses higher than 1000 mg/m^2 [55,56]. Table 3 summarizes phase III and neurotoxicity-focused oxaliplatin trials published in the literature. It has been suggested that sensory excitability techniques provide an early predictor of the chronic neurotoxicity of oxaliplatin [54]. Furthermore, sustained thermal hyperalgesia after the third oxaliplatin cycle has also been identified as an early predictor of chronic PN [57]. Few studies have attempted to detect distinctive gene polymorphisms that identify patients at high-risk of developing PN [51–53,58–66]. Among

others, glutathione S-transferase P1 polymorphism (Ile105Val) and SCNA 1A polymorphism (T1067A T/T) have been suggested as identifying subjects at increased risk of developing oxaliplatin-induced PN. Integrin beta-3 (IGTB3) polymorphism at residue 33 might also represent a specific biomarker that predicts the incidence and severity of chronic PN [67].

2.5. Options for neuroprotection

Several chemoprotective agents have been tested for their ability to limit or prevent the PN induced by cisplatin or carboplatin. Two recent Cochrane Library reviews involving amifostine, diethyl-dithio-carbamate, glutathione, Org 2766 and vitamin E concluded that data were insufficient to support their effectiveness in cisplatin PN [68,69]. A recent trial with vitamin E provided class II evidence about its benefit in preventing cisplatin-induced PN [70], but the results need to be confirmed in a larger setting. Until then, dose-reduction, or a longer interval between cycles, represent the only way to ameliorate cisplatin-induced PN.

Similarly, schedule modification and eventually, treatment withdrawal and later reintroduction when the PN has been resolved, are the methods to minimize oxaliplatin-induced PN [71,72]. "Chronomodulation" (i.e. a dosing schedule based on circadian rhythm) has recently been evaluated in a meta-analysis, but results are conflicting [73]. Several agents have been tested as neuroprotectants against oxaliplatin-induced PN [32], including glutathione [74], glutamine [75], xaloproden [76] and oxcarbazepine [77] Another, still controversial, pharmacological approach is the intravenous administration of calcium gluconate and magnesium sulfate before and after oxaliplatin infusion [78,79]. A large phase III trial (Combined Oxaliplatin Neuropathy Prevention Trial, CONCEPT) was terminated early because it has been suggested that Ca/Mg intravenous supplementation may interfere with the cytotoxic activity of oxaliplatin-based schedules [80]. However, a later results review of this study performed by an independent board did not find differences in response rate between patients receiving placebo or calcium and magnesium infusion. In line with this, a multicentre study, reported as an abstract, confirmed the safety use of calcium and magnesium salts and their benefit in lowering as the frequency and severity of chronic oxaliplatin neuropathy [78]. In view of the conflicting results obtained in the two most recently published studies [79,81] the usefulness of the use of calcium/magnesium infusion in oxaliplatin-treated patients is still controversial.

3. Vinca alkaloids

This group of chemotherapeutic agents includes both natural alkaloids, such as vincristine and vinblastine, and semi-synthetic compounds, such as vindesine, vinorelbine and vinflunine. These drugs have a broad spectrum of indications in the treatment of haematologic and lymphatic malignancies as well as of solid tumours such as breast, ovarian, testicular, brain and non-small cell lung tumours and sarcomas. Vinca alkaloids have a different toxicity profile; vincristine being the most neurotoxic drug.

3.1. Pathogenesis

Vinca alkaloids exert their antineoplastic effect by inhibiting microtubule dynamics in mitotic spindles, resulting in an arrest of dividing cells at the metaphase stage and ultimately leading to cell death [82]. Vinca alkaloids form a stable complex in the GTPase domain of β-tubulin, inhibiting the GTP hydrolysis, which prevents its polymerization from soluble dimers into microtubules. The affinity for tubulin differs among vinca alkaloid compounds (vincristine > vinblastine > vinorelbine > vinflunine) and this biochemical property could explain the distinct neurotoxic profile of these drugs [83]. The effect on tubulin dimers produce loss of axonal microtubules and alteration in their length, arrangement and orientation leading to axonal swelling in both myelinated and unmyelinated fibers [84–86]. These alterations of the neuronal cytoskeleton lead to abnormalities in axonal transport, build up of neurofilaments in the cell bodies and proximal axons and progressive accumulation of axoplasmic organelles and vesicles [86,87]. Secondary to axonal damage, reduction of myelin thickness [88], shortening of inter-nodal length and segmental demyelination can occur [89,90].

3.2. Clinical and electrophysiological characteristics

The first manifestation of vincristine-induced PN is the decrease/loss of DTR followed by paresthesias [91,92]. If treatment continues muscular weakness can occur. Objective touch and two-point discrimination sensory loss are usually infrequent or mild and limited to fingers and toes; however 70% of asymptomatic patients have increased touch detection thresholds [93]. Vibration perception is rarely more than mildly impaired [91,92,94], but when measured with quantitatively methods it is reduced in 20% of patients [95]. Joint position sense usually remains intact [95]. Pain can be observed in some patients, usually restricted to the glabrous skin of the fingertips and toes, with elevated sharpness and warm detection thresholds in these areas [94,96]. When motor weakness appears, it is most evident in the dorsiflexors of ankles and toe/fingers extensors muscles [94,95]. In high-intensity treatments muscle cramps are frequent. Symptoms attributable to severe autonomic dysfunction, e.g. colicky abdominal pain and constipation, can occur even within a few days of drug administration and precede paresthesias or DTR reduction [91]. Impotence has been reported to occur in between 15% and 24% of patients [97]. Other dysautonomic manifestations such as urinary retention and orthostatic hypotension have been reported anecdotally.

At neurophysiological examination, both SNAP and CMAP have been reported to decrease with treatment duration. Spontaneous fibrillation associated with reduction in the interference pattern is found in all distal muscles [92]. Although sensory symptoms and signs improve when the treatment is withdrawn, SNAP remains altered in most patients [92,98]. Only slight reduction of NCV is demonstrated in either sensory or motor fibers even in the setting of severe PN [89,94].

3.3. Incidence, severity and risk factors

Although vincristine is one of the oldest neurotoxic antineoplastic drugs, the exact incidence of PN is still unknown due to the heterogeneity in chemotherapeutic regimens that are employed for the treatment of different type of tumours. Table 4 reports the results obtained in the most relevant trials.

The severity of vincristine-induced PN is dose-related. All patients receiving at least 4 mg/m^2 have reduction or loss of ankle reflexes and most patients who have received total doses of 2–6 mg/m^2 also report mild distal paresthesias. A relevant number of patients receiving a total dose of 8 mg/m^2 develop motor weakness or gait impairment [91]. Neuropathic pain is relatively frequent in high-dose vincristine-treated patients [91,96]. From the literature, two conditions emerged as possible high-risk situations for a severe course and early onset of vincristine PN: unrecognized hereditary peripheral neuropathies [99] and hepatic insufficiency [91]. Age and poor nutritional status have not been well demonstrated as risk factors [100]. A few cases of severe fulminating neuropathy during vincristine treatment, mimicking Guillain-Barré syndrome features, have also been described [101]. Intrathecal administration of vincristine can cause a severe ascending radiculomyeloencephalopathy, fatal in most cases [102].

3.4. Outcome

Vincristine neuropathy is usually reversible when therapy is discontinued. The median duration of paresthesias and motor weakness after treatment discontinuation is 3 months. However, a coasting effect has been reported in the first month after finishing therapy in up to 30% of patients, being more prevalent in high-dose intensity regimens [95]. Although most DTR reappear, ankle reflexes recovery is uncommon [93].

3.5. Options for neuroprotection

To minimize the potential neurotoxic effects of vincristine the usual recommended vincristine dose is 1.4 mg/m^2 per single dose with an upper limit of 2 mg on single doses, regardless of body surface area. In addition, there are no pharmacological treatments to reduce or prevent vinca alkaloid-induced PN. Randomized, double-blind trials assessing the efficacy of Org2766 [103] and gangliosides [94] have not shown benefit. Up to now, reduction in dose level and frequency of administration, or even treatment discontinuation, are the only proven methods to ameliorate and stop the progression of vinca alkaloid-induced PN.

3.6. Other vinca alkaloids

Vinblastine-induced PN is similar, but less severe, to that observed with vincristine; however haemathological toxicity associated with vinblastine administration usually precedes neurotoxicity, and represents the main dose-limiting adverse effect.

Vinorelbine-treated patients usually develop a mild, distal axonal sensory-motor neuropathy, involving small and large fibers without evidence of C-fiber dysfunction, as evaluated by sympathetic skin response. The most common symptoms and signs include DTR impairment (94%), paresthesias (50%) and hypopallaesthesia (9%) with mild overall neurotoxicity [104]. Vinorelbine-induced PN is also dose-dependent and reversible after drug discontinuation [104].

NCI-CTC scale grade 1–2 sensory-motor neuropathy has been reported in 10% of patients treated with vinflunine. Notably, autonomic neuropathy characterized by constipation and abdominal pain is the most frequently reported feature, occurring in 20% of patients, and being severe grade in 8% [105,106].

Liposomal vincristine is a novel formulation developed with the aim of improving the efficacy and pharmacokinetic profile without increasing neurotoxicity. Conflicting results about its PN safety have been reported in phase I and II trials. Given at doses ranging between 2 and 2.8 mg/m^2, sensory-motor neuropathy has been reported in 12–55% of treated patients, being severe in 7–34% [107].

4. Taxanes

Taxanes (paclitaxel and docetaxel) belong to the family of chemotherapy agents classified as microtubule-stabilizing agents (MTSAs) and they are effective in treating various types of solid tumours. PN is considered as being the main non-haematological toxicity of taxanes, usually resulting in dose modification due to its severity [108].

4.1. Pathogenesis

Through their action of disrupting microtubules of the mitotic spindle and the subsequent interference in axonal transport, taxanes are able to affect the soma of sensory neurons as well as axons. In addition, it has been demonstrated that they evoke a “dying back” process starting from distal nerve endings followed by disturbed cytoplasmatic flow in the affected neurons [109–111].

Injury of neuronal and non-neuronal cells within the peripheral nervous system, macrophage activation in both the DRG and peripheral nerve, and microglial activation within the spinal cord are all cellular processes that also seem

Table 4
Incidence of vinca alkaloids-induced neuropathy. Selection criteria: prospective studies including information about neuropathy and neurological assessment tools.

Author (year)	Patients (*n*)	Schedule	Dose-intensity Planned (mg/m^2/week)	Neurological Assesment	Neuropathy Incidence
Vincristine					
Holland [215]	393	Induction:	NA	Not validated neuropathy related symptoms scale, similar to NCI-CTC	Grade 1-2: 23%
		75 μg/kg/week for 4 months.			Grade 3: 33%
					Grade 4: 25%
					Grade 5: 10%
Watkins [226]		Mean dose:	NA	Neurologic clinical assessment	Patients with PN:
	10	0.28 (0.06–0.74) mg/kg			60%
	13	0.23 (0.05–0.75) mg/kg			61.5%
	23	0.3 (0.05–0.57) mg/kg			4.3%
	9	0.42 (0.07–1.47) mg/kg			22%
	5	0.23 (0.06–0.51) mg/kg			40%
DeAngelis [94]	27	0.5 mg/m^2/day during 4 days every 7 days for 12 cycles	2	NCS	All patients had moderate to severe signs and symptoms of sensory-motor neuropathy.
				Neurologic clinical assessment	
Rea [217]	31	2 mg every 7 days for 4 cycles	2	NCI-CTC	Grade 2–3: 45%
Reinders-Messelink [216]	11	1.5 mg/m^2/week for 8 cycles	1.5	WHO	Grade 1: 9%
				VPT	Grade 2: 73%
				NCS	Grade 3–4: 0%
					33% sensory abnormalities
					22% VPT abnormalities
Broun [227]	32	1.4 mg/m^2 was weekly as iv bolus for 4 weeks, then every other week	1.4	NCI-CTC	Grade 2–3: 34%
Verstappen [95]	47	2 mg every 21 days	0.67	Neuropathy scales Symptoms scales	Paresthesias 34% Numbness 43%
	67	4 mg every 21 days	1.33	VPT	Pain 14% Paresthesias 60% (10%severe) Numbness 70% (4% severe) Pain 62% (16% severe)
Jackson [219]	25	0.5 mg plus 0.25 mg/m^2/day during 5 days every 21 days	0.58	Neurologic clinical assessment	Patients with PN: 48%
					Depression reflexes: 36%
					Motor weakness: 4%
					Sensory disturbances: 24%
Taylor [224]	27	1.6 mg/m^2 every 21 days for 6 cycles	0.53	SWOG criteria	Grade 1-2: 22% (mild paresthesias)
Sandler [91]	50	2 mg/m^2 every 14 days	0.5	NCS	Depression reflexes: 100%
				Neurologic clinical assessment	Motor weakness: 34%
					Sensory disturbances: 46%
Powles [225]	105	1.4 mg/m^2 every 21 days	0.47	WHO	Grade 1: 24%
					Grade 2: 14%
					Grade 3–4: 5%
Walewski [220]	24	1.4 mg/m^2 every 21 days for 6–8 cycles	0.47	NCI-CTC	No peripheral neuropathy

Table 4 (*Continued*)

Author (year)	Patients (*n*)	Schedule	Dose-intensity Planned (mg/m^2/week)	Neurological Assesment	Neuropathy Incidence
Thant [100]	11	1.4 mg/m^2 twice every 21 days for 3 cycles	0.47	NCS	Grade 1–2: 63.6%
				Neurologic clinical assessment	Grade 3–4: 36.4%
Klasa [223]	44	1.2 mg/m^2 every 21 days	0.4	WHO QLQ-C30	Grade 1: 54% Grade 2: 11% Grade 3–4: 2%
Katsumata [221]	23	1.2 mg/m^2 every 21 days	0.4	NCI-CTC	Grade 1: 0% Grade 2: 26.1% Grade 3–4: 0%
Leighl [222]	31	1.2 mg/m^2 every 21 days	0.4	NCI-CTC	Grade 3–4: 13%
van Kooten [228]	15	1.4 mg/m^2 twice every 28 days for 6 cycles	0.35	VPT Neurologic clinical assessment	Patients with PN: 80%
Liposomal vincristine					
Sarris [107]	35	2 mg/m^2 every 14 days for 12 cycles	1	NCI-CTC	Grade 3–4: 31.4%
Rodriguez [246]	119	2 mg/m^2 every 14 days for12 cycles	1	NCI-CTC	Grade 3–4: 32%
Vinblastine					
Druker [218]	24	6 mg/m^2 on days 1 and 8 every 28 days	3	Neurologic clinical assessment	Mild PN: 8%
Vinorelbine					
Pace [104]	23	25 mg every 7 days for 24 cycles	NA	NSS	WHO>2: 0%
				NCS WHO	Moderate (5–10): 66.6% Severe (>10): 33%

NA: not available. NCI-CTC: National Cancer Institute-Common Toxicity Criteria; PN: peripheral neuropathy; WHO: World Health Organization; NCS: nerve conduction studies; SWOG: South West Oncology Group; QLQ-C30: European Organization for the Treatment of Cancer quality of life questionnaire-30 items; VPT: Vibration perception threshold; NSS: Neurological Symptom Score. For a description of the scales see Cavaletti et al. [242].

to contribute greatly to the genesis of taxane-induced PN through signal transduction-mediated events [112]. Results from a recently published experimental study showed that paclitaxel leads to massive polar reconfiguration of axonal microtubules, accompanied by impaired organelle transport in cultured *Aplysia* neurons [113] and another animal study evidenced that paclitaxel degeneration of the intraepidermal terminal arbors of primary afferent neurons is associated with an increased incidence of swollen and vacuolated axonal mitochondria in A-fibers and C-fibers [114].

4.2. Clinical and electrophysiological characteristics

Taxane-based therapy usually induces paresthesias, numbness and/or pain in a stocking-and-glove distribution. Decreased vibration perception and sense of position, loss of pain and temperature sensation and DTR impairment are also evident at clinical examination [115]. Electrophysiological abnormalities, mainly involving the decrease of SNAP or abolishment of sensory responses, indicate axonal sensory PN. Reduction of CMAP occurs at the highest cumulative doses, while sensory and motor NCV are usually spared. Motor involvement with distal or proximal weakness and myopathy are less frequently seen [108].

4.3. Incidence, severity and risk factors

Besides the NCI-CTC, the 11-item neurotoxicity subscale Functional Assessment of Cancer Therapy/Gynaecological Group Neurotoxicity (FACT/GOG-Ntx) and the FACT/Taxane scale have also been employed to evaluate the incidence and grade the severity of taxanes-induced PN. Recently, the TNS [116–118] as well as other modifications of the TNS have been used to assess the neurotoxicity secondary to paclitaxel-based treatment [25].

The administration of paclitaxel-containing regimens is consistently associated with an increased incidence of PN compared with docetaxel [119]. The incidence and severity of taxanes-induced PN is mainly related to the cumulative dose. Generally, severe taxane-induced PN occurs in patients receiving cumulative doses around 1000 mg/m^2 for paclitaxel and 400 mg/m^2 for docetaxel [120]. Table 5 summarizes phase III and neurotoxicity-focused taxanes trials.

Prior or concomitant administration of platinum compounds and pre-existing PN due to various medical conditions are also considered to increase the incidence and severity of taxane-induced PN. Moreover, the risk appears to be related to treatment schedules (weekly versus every three weeks treatment schedule) for paclitaxel [121]. However, a recent metanalysis does not support this view as it has been

Table 5
Incidence of taxanes-induced neuropathy. Selection criteria: phase III studies with specific neurologic assessment tools.

Author (year)	*n*	Chemotherapy schedule	Neurological assessment	Neuropathy incidence
Vasey [229]	532	Paclitaxel 175 mg/m^2 and carboplatin AUC 5 every 21 days for 6 cycles	NCI-CTC	Sensory Grade 1–2: 70 pts
			Neurotoxicity Score	Grade 3–4: 8 pts Motor Grade 1–2: 14 pts Grade 3–4: 2 pts
Winer [230]	152	Paclitaxel 210 mg/m^2 administered as a 3-hour infusion every 3 weeks	NCI-CTC	Grade 3–4 sensory: 19% Grade 3–4 motor: 11%
	149	Paclitaxel 250 mg/m^2 administered as a 3-hour infusion every 3 weeks	NCI-CTC	Grade 3–4 sensory: 33% Grade 3–4 motor: 14%
Smith [232]	279	Paclitaxel 250 mg/m^2 administered as a 24-hour infusion every 3 weeks	NCI-CTC	Grade 3–4 sensory: 7% Grade 3–4 motor: 7%
Argyriou [44]	21	Paclitaxel 175 mg/m^2 and carboplatin AUC 5 every 21 days for 6 cycles	TNS	Overall neurotoxicity: 14/21 pts (66.6%).
			NCS	TNS: 1–11 (mild): 28.6% 12–23 (moderate): 50% ≥ 24 (severe): 21.4%
Krzakowski [105]	275	Docetaxel 75 mg/m^2 1-hour infusion every 21 days for 6 cycles	NCI-CTC	Overall neurotoxicity: Grade 1–4: 15%.
Kruijtzer [231]	253	Docetaxel 100 mg/m^2 1-hour infusion every 21 days for 6 cycles	NCI-CTC	Grade 3–4 sensory: 2% Grade 3–4 motor: 1%
Vasey [229]	539	Docetaxel 75 mg/m^2 1-hour infusion every 21 days for 6 cycles	NCI-CTC	Sensory Grade 1–2: 44 pts
			Neurotoxicity Score	Grade 3–4: 2 pts Motor Grade 1–2: 8 pts Grade 3–4: 1 pts

NCI-CTC: National Cancer Institute-Common Toxicity Criteria; TNS: Total Neuropathy Score; NCS: nerve conduction studies; PN: peripheral neuropathy. For a description of the scales see Cavaletti et al. [242].

demonstrated that the incidence of serious adverse events, including PN, was significantly lower in weekly taxane schedules [122]. Recently published data show that elderly cancer patients are not at increased risk of developing taxanes-induced PN [123].

The use of an albumin-bound paclitaxel formulation (nab-paclitaxel) has been reported to induce a less severe course of CIPN. However, the results are still conflicting and the number of patients as well as the duration of their follow-up are rather limited [124–126].

4.4. Outcome

Generally, symptoms of taxane-induced PN improve or resolve within the first 3–6 months after the discontinuation of treatment [44]. However, severe symptoms may persist for a much longer time.

4.5. Options for neuroprotection

Strategies to prevent taxane-induced PN evaluated the use of several neuroprotective drugs, including thiols, neurotrophic factors and antioxidants, but none of them proved effective in large, controlled, clinical trials [127]. The clinical efficacy of amifostine has been tested with conflicting overall negative, results [115,128]. It was demonstrated in a small phase II clinical trial that glutamine is potentially able to preventing high dose paclitaxel-induced PN [129]. In a pilot phase II clinical trial, acetyl-L-carnitine significantly reduced the severity of paclitaxel-induced PN [130]. Two phase II randomized controlled trials, have shown that vitamin E at a daily dose of 600 mg bid may also exert neuroprotective effects in patients either treated with paclitaxel-based regimens alone or with paclitaxel/cisplatin regimens [131,132]. The results of a large international clinical phase III trial that sought to assess the efficacy of vitamin E against chemotherapy-induced PN, including taxane-induced PN, have recently been reported. In that setting, vitamin E did not appear to reduce the incidence of sensory neuropathy in patients receiving neurotoxic chemotherapy [133]. However, the negative results of this study conducted by researchers of the Mayo Clinic should be considered with caution, because of some significant methodological problems [134].

5. Epotilones

It was only in 1995 that a second class of MTSAs was discovered [135,136]. These macrolides were secondary

metabolites produced by mycobacteria and they were called "epothilones" to reflect their basic structural features, which include an *epo*xide moiety, a *thi*azole-containing side chain and a single ket*one* function. Two major fermentation products were reported in the *myxobacterium Sorangium cellulosum Soce 90*, epothilone A and epothilone B (also known as patupilone) [137,138].

In addition to these principal compounds, other metabolites were subsequently isolated from the fermentation of *Soce 90*: epothilones C-F. Of these, only the 12, 13-desoxyepothilones, called respectively epothilone C (desoxyepothilone A) and D (desoxyepothilone B, also known as KOS-862), were produced in significant amounts.

Although epothilones were originally described as natural fungicidal macrolides, interest significantly increased when their capacity to induce microtubule polymerization at submicromolar concentrations was identified in an *in vitro* screening program. These *in vitro* studies indicated that epothilones have a taxane-like effect on tubulin, inducing microtubule bundling, the formation of multipolar spindles, and mitotic arrest [139].

5.1. Pathogenesis

Epothilones, like paclitaxel, are able to induce *in vitro* the polymerization of tubulin dimers in microtubules in the absence of either guanosine triphosphate (GTP) and/or microtubule-associated proteins, and to stabilize preformed microtubules against conditions favoring depolymerization, including dilution, cold temperatures or Ca^{2+} [135,137,140].

Competition studies with paclitaxel have demonstrated that epothilones share either the same or an overlapping binding site on tubulin [135,137]. Kinetic experiments have revealed that these compounds are competitive inhibitors of paclitaxel binding to tubulin polymer. These findings suggest that the microtubule binding sites of paclitaxel and epothilones are largely overlapping or even identical, thus supporting the view that the mechanism of their neurotoxicity might be similar.

5.2. Clinical and electrophysiological characteristics

Similar to the observations performed with taxanes since the first phase I studies, PN has emerged as a potentially epothilone-associated severe toxicity [141]. However, its real impact and clinical features, as well as the identification of possible risk factors for severe neurotoxicity and the assessment of the final outcome of symptoms and signs, are still unclear. Despite the fact that sensory peripheral neurotoxicity is definitely the main toxic effect on the nervous system, the occurrence of motor or autonomic neuropathies also has occasionally been reported in the clinical trials (mostly phase II, Table 6).

No neurophysiological investigation has so far been reported in epothilone-treated patients.

5.3. Incidence, severity and risk factors

In the case of epothilones, PN has also generally been assessed using common toxicity scales, although the real incidence is difficult to assess since most of those patients exposed to the drug had been previously treated with other potentially neurotoxic drugs.

Moreover, this family of compounds is new and therefore the data reported so far are relatively scarce. Most of them concern ixabepilone (BMS-247550), a semi-synthetic epothilone B used in refractory breast cancer. A partial description of the ixabepilone-induced PN has been reported in more than 1000 patients [142–150].

In these studies the incidence of severe (i.e. grade 3/4 according to the NCI-CTC scale) sensory neuropathy ranged between 6% and 21%, while overall sensory neuropathy was reported in up to 71% of exposed patients. The relationship existing between the schedule of ixabepilone administration and the incidence of sensory neuropathy is not clear: for example, using the same schedule (40 mg/m^2 delivered via a 3-hour infusion every 3 weeks) an incidence of grade 3/4 sensory neuropathy of 6% has been reported in one study [150], while this value increased to 12% and 20–21% in other studies [142–148]. A possible reason for this discrepancy may reside in the schedule modifications that occurred during the course of the clinical trials, based on the emerging safety data.

The important issue of predicting the risk of severe ixabepilone-induced peripheral neurotoxicity was specifically addressed in breast cancer patients [151], but several methodological limitations in this study prevent the results obtained being considered as generally applicable in the management of epothilone-treated patients.

5.4. Outcome

A very important issue of ixabepilone-induced sensory neuropathy relates to its long-term outcome, since evidence from the published data show that symptoms and signs are reversible in most of the patients displaying neuropathy within weeks of drug treatment withdrawal, i.e. much earlier that in taxane-treated patients. However, the numerical data supporting this assumption are still relatively limited and observations should be extended. Moreover, no details regarding the outcome of patients with the poorest neurological profile are available, so that identification of potential risk factors for an unfavorable long-term course is not possible. In one study, it was stated that ixabepilone PN is irreversible, but the duration of observation after drug withdrawal was not indicated [142].

5.5. Other epothilones

The synthesis of epothilones has been the subject of extensive efforts by chemists and currently there have been more than 30 reports of synthesis of epothilones A and B in the literature.

Table 6
Incidence of ixabepilone-induced neuropathy. Selection criteria: prospective studies with neurologic assessment.

Author (year)	Patients (*n*)	Chemotherapy schedule	Neurological Assessment	Neuropathy Incidence
Eng [142]	25	40–50 mg/m^2, 1-hour infusion/3-hour infusion, every 3 weeks	NCI-CTC	Sensory Grade 1: 28% Grade 2: 4% Grade 3: 20%
Denduluri [143]	12	8–10 mg/m^2/day 1-hour infusion for 3 days, every 3 weeks	NCI-CTC	Sensory Grade 1: 16% Grade 2: 25%
Denduluri [144]	23	6 mg/m^2/day 1-hour infusion for 5 days, every 3 weeks	NCI-CTC	Sensory Grade 1: 39% Grade 2: 13% Motor Grade 2: 4% Grade 3: 4% Autonomic Grade 2: 4%
Dreicer [150]	45	40 mg/m^2, 3-hour infusion, every 3 weeks	NCI-CTC	Sensory Grade 3–4: 6% (no data about other grades)
Roche [146]	65	40–50 mg/m^2, 1-hour infusion/3 hour infusion, every 3 weeks	NCI-CTC	Sensory Grade 1-2: 51% Grade 3: 20% Motor Grade 2: 1% Grade 3: 5%
Perez [145]	126	40 mg/m^2, 3-hour infusion, every 3 weeks	NCI-CTC	Sensory Grade 1: 29% Grade 2: 30% Grade 3: 13% Motor Grade 1: 1% Grade 2: 7% Grade 3: 1%
Vansteenkiste [149]	146	50-40-32 mg/m^2, 3-hour infusion, every 3 weeks or 6 mg/m^2/day 1-hour infusion for 5 days, every 3 weeks	NCI-CTC	Sensory Grade 3: 5% Grade 4: 1% Any grade sensory neuropathy: 40%
Thomas [147]	49	40–50 mg/m^2, 1-hour infusion/3-hour infusion, every 3 weeks	NCI-CTC	Sensory Grade 1: 18% Grade 2: 33% Grade 3: 12% Motor Grade 2: 2% Autonomic Grade 2: 2%
Thomas [148]	369	40 mg/m^2, 3-hour infusion, every 3 weeks	NCI-CTC	Sensory Grade 1: 17% Grade 2: 27% Grade 3: 20% Grade 4: 1% Motor Grade 1: 4% Grade 2: 7% Grade 3: 5%
Burtness [236]	85	6 mg/m^2/day 1-hour infusion for 5 days, every 3 weeks or 20 mg/m^2/day 1-hour infusion on days 1, 8 and 15, every 4 weeks	NCI-CTC	Sensory Grade 3: 6% Sensory Grade 3: 20% Motor Grade 3: 11%
Bunnell [237]	62	40 mg/m^2, 3-hour infusion, every 3 weeks	NCI-CTC	Sensory Grade 1: 24% Grade 2: 31% Grade 3: 19% Motor Grade 3: 2%
O'Connor [238]	28	25 mg/m^2, 1-hour infusion weekly for 3 weeks, every 4 weeks	NCI-CTC	Sensory Grade 1: 21% Grade 2: 0% Grade 3: 7%
Baselga [239]	161	40 mg/m^2, 3-hour infusion, every 3 weeks	NCI-CTC	Overall neuropathy Grade 1:26% Grade 2:14% Sensory Grade 3: 1%
Ott [240]	24	20 mg/m^2/day 3-hour infusion on days 1, 8 and 15, every 4 weeks	NCI-CTC	Overall neuropathy Grade 1:29% Grade 2:8% Grade 3: 4%
Huang [241]	87	6 mg/m^2/day 1-hour infusion for 5 days, every 3 weeks	NCI-CTC	Sensory Grade 1: 32% Grade 2: 40% Grade 3: 16%

NCI-CTC: National Cancer Institute-Common Toxicity Criteria. For a description of the scale see Cavaletti et al. [242].

Only one phase II clinical trial has been performed using KOS-862; in that study 71% of the treated patients developed sensory neuropathy (NCI-CTC grade 3 = 10%). Motor neuropathy was present in about 5% of the patients and ataxia (of unknown origin) in 8% of the patients [152].

The first fully synthetic third-generation epothilone B derivative, ZK-EPO (sagopilone) is currently under clinical investigation in a trial designed to assess its activity and PN.

6. Bortezomib

Bortezomib, a modified dipeptidyl boronic acid, is one of the first-line treatments in multiple myeloma (MM) patients. Bortezomib inhibits the 20S proteasome complex and acts by disruption of various cell signaling pathways. However, its use is frequently associated with the development of significant neurotoxic effects. Bortezomib-induced PN is, in most cases, painful and when it occurs it can potentially lead to dose modification and severe disability, thereby compromising the outcome of patients with MM [153].

6.1. Pathogenesis

The pathogenesis of bortezomib-induced PN remains largely unknown and only recent results from experimental studies have contributed to a better understanding. Overall, it seems that DRG represent the anatomical structures which are particularly susceptible to significant cellular changes secondary to bortezomib administration. This is because of the structure of their capillaries that allow free passage of molecules between the circulation and the extracellular fluid [153–155].

This hypothesis has been supported by a study in animal models demonstrating that bortezomib exerts significant neuronal dysfunction characterized by interference with transcription, nuclear processing and transport, and cytoplasmic translation of mRNAs in DRG neurons [156]. Histopathological and neurophysiological findings show that the neuronopathy secondary to bortezomib use mostly evokes a dose-dependent reduction of large and C-fibers with abnormal vesicular inclusion body in unmyelinated axons [157,155].

Mitochondrial and endoplasmic reticulum damage and also dysregulation of neurotrophins, mainly by either bortezomib-induced activation of the mitochondrial-based apoptotic pathway or inhibition of the transcription of nerve growth factor-mediated neuron survival (through interference with the NF-κB pathway), have also been proposed as potentially playing a significant role in the genesis of bortezomib-induced PN [158,159].

6.2. Clinical and electrophysiological characteristics

Distal painful sensory PN represents the clinical hallmark of bortezomib-induced PN. Affected patients experience neuropathic pain, distal sensory loss in a stocking-and-glove distribution, hyporeflexia or even areflexia and disturbed proprioception [160]. Most cases will self-report neuropathic pain as of moderate to severe intensity with a mean VAS of 8 [161].

Quantitative sensory testing shows abnormalities consistent with a painful neuropathy due to dysfunction in all three major fiber types (i.e. Aβ, Aδ, and C) of the sensory nerves [161]. Common findings from the conventional nerve conduction studies are SNAP changes [153]. Myelin changes with distal slowing of sensory NCV may also be present as part of degeneration of fast-conducting fibers secondary to an initial dysfunctional neuronopathy [157]. Motor nerve conduction studies evidencing reduced CMAP can be occasionally observed.

6.3. Incidence, severity and risk factors

Most of the available studies assessing bortezomib-induced PN have employed the NCI-CTC criteria or the FACT/GOG-Ntx subscale [162,163]. Recent studies have also adopted different TNS versions to assess the severity of bortezomib-induced PN [164,165].

The reported data (Table 7) indicate that bortezomib-induced PN (any grade) may occur in about half of the patients receiving treatment with this drug [166]. The cumulative dose of bortezomib has also been consistently associated with an increased incidence of bortezomib-induced PN. However, patients with recurrent disease may develop more severe bortezomib-induced PN than those with newly diagnosed disease. In fact, pooled safety data reveal that up to 75% of patients receiving bortezomib for recurrent disease are at risk of developing NCI-CTC grade 1–2 bortezomib-induced PN as opposed to 33% of patients with newly diagnosed MM. Severe neurotoxicity (grade 3–4) may occur in up to 30% of patients with recurrent MM as opposed to about 20% of patients with newly diagnosed disease [167].

Several risk factors have been proposed to be associated with increased incidence of bortezomib-induced PN, including MM-associated neuropathy, pre-existing neuropathy of other origin, age and comorbidities such as diabetes mellitus [163,164,168]. A recently published study enrolling 58 relapsed/refractory MM patients treated with bortezomib highlighted that that absence of neurological monitoring and prior treatment with vincristine were associated with greater risk of bortezomib-induced PN. From this study it also emerged that patients with a baseline total neurology score – clinical version (TNSc) score above 2 are at increased risk for developing more severe bortezomib-induced PN [165].

6.4. Outcome

Symptoms of bortezomib-induced PN usually improve or completely resolve after three to four months following discontinuation of treatment, as evidenced by the long-term data of the phase III APEX trial analyzed to assess the reversibility of bortezomib-associated PN in MM patients.

Table 7
Incidence of bortezomib-induced neuropathy. Selection criteria: phase III studies with specific neurologic assessment tools.

Author (year)	Patients (*n*)	Chemotherapy schedule	Neurological assessment	Neuropathy incidence
Richardson [2]	256	1.0 or 1.3 mg/m^2 iv bolus on days 1, 4, 8, and 11, every 21 days, for up to eight cycles	FACT/GOG-Ntx	Grade 1–2: 22%
			Neurological examination	Grade 3–4: 13.4% dose reduction in 12% and discontinuation in 5% of pts
Richardson [233]	331	1.3 mg/m^2 iv bolus on days 1, 4, 8, and 11 for eight three-week cycles, followed by treatment on days 1, 8, 15, and 22 for three five-week cycles	FACT/GOG-Ntx	Any grade: 124 (37%)
			Neurological examination	Grade 3–4: 30 (9%) discontinuation in 8% of pts
Richardson [234]	64	1.3 mg/m^2 on days 1, 4, 8, and 11, for up to eight 21-day cycles	Neurologic and neurophysiological examination, measurement of intraepidermal nerve fiber density	64% of patients (grade 3 in 3%)
Chaudhry [235]	27	1.3 mg/m^2 on days 1, 4, 8, and 11, for up to eight 21-day cycles; median cumulative dose of 35.6 mg/m^2	TNSr NCS	26/27 pts; median TNSr 10, range 1–24; 11 were grade 1, 10 grade 2, 5 grade 3, and none grade 4 (TNSr 2–8 = grade 1; TNSr 9–16 = grade 2; TNSr 17–24 = grade 3; and TNSr 25–32 = grade 4)
Velasco [165]	24	1.3 mg/m^2 on days 1, 4, 8, and 11, for up to eight 21-day cycles; cumulative dose of 27.37 ± 11.42	TNSc	Grade 1–2: 10(41.7%)
			TNSr NCS NCI-CTC	Grade 3–4: 4 (16.6%) Dose reduction in 8 (33%) Median TNSc 7 (range 5–12) Median TNSr 11 (range 5–16)

FACT/GOG-Ntx: Functional Assessment of Cancer Therapy Scale/Gynecologic Oncology Group-Neurotoxicity; NCS: nerve conduction studies; TNS: Total Neuropathy Score; TNSc: TNS-clinical versión; TNSr: TNS reduced version PN: peripheral neuropathy; NCI-CTC: National Cancer Institute-Common Toxicity Criteria. For a description of the scales see Cavaletti et al. [244].

This study demonstrated that 64% of patients with at least NCI-CTC grade 2 bortezomib-induced PN experienced improvement or resolution of symptoms compared to baseline at a median of 110 days [169].

6.5. Options for neuroprotection

Several pharmacological agents have been tested to treat bortezomib-induced PN, including the administration of various opioids, tricyclic antidepressants, anticonvulsants, serotonin-norepinephrine reuptake inhibitors, non-steroidal anti-inflammatory agents, vitamins and nutritional supplements, given either as monotherapy or in combination regimens [153], but no evidence-based conclusions can be provided regarding their effectiveness.

Since there is no evidence for any effective prophylactic treatment, strict adherence to the dose modification guidelines, as presented in Table 8, is recommended to reduce the risk of severe bortezomib-induced PN [170].

Table 8
Recommended[a] schedule modifications for bortezomib-related neuropathy.

Severity of neuropathy	Schedule modification
Grade 1 (paresthesias, weakness and/or loss of reflexes) with no pain or loss of function	No action
Grade 1 with pain or Grade 2 (interfering with function but not with activities of daily living)	Reduce to 1.0 mg/m^2
Grade 2 with pain or Grade 3 (interfering with activities of daily living)	Withhold bortezomib treatment until symptoms of toxicity have resolved. When toxicity resolves re-initiate bortezomib treatment and reduce dose to 0.7 mg/m^2 and change treatment schedule to once per week.
Grade 4 (sensory neuropathy which is disabling or motor neuropathy that is life threatening or leads to paralysis) and/or severe autonomic neuropathy	Discontinue bortezomib

Adapted from: http://www.ema.europa.eu/humandocs/PDFs/EPAR/velcade/emea-combined-h539en.pdf.

[a] Based on schedule modifications in Phase II and III multiple myeloma studies and post-marketing experience.

7. Thalidomide

Thalidomide was first introduced into European markets in the 1950s as a sleep aid and antiemetic drug for pregnant

women. It was withdrawn from the market soon after when its teratogenic effects were discovered. It has re-emerged recently as an effective treatment for several dermatological, gastrointestinal, and oncological conditions. In May 2006 the US Food and Drug Administration (FDA) granted approval to thalidomide for use in combination with dexamethasone in newly diagnosed MM patients. PN is now recognized as one of the most significant side effects of this medication.

7.1. Pathogenesis

The exact mechanism of the anti-malignancy action of thalidomide is not known, but it is possible that it acts through angiogenesis inhibition, immunomodulation and cytokine modulation – individually or in combination. Similarly, the pathogenesis of thalidomide-induced PN damage is also unknown. The structure of thalidomide is characterized by a 3-substituted glutarimide ring and a phthalimide ring. Both rings are prone to enzymatic or non-enzymatic hydrolysis [171,172]. Several *in vitro* and *in vivo* studies have been performed to elucidate the structures of the metabolites formed, identify the enzymes responsible for their production and assess interspecies differences in metabolism, which may account for differences in the activity and toxicity of thalidomide. However, no conclusive results have been achieved regarding PN.

7.2. Clinical and electrophysiological characteristics

The most common presentation of thalidomide-induced PN is distal paresthesias and/or dysesthesias with or without sensory loss [172]. Physical examination may be normal or show mild reduction in sensation in distal limbs. Strength is usually preserved, although mild weakness may be present. DTR, particularly ankle jerks, may be depressed or absent. Symptoms can be disabling and often necessitate discontinuation of the drug despite disease control. Onset is usually delayed after initiating thalidomide, mostly depending on the schedule of treatment. On a neurophysiological basis, reduction in SNAP with relative preservation of CMAP and NCV are typical findings [173], suggesting a sensory axonal neuropathy as the predominant pathological event.

Alternatively, some evidence suggests that thalidomide may also cause a DRG neuronopathy. In these patients the clinical presentation is rather different, with an early involvement of all four limbs. Using MRI, T2 hyperintense, non-mass-like, non-enhancing lesions may be seen in the posterior columns of the spinal cord of these patients, indicating the involvement of the centripetal branch of the primary sensory neuron axons [174].

7.3. Incidence, severity and risk factors

Somewhat conflicting results regarding the relationship of thalidomide dosage and incidence of PN have been reported. Although some studies found a relationship between cumulative dose and occurrence of PN [173], others failed to do so [175–177]. Alternatively, increased risk of thalidomide-induced PN has been related to daily dose of thalidomide [175]. Risk factors for PN in thalidomide-treated patients have not been elucidated and most studies failed to find a correlation between age, thalidomide administration and occurrence of CIPN [175–178]. Preliminary clinical data suggest that the thalidomide analogues lenalidomide and pomalidomide are more potent and have a better toxicity profile, including reduced risk of inducing CIPN [172].

7.4. Outcome

The issue of the long-term course and final outcome of thalidomide-induced PN has not yet been extensively investigated. However, when specifically studied it appeared that the neuropathic symptoms may frequently improve on discontinuation of thalidomide [179,180].

7.5. Options for neuroprotection

No effective pharmacological intervention is available to prevent thalidomide-induced PN and dose reduction or withdrawal is warranted if it occurs.

7.6. Immunomodulatory drugs (IMiDs)

Newer classes of α-phthalimidoglutarimides are designated as IMiDs and they include lenalidomide and pomalidomide.

In 2005, lenalidomide was FDA-approved for the treatment of myelodysplastic syndrome in patients with deletion 5q cytogenetic abnormality and in 2006 for use in combination with dexamethasone in patients with relapsed MM. Pomalidomide is currently in clinical development for the treatment of haematological malignancies.

An open-label randomized phase II study in 102 heavily pre-treated, relapsed or refractory MM patients evaluated the efficacy and safety of two lenalidomide dosing regimens, 15 mg twice daily or 30 mg once daily on days 1–21 of a 28 day cycle [181]. The incidence of treatment-emergent PN was 23% in the twice-daily arm versus 10% for the once-daily arm, with 3% NCI-CTC grade 3 neuropathy.

The use of lenalidomide plus dexamethasone was also investigated in two phase III studies. In the lenalidomide/dexamethasone groups the incidence of grade 3–4 PN was 1.7% versus 1.1% in the placebo/dexamethasone arm in one study and 6.9% in both arms in the second ones [182–184].

In a single-centre study, 78 patients with recurrent MM who had received bortezomib were retrospectively reviewed for the incidence, severity and risk factors for PN [160]. In 6 out of 9 patients who experienced PN with bortezomib, two weeks after switching treatment to lenalidomide, a significant unexpected improvement in PN symptoms was observed, including 3 patients who were able to stop analgesics,

although a clear causal relationship between lenalidomide treatment and PN course could not be established.

8. Conclusion

From this review it appears that CIPN understanding still represents an unmet clinical need in the modern approach to cancer patients. Its importance has become even clearer in view of the better results obtained with more effective schedules of treatment allowing longer survival and cancer cure, thus making the occurrence of a disabling and long-lasting peripheral neurotoxicity even less tolerable.

To give some relief to CIPN patients, several palliative care attempts have been suggested, but their effectiveness is still unclear, but overall unsatisfactory. Generally, pharmacological symptomatic treatments have targeted the painful component of CIPN, with some partial demonstration of efficacy [69,185]. More recently, non-pharmacological treatment [186] and physical therapy have also been suggested in an attempt to achieve a functional recovery from severe limitations secondary to sensory impairment and not only to increase strength [187]. This sensory training is an intriguing rehabilitative approach in ataxic patients [188], although the investigation on its validity in CIPN is still at its earliest stages.

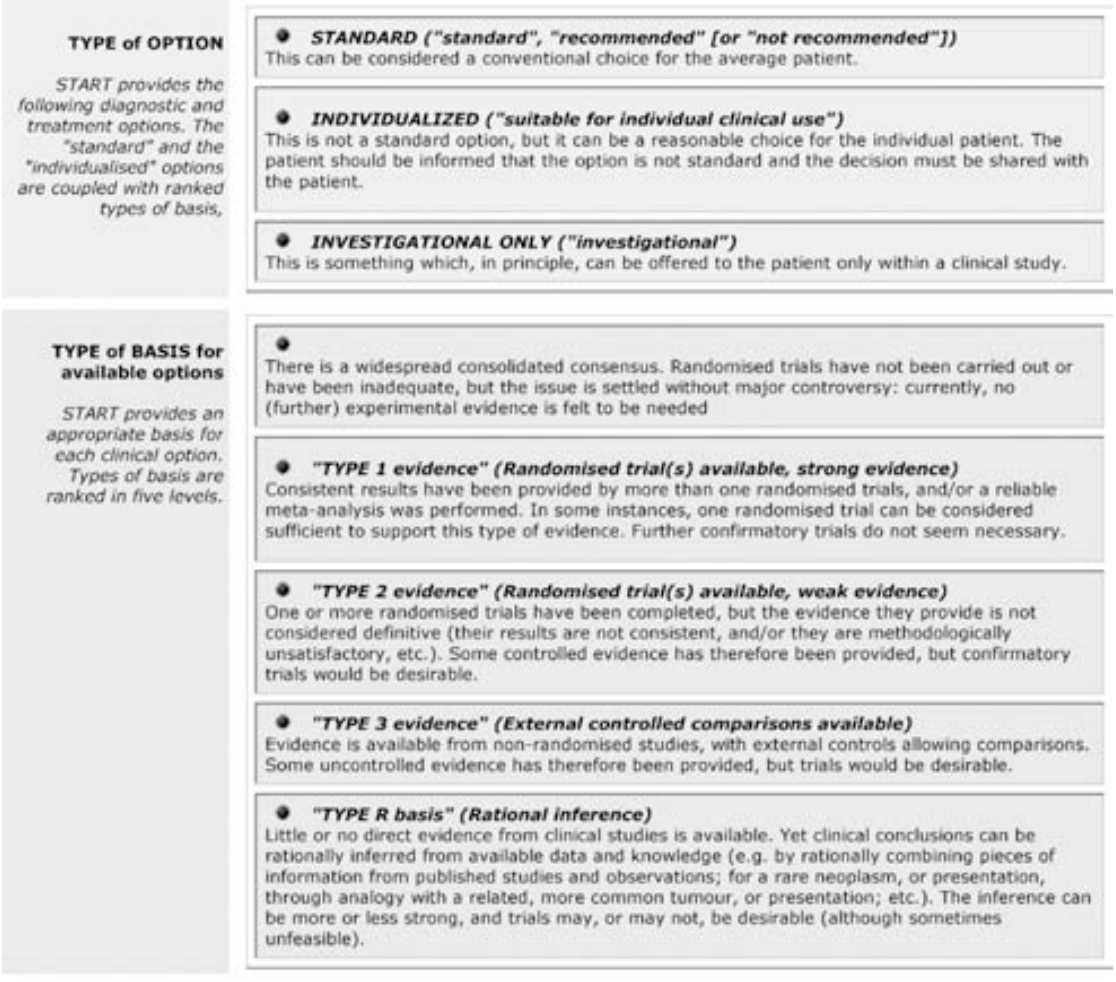
A deeper knowledge should, therefore, be achieved and this goal may be accomplished only through an extensive collaboration between all the healthcare professionals involved in cancer patient management, focused on the common aim to optimize cancer treatment while minimizing potentially disabling side effects such as CIPN.

Reviewers

Professor David Cornblath, Johns Hopkins University, Department of Neurology, Baltimore, MD, United States.

Professor Paolo Bidoli, Chief of Medical Oncology, S. Gerardo Hospital, Department of Medical Oncology, Monza, Italy.

Professor Wolfgang Grisold, Kaiser-Franz-Josef-Spital, Department of Neurology, Kundratstrasse 3, A-1100 Vienna, Austria.



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Biographies

Andreas A. Argyriou is Consultant Neurologist at the Department of Neurology, "Saint Andrew's" General Hospital of Patras and researcher at the Division of Clinical Oncology-Department of Medicine of the University Hospital of Patras, Greece

Jordi Bruna is a specialist of the Unit of Neuro-Oncology, Department of Neurology, Bellvitge University Hospital, Hospitalet del Llobregat and Group of Neuroplasticity and Regeneration, Department of Cell Biology, Physiology and Immunology, Universitat Autonoma of Barcelona.CIBERNED. Spain

Paola Marmiroli is a Neurologist and assistant professor at University of Milan-Bicocca, Monza, Italy Department of Neuroscience and Biomedical Technologies.

Guido Cavaletti is the head of the Experimental Neurology Unit at University of Milan-Bicocca, Department of Neuroscience and Biomedical Technologies and Senior Consultant Neurologist at the Department of Neurology of the S. Gerardo Hospital in Monza, Italy.

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3.1. Platinum derivatives-induced peripheral neuropathy

Jordi Bruna Escuer
Unit of Neuro-Oncology, Department of Neurology, Hospital Universitari de Bellvitge Hospitalet de Llobregat, and Group of Neuroplasticity and Regeneration (CIBERNED) Institute of Neurosciences, Universitat Autònoma de Barcelona, Spain

Drug history and clinical use

Since platinum compounds, inorganic heavy metal drugs, were identified as antineoplastic agents, their use has been increasing in routine oncological clinical practice over the years. It is estimated that as many as 50-70% of cancer patients are treated with platinum compounds (Dyson and Sava, 2006). The first employed platinum drug, cisplatin, has been used since 1969 (Windebank, 1999) and remains the first-line treatment of germ cell tumors. Moreover, it is used as an adjunctive therapy in ovarian, cervical, medulloblastoma, bladder, head and neck, esophageal and lung cancers. Cisplatin is administered intravenously, mostly in a dosage range of 20-100 mg/m^2 in different schedules depending on the primary tumor and the adjunctive therapy (Table 1) (Chu and DeVita, 2009). Since the 1970s, several platinum analogs have been tested for anti-tumor-cell activity and to improve the adverse event profile.

Correspondence/Reprint request: Dr. Jordi Bruna Escuer, Unit of Neuro-Oncology, Department of Neurology Hospital Universitari de Bellvitge, Hospitalet de Llobregat, and Group of Neuroplasticity and Regeneration (CIBERNED), Institute of Neurosciences, Universitat Autònoma de Barcelona, Spain.
E-mail:

Table 1. Schedules with platinum-drug treatments in ordinary clinical practice.

CISPLATIN	
Ovarian cancer:	75 mg/m^2 iv on day 1 every 21 days combined with paclitaxel 100 mg/m^2 iv on day 1 every 21 combined with cyclophosphamide
Testicular cancer:	20 mg/m^2 iv on days 1-5 every 21 days combined with etoposide and bleomycin
Non-small cell lung cancer:	60-100 mg/m^2 iv on day 1 every 21 days combined with etoposide or gemcitabine
Head and neck cancer:	20 mg/m^2 /day in continous iv infusion for 4 days
CARBOPLATIN	
Previously untreated patients:	Target AUCs are between 5 and 7 mg/ml/min
Previous treated patients:	Target AUCs are between 4 and 6 mg/ml/min
OXALIPLATIN	
Colon cancer:	85 mg/m^2 iv on day 1 every 14 days combined with leucovorin and 5Fu or combined with leucovorin, 5-Fu and bevacizumab Alternatively can be used 100-130 mg/m^2 iv every 21 days

One of the second generation of platinum drugs, carboplatin, emerged and underwent its first clinical trials in 1985 being fully implemented into clinical practice in 1989. At present, it is the drug of choice for ovarian cancer treatment as well being extensively used in breast and metastatic lung cancer. Moreover, some treatments for germ cell, bladder, endometrial and head and neck tumors may include schedules with carboplatin as adjuvant therapy. Carboplatin is also administered intravenously, and dosage is calculated according to a target area under the curve (AUC) based on the glomerular filtration rate instead of mg/m^2 of body surface. Target AUC is usually between 5 and 7 mg/mL/min for previously untreated patients and between 4 and 5 mg/mL/min for previously treated patients (Table 1) (Chu and DeVita, 2009).

Oxalipatin, a third generation platinum analog and among the most recent platinum drugs to emerge, has been incorporated in combination as a first-line drug for the treatment of colorectal cancer during the last decade. In addition, it is being extensively evaluated for the treatment of gastrointestinal and ovarian malignancies. Oxaliplatin is administered intravenously and recommended doses range from 85 to 130 mg/m^2, depending on the treatment cycle duration (Table 1) (Chu and DeVita, 2009).

While the general toxicity profile differs among these compounds, platinum-induced peripheral neuropathy is a common event in all of them, with only some patterns being distinctive to the individual agents. In an attempt to reduce the toxic profile of these drugs, other platinum drugs have been developed and are currently under assessment. At present, satraplatin, an orally administered compound tested against hormone refractory prostate

cancer, and lipoplatin, a liposomal cisplatinum formulation, are being assessed in phase III trials (Boulikas, 2008; Sternberg et al., 2009, Stathopoulos et al., 2010). Finally, at least five other platinum drugs (aroplatin, miriplatin, prolindac, picoplatin and nedaplatin) are currently undergoing phase I and II assessment.

Clinical aspects of neurotoxicity

The earliest signs and symptoms observed in platinum-treated patients are reduced vibratory sensitivity in the toes and loss of ankle jerks, associated with numbness, tingling or paresthesias in fingers and toes. Prolonged treatment may worsen symptoms and signs, with a generalized loss of deep tendon reflexes and more proximal vibratory sensitivity impairment. Feet and legs are usually more severely affected than fingers and hands. Pin and temperature sensation, joint position and light touch perception are less severely affected. In the worst cases the loss of propioception may result in ataxic gait (Roelfols et al., 1984; Thomson et al., 1984; Daugaard et al., 1987; Mollman et al., 1988; Krarup-Hansen et al., 2007). Lhermitte's phenomenon secondary to spinal cord dorsal column injury by anterograde degeneration of sensory fibers from damaged dorsal root ganglia cells is occasionally observed (Eeles et al., 1986; Argyriou et al., 2007a; Jurado et al., 2008; Park et al., 2009). This phenomenon occurs more frequently with oxaliplatin- than cisplatin-treated patients. Motor impairment is not detectible, although patients with clumsiness due to sensory disturbances may report this sensation as muscular weakness. Dysautonomic symptoms have not developed with routine clinical schedules (Vandertrop et al., 1996, Krarup-Hansen et al., 2007), although impotence has anecdotally been reported in combined regimens with cisplatin and vinblastine (Hansen, 1990).

Oxaliplatin-induced peripheral neuropathy presents as two distinct clinical syndromes: a chronic cumulative sensory neuropathy with the typical features of platinum drug peripheral neuropathy, and an acute transient syndrome characterized by transient paresthesias in the distal extremities and perioral region, typically triggered or exacerbated by exposure to cold that usually appears during or within hours after infusion. Although cold hyperalgesia is manifested during and between oxaliplatin infusions (sustained from an accumulated dose of 255 mg/m^2), neither hyperalgesia nor allodina to mechanical stimuli is presented (Attal et al., 2009).

A smaller number of patients present slurred speech, jaw pain during chewing, dysesthesias of the pharynx that may cause subjective dysphagia or difficulty in breathing, and paresthesias in the extremities or calf cramps with

walking that tend to persist for days to weeks (de Gramont et al., 2000; Wilson et al., 2002; Argyriou et al., 2008; Park et al., 2009; Attal et al., 2009). Less frequently, this acute sensory syndrome is accompanied by acute motor symptoms (13%) such as fasciculations, episodic muscle cramps, and delayed relaxation of the finger extensor muscles after nerve percussion. Neurologic examination within the first days after oxaliplatin infusion typically shows no change in strength, sensation or deep tendon reflexes (Wilson et al., 2002; Lehky et al., 2004; Krishnan et al., 2005).

Incidence

Platinum-induced peripheral neuropathy is related to total cumulative dose (Thompson et al., 1984; van der Hoop et al., 1990a; Gregg et al., 1992; Glendenning et al., 2010) and to dose-intensity treatment (Mollman et al., 1988; Cavaletti et al., 1992).

Using cisplatin, peripheral neuropathy ensues after the administration of 250-350 mg/m^2 (Thompson et al., 1984; Glendenning et al., 2010) while, at a cumulative dose of 500-600 mg/m^2, almost all patients have objective evidence of neuropathy (Roelofs et al., 1984) which is severely disabling in at least 10% of patients (van der Hoop et al., 1990b). Although preventive therapeutic strategies such as hypertonic saline, antiemetics and use of granulocyte-colony stimulating factor minimize most of the hematological and non-hematological adverse events of cisplatin, peripheral neurotoxicity remains one of the the major dose-limiting side effects. Recently, reduced neurotoxicity with the use of a liposomal formulation of cisplatin (lipoplatin) has been reported (Jehn et al., 2008), although this result has not been clearly shown in another phase III trial with lipoplatin (Stathopoulos et al., 2010). Likewise, the new platinum compound satraplatin has shown no significant differences in the incidence of neuropathy with regard to any chemotherapeutic treatment arm (Sternberg et al., 2009). The peripheral neuropathy incidence ranges of the main studies with cisplatin are summarized in Table 2.

Carboplatin-induced peripheral neuropathy is less frequent (occurring in about 10-20% of treated patients) and severe at conventional doses than in cisplatin-treated patients, with myelosupression being the main dose-limiting side effect (Adams et al., 1989; Gurney et al., et al., 1990; van der Hoop et al., 1990b; Swenerton et al., 1992). However, at high dose areas under the curve (AUC)>6, carboplatin produces higher rates of neuropathy with the characteristics of cisplatin neuropathy (Cavaletti et al., 1998).

Table 2. Incidence of cisplatin-induced neuropathy. Selection criteria: phase III studies and prospective studies with specific neurologic assessment tools, excluding patients with other neurotoxic agents.

Author (year)	Patients (n)	Schedule	Dose-intensity Planned (mg/m^2/week)	Neurological Assessment	Neuropathy Incidence
PHASE III STUDIES					
Crinò (1988)	150	120 mg/m^2 every 21 days	40	WHO	PN in 3.3%
van der Hoop (1990)	195	100 mg/m^2 every 36 days	20	WHO	Grade 1: 20.5% Grade 2: 23.5% Grade 3: 3.6%
	97	75 mg/m^2 every 21 days	25		Grade 1: 25% Grade 2: 15% Grade 3: 5%
Kawahara (1991)	80	80 mg/m^2 every 28 days	20	WHO	Grade 2: 2.5% Grade 3: 0%
Swenerton (1992)	210	75 mg/m^2 every 28 days	25	ECOG criteria	Grade 1: 18.1% Grade 2: 7.3% Grade 3: 2.4%
Cartei (1993)	52	75 mg/m^2 every 21 days	25	NCI-CTC	Grade 2: 11.5% Grade 3: 3.8%
Gandara (1993)	103	50 mg/m^2 on days 1 and 8 every 28 days for 8 cycles	25	SWOG criteria	PN in 4%
	216	100 mg/m^2 on days 1 and 8 every 28 days for 4 cycles	50		PN in 2.8%
Kemp (1996)	120	100 mg/m^2 every 21 days for 6 cycles	33.3	NCI-CTC	Grade 1: 25.8% Grade 2: 29.2% Grade 3: 12.5%
Wozniak (1998)	200	100 mg/m^2 every 28 days for 4 cycles	25	SWOG criteria	Grade 1-2: NA Grade 3-4: 0.5%
Sutton (2000)	90	20 mg/m^2 during 5 every 21 days for 8 cycles	33.3	GOG criteria	Grade 2: 7.8% Grade 3: 12.2% Grade 4: 1.1%
Gatzemeier (2000)	206	100 mg/m^2 every 21 days	33.3	NCI-CTC QLQ-C30	Grade 1-2: 11.6% Grade 3-4: 0.4%
Wachters (2003)	119	80 mg/m^2 every 21 days for 5 cycles	26.7	NCI-CTC QLQ-C30	Grade 1: 14% Grade 2 : 8% Grade 3-4: 1%
Bacon (2003)	73	75 mg/m^2 every 21 days	25	NCI-CTC QLQ-C30	Grade 1-2: 46.6% Grade 3-4: 2.7%
Fleming (2004)	129	50 mg/m^2 every 21 days for 7 cycles	16.7	NCI-CTC FACT/GOG-Ntx	Grade 1-2: 3.1% Grade 3-4: 0.8%

Table 2. Continued.

Jehn (2008)	21	100 mg/m^2 every 21 weeks for 6 cycles	33.3	NCI-CTC	Grade 1: 33.3% Grade 2: 33.3% Grade 3: 19% Grade 4: 0%
Homesley (2009)	261	50 mg/m^2 every 21 days for 6 cycles	16.7	NCI-CTC FACT/GOG-Ntx	Grade 1: 24.9% Grade 2: 3.1% Grade 3: 1.9% Grade 4: 0%
Liposomal cisplatin					
Jehn (2008)	25	100 mg/m^2 on days 1,8,15 every 21 days for 6 cycles	100	NCI-CTC	Grade 1: 27% Grade 2: 0% Grade 3: 0% Grade 4: 4%
Stathopoulos (2010)*	114	200 mg/m^2 every 2 weeks for 9 cycles. After first cycle 100 mg/m^2	50 first cycle 25 resting cycles	NCI-CTC	Grade 1-2: 44.7% Grade 3-4: 0.9%
STUDIES WITH SPECIFIC NEUROLOGICAL ASSESSMENT TOOLS					
van der Hoop (1990)	13	75 mg/m^2 every 21 days for 6 cycles	25	Neurological Clinical scale VPT	Symptoms Sum Score:1.75±0.55 Signs Sum Score: 7.42±1.3
Thompson (1984)	11	50 mg/m^2 every 28 days	12.5	Neurological clinical assessment NCS	PN in 91%
Roelofs (1984)	24	60 mg/m^2 every 28 days	15	Neurological clinical assessment NCS Sural biopsy	PN in 92%
Daugaard (1987)	8	40 mg/m^2 every 7 days for 3 cycles	40	NCS	PN in 100%
Mollman (1988)	19 11	50 mg/m^2 every 28 days for 9 cycles 40 mg/m^2 every 28 days for 9 cycles	12.5 10	Neurological clinical assessment NCS	PN in 47% PN in 100%
Sebille (1990)	52	25 mg/m^2 during 4 days every 21 days	33.3	NCS	14% had an increase in the latency H reflex 9% had a decrease in the sensory conduction velocity
Cavaletti (1992)	20 40	75 mg/m^2 every 21 days for 6 cycles 50 mg/m^2 every 7 days for 9 cycles	25 50	NSS NDS NCS	PN:38.6% PN: 16.6%

Table 2. Continued.

LoMonaco (1992)	16	40 mg/m^2 during 5 days every 28 days for 3 cycles	50	Neurological clinical assessment NCS	PN in 89%
van den Bent (2002)	33 47	50 mg/m^2 on days 1, 8, 15 and 29, 36, 43 70 mg/m^2 on days 1, 8, 15 and 29, 36, 43	42 58.8	Questionnaire for neurological symptoms VPT NCI-CTC	NCI.CTC all group Grade 1: 55% Grade 2: 15% Grade 3: 3.8%
Pace (2003)	14	Several	--	NSS WHO	PN in 85.7% NSS: 4.7±2.9
Pace (2010)	24	NA	NA	TNS NCS	TNS: 4.1±4.5

WHO: World Health Organization. ECOG: Eastern Cooperative Oncology Group. NCI-CTC: National Cancer Institute-Common Toxicity Criteria. SWOG: South West Oncology Group. GOG: Gynecologic Oncology Group. PN: peripheral neuropathy. QLQ-C30: European Organization for the Treatment of Cancer quality of life questionnaire-30 items. FACT/GOG-Ntx: Functional Assessment of Cancer Therapy Scale/Gynecologic Oncology Group-Neurotoxicity scale. VPT: vibration perception threshold. NCS: nerve conduction studies. NSS: Neurological Symptom Score. NDS: Neurological Disability Score. TNS: Total Neuropathy Score. NA: not available.*: In this study, both treatment arms received identical dose of adjuvant paclitaxel.

Combination schedules of platinum compounds with other neurotoxic antineoplastic drugs have proved to be effective in several tumors, becoming first-line therapies for treatment of ovarian and lung cancer in many centers. However, the use of cisplatin or carboplatin with taxanes has been reported to induce higher peripheral neuropathy incidence and severity even at lower cumulative doses of each antineoplastic agent (Rowinsky et al., 1993; Chaudhry et al., 1994; Hilkens et al., 1997; Gatzemeier et al., 2000; Markman et al., 2001; Bacon et al., 2003; Argyriou et al., 2005 and 2007b; Homesley et al., 2009), although this result has not been confirmed by other authors (Cavaletti et al., 1997; Verstappen et al., 2003). In the same way, when platinum compounds are combined with vinorelbine, a higher rate of mild neuropathy is observed (Comella, 2001; Winton et al., 2005). However, which drug accounts for the main neuropathic effect, or whether this increased incidence is due to synergistic drug effects, are unresolved questions.

Retreatment with platinum drugs is a feasible option in some patients. One study demonstrated that weekly cisplatin schedules (50-70 mg/m^2) up to a cumulative dosage of 450 mg/m^2 were safe in patients previously treated with carboplatin or cisplatin, despite prior higher cumulative dosages, if the

retreatment was given with an interval in between. However, the duration of this safety interval was not specified in the study (van den Bent et al., 2002).

Frequency and severity of neuropathy induced by oxaliplatin vary depending on the extent of acute or chronic neurotoxicity. Oxaliplatin-induced acute symptoms are transiently present in the vast majority of patients and are usually reversible within hours or days; there is, however, an increase in both duration and severity with repeated exposure to oxaliplatin, and the severity becomes more difficult to establish. Several explanations must be considered for this. Firstly, cold-related symptoms are not well reflected in commonly employed neurotoxicity scales, and the scales addressed to this specific issue have not been validated; secondly, some studies do not differentiate between acute and established neurotoxicity (Andre et al., 2004). Conversely, chronic peripheral neuropathy persists between cycles and severity increases with cumulative dose; its incidence is routinely evaluated in most oxaliplatin-trials (de Gramont et al., 2000; Rothenberg et al., 2003; Argyrou et al., 2007a; Al-Batran et al., 2008; Park et al., 2009). Severe neuropathies (grade ≥ 3 using NCI.CTC scale) have been observed with cumulative doses ranging from 510-765 mg/m^2 in up to 10% of patients, between 765-1020 mg/m^2 in up to 30%, and in 50% of patients with doses higher than 1000 mg/m^2 (de Gramont et al., 2000; Souglakos et al., 2002). Interestingly, combinatory oxaliplatin regimens adding bevacizumab for metastatic colorectal cancer, an anti-angiogenic drug without a neurotoxic profile when administered alone, show an increased incidence of total and high grade neuropathy with respect to an oxaliplatin without bevacizumab treatment arm (Giantonio et al., 2007; Allegra et al., 2009). Table 3 summarizes the incidence and severity ranges of phase III and neurotoxicity-focused oxaliplatin trials published in the literature.

Cumulative dose, dose-intensity and combinatory therapies are the main risk factors for developing platinum-induced neurotoxicity. However, other aspects may influence the occurrence of neuropathy.

In cisplatin treatment, low magnesium serum levels have also been related to higher neuropathy incidence and severity (Bokemeyer et al., 1996; Glendenning et al., 2010). Other risk factors including alcohol ingestion, smoking, familial diabetes, serum creatinine levels, age, tumor characteristics or performance status are not considered as being related to the onset or severity of peripheral neuropathy (Mollman et al., 1988; van der Hoop et al., 1990b).

In oxaliplatin treatment, it has been suggested that sensory excitability techniques provide an early predictor of chronic neurotoxicity of oxaliplatin (Park et al., 2009). So reductions in nerve superexcitability observed during the first cycles of treatment are indicators of an increased subsequent

Table 3. Incidence of oxaliplatin-induced neuropathy. Selection criteria: phase III studies and prospective studies with specific neurologic assessment tools.

Author (Year)	Patients (n)	Chemotherapy schedule	Dose-Intensity planned (mg/m^2/week)	Neurological Assessment	Neuropathy Incidence
PHASE III STUDIES					
Giacchetti (2000)	100	125 mg/m^2 every 21 days, as a continuous 6-hour intravenous infusion	41.67	Specific scale of peripheral neuropathy	Grade 1: 32% Grade 2: 13% Grades 3-4: 10%
de Gramont (2000)	209	85 mg/m^2 every 14 days, until progression disease	42.5	NCI-CTC	Grade 1: 20.6 % Grade 2: 29.2 % Grade 3: 18.2 % Grade 4: 0%
Cascinu (2002)	26	100 mg/m^2 every 14 days	50	NCI-CTC	After 8 cycles: Grade 1: 21.1% Grade 2: 31.6% Grade 3-4: 26.3%
Rothenberg (2003)	153	85 mg/m^2 every 14 days, median cycles 3 (1-18)	42.5	specific scale (similar to NCI-CTC)	Acute: Grade 1-2: 51% Grade 3-4: 7% Chronic: Grade 1-2: 49% Grade 3-4: 2%
	150	85 mg/m^2 every 14 days, median cycles 3 (1-16)			Acute: Grade 1-2: 50% Grade 3-4: 3% Chronic: Grade 1-2: 48% Grade 3-4: 3%
André (2004)	1108	85 mg/m^2 every 14 days for 12 cycles	42.5	NCI-CTC	Grade 1: 46% Grade 2: 33.6% Grade 3: 12.4%
Louvet (2005)	157	100 mg/m^2 every 14 days, until progression disease	50	NCI-CTC	Grade 1-2: 76% Grade 3: 19.1%
Tournigand (2006)	309	85 mg/m^2 every 14 days for 12 cycles	42.5	NCI-CTC	Grade 1: 34% Grade 2: 37% Grade 3: 18% Grade 4: 0 %
	303	130 mg/m^2 every 21 days x6 cycles, stop OXL 6 weeks and reintroduction other 6 OXL cycles	37.14		Grade 1: 36% Grade 2: 42% Grade 3: 13% Grade 4: 0 %

Table 3. Continued.

Gibson (2006)	324	85 mg/m^2 every 14 days	42.5	NCI-CTC	Grade 1: 38% Grade 2: 18.8% Grade 3-4: 16.7%
Land (2007)	189	85 mg/m^2 at week 1,3,5 of a 8 weeks cycle for 3 cycles	31.875	FACT/GOG-Ntx NCI-CTC NCI-Sanofi grade	At week 12: NCI-Sanofi. Grade 1-2: 68.3% Grade 3-4: 3% NCI-CTC Grade 1-2:61.3% Grade 3-4:2.6%
Wang (2007)	44	85 mg/m^2 every 14 days	42.5	WHO NCS	Grade 1-2: 40.9% Grade 3-4: 31.8%
Giantonio (2007)	285	85 mg/m^2 every 14 days	42.5	NCI-CTC	Grade 3: 8.8% Grade 4:0.4%
Schmoll (2007)	944	130 mg/m^2 every 21 days	43.33	NCI-CTC	Grade 1-2: 67% Grade 3-4: 11%
Al-Batran (2008)	112	85 mg/m^2 every 14 days, until progression disease	42.5	NCI-CTC OXL-specific scale	Grade 1-2: 49.2% Grade 3-4: 14.3%
Cassidy (2008)	655 649	130 mg/m^2 every 21 days, maximum 16 cycles 85 mg/m^2 every 14 days, maximum 24 cycles	43.33 42.5	NCI-CTC	Equal in both groups Grade 1: 11 % Grade 2: 5 % Grade 3: 4 % Grade 4: 0 %
Cunningham (2008)	452	130 mg/m^2 every 21 days, maximum 8 cycles	43.33	NCI-CTC QOL-QC30	Grade 1-2: 75.2% Grade 3-4: 6.4%
Allegra (2009)	1321 1326	85 mg/m^2 every 14 days for 12 cycles 85 mg/m^2 every 14 days for 12 cycles, plus bevacizumab	42.5	NCI-CTC	Grade 2: 29% Grade 3: 14% Grade 2: 33% Grade 3: 16%
Cathomas (2009)	20	130 mg/m^2 every 21 days, maximum 6 cycles heated at 37ºC	43.33	NCI-CTC Neurological symptoms questionnaire (NCI)	NCI-CTC Grade 1: 85% Grade 2: 15% Questionnaire : Dysestesias/ Paresthesias/ Acute Grade 1: 35% / 40%/ 35% Grade 2: 10% / 20%/ 5% Grade 3: 30% / 20%/ 30% Grade 4: 5% / 10%/ 20%

Table 3. Continued.

Poplin (2009)	272	100 mg/m^2 every 14 days, until progression disease	50	NCI-CTC	Grade 3: 9.5%
Qvortrup (2010)	69 70	130 mg/m^2 every 21 days for 8 cycles 130 mg/m^2 every 21 days for 8 cycles, chronotherapy	43.33	NIC-CTC	Grade 2: 42% Grade 3: 16% Grade 2: 27% Grade 3: 19%
Ishibashi (2010)	16	85 mg/m^2 every 14 days	42.5	NCI-CTC Debiopharm neurotoxicity scale	Grade 1: 94% Grade 2: 6% Grade 3-4: 0%
STUDIES WITH SPECIFIC NEUROLOGICAL ASSESSMENT TOOLS					
Krishnan (2005)	16	100 mg/m^2 every 14 days	50	NCI-CTC NSS Oxaliplatin-specific neurotoxicity score NCS	NCI.CTC: Grade 1-2: 12.5% Grade 3-4: 37.5% NSS: 1: 25% 2: 12.5% 3: 12.5%
Argyriou (2006)	20	85 mg/m^2 every 14 days for 12 cycles	42.5	NSS NDS NCS	PN: 75% TNS: 11.2±9.05 NDS:20±23.1 NSS: 1.5±1.3
Argyriou (2007)	25	85 mg/m^2 every 14 days for 12 cycles	42.5	NSS NDS FGS NCS TNS WHO CIPN (1-3)	TNS: 1-11 (mild): 24% 12-23 (moderate): 32% ≥ 24 (severe): 8% Mean NDS: 21.1±17.5 Mean NSS: 1.8±0.8

FACT/GOG-Ntx: Functional Assessment of Cancer Therapy Scale/Gynecologic Oncology Group-Neurotoxicity scale. NCI-Sanofi: oxaliplatin Sanofi-developed specific questionnaire. NCI-CTC: National Cancer Institute-Common Toxicity Criteria. WHO: World Health Organization. QLQ-C30: European Organization for the Treatment of Cancer quality of life questionnaire-30 items PN: peripheral neuropathy. NSS: Neurological Symptom Score. NDS: Neurological Disability Score. FGS: Functional Grading Scale. NCS: nerve conduction studies. TNS: total neuropathy score.

likelihood of developing moderate to severe neurotoxicity, predicting clinical outcome prior to treatment completion (Park et al., 2009). Furthermore, sustained thermal hyperalgesia after the third oxaliplatin cycle has also been identified as an early predictor of chronic peripheral neuropathy

(Attal et al., 2009). Other clinical risk factors such as diabetes have not been related to the incidence, severity or course of oxaliplatin-induced neuropathy after a review of the data from 3 phase III clinical trials (Ramanathan et al., 2010). In addition, strategies focused on dose-intensity schedules based on circadian rhythms, called chronomodulation, have shown conflicting results (Liao et al., 2010; Qvortrup et al., 2010).

Polymorphisms in genes involved in platinum biotransformation (McWhinney et al., 2009) and in the mechanisms for repairing platinum-DNA adducts (Dzagnidze et al., 2007) could modulate and partially explain the individual differences in the severity of the peripheral neuropathy induced by these drugs (Kweekel et al., 2005). A broad spectrum of genes has been evaluated although, up to now, few studies have attempted to detect distinctive gene polymorphisms that identify patients at high-risk of developing peripheral neuropathy (Kweekel et al., 2005; McWhinney et al., 2009). Glutatione S-Transferase P1 polymorphism (Ile105Val) and SCNA 1A polymorphism (T1067A T/T) have been suggested as identifying subjects at increased risk of developing oxaliplatin-induced peripheral neuropathy. In contrast, neuropathy is less frequent among patients with the GSTM1-null or GSTM3 intron 6 AGG/AGG genotypes. These polymorphisms encode metabolizing enzymes that decrease the reactivity of toxins with substrates in the body and voltage gate sodium channel subunits, respectively (Chen et al., 2010; Khrunin et al., 2010). Integrin beta-3 (IGTB3) polymorphism at residue 33 might also represent a specific biomarker to predict the severity but not the incidence of chronic peripheral neuropathy. A possible explanation of this feature is that proteins encoded by this polymorphism might play a role in neuronal survival, enhancing the activation of the ERK1/2 and MAPK pathways (Antonacopoulou et al., 2010).

Neurophysiological results

Nerve conduction studies performed in patients treated with platinum drugs have consistently evidenced sensory axonal damage with reduced amplitude of the sensory nerve potentials. With the use of cisplatin there has also been observed a progressive decrease in the amplitude of H waves from accumulated doses of 300 mg/m^2. Moreover, the distal sensory latencies and sensory nerve velocities become mildly prolonged at greater cumulative doses (400 mg/m^2). In contrast, in oxaliplatin-treated patients changes have not been observed in conduction nerve velocities (Thompson et al., 1984; Daugaard et al., 1987; Cavaletti et al., 1992; Krishnan et al., 2005; Krarup-Hansen et al., 2007; Argyriou et al., 2007a). With regard to motor nerve

conduction studies in platinum-treated patients, the nerve conduction velocities, compound muscle action potentials and F-wave latencies remain unchanged throughout treatment. In relation to autonomic evaluations, no alterations have been reported in oxaliplatin- and carboplatin-treated patients, but conflicting findings have been observed in autonomic tests of cisplatin-treated patients: while some studies identified abnormal values in heart-rate response to breathing or Valsalva manoeuvre (Hansen et al., 1990; Bogerd et al., 1990; Earl et al., 1998), in another study these results were not reproduced (Krarup-Hansen et al., 2007). One explanation is that the former studies included patients treated with adjuvant vinblastine or doxorubicin, drugs known to induce autonomic neuropathy and cardiotoxicity, respectively, and that the negative study only evaluated patients treated with cisplatin.

In contrast, neurophysiological studies of oxaliplatin acute neuropathy 24-48h after oxaliplatin infusion showed neuromyotonic discharge and repetitive compound muscle action potentials, whereas compound muscle action potential amplitudes, conduction velocities, and F-wave latencies remained unchanged. All these abnormalities resolved within 3 weeks after oxaliplatin administration (Lehky et al., 2004). No acute abnormalities were seen in conventional sensory nerve conduction studies. However, a reduction in refractoriness parameters and superexcitability has been demonstrated in axonal excitability studies. These changes are greater in first cycles (1-4) compared with later treatment (Park et al., 2009). Electrophysiological changes in acute oxaliplatin neurotoxicity are thought to be caused by the dysfunction of nodal axonal voltage-gated transient Na^+ channels (Krishnan et al., 2005), probably Ca^{2+} dependent Na^+ channels. Oxaliplatin is metabolized by the neuron into oxalate and dichloro-diaminocyclohexane platinum (Kweekel et al., 2005). Oxalate is a well known chelator of both Ca^{2+} and Mg^{2+} whose action could interfere with channel kinetics (Adelsberger et al., 2000) and could reduce the overall Na^+ current (Grolleau et al., 2001), reducing axonal refractoriness (Park et al., 2009; Krishnan et al., 2005) and predisposing to ectopic activity. Conversely, it has been demonstrated that dichloro-diaminocyclohexane platinum does not induce acute symptoms (Sakurai et al., 2009), nor does it affect neuronal membrane properties (Grolleau et al., 2001).

Histopathological findings

The main target of platinum compound damage is dorsal root ganglia. The levels of cisplatin in treated patients are higher in the dorsal root ganglia

than other nervous system tissues and they show a positive correlation with the degree of clinical and histopathologic neurotoxicity. Moreover, the levels of platinum within the dorsal root ganglia, dorsal roots and peripheral nerves are correlated with the cumulative dosage of cisplatin (Gregg et al., 1992; Dzagnidze et al., 2007). Dorsal root ganglia of some, but not all, patients have been found to contain necrotic neurons and nodules of Nageotte, and the mean volume of the somata show a significant reduction (Thomson et al., 1984; Krarup-Hansen et al., 1999). At the same time, other necropsic studies have also found morphologic alterations in posterior columns of the spinal cord and the peripheral nerves, respectively (Walsh et al., 1982; Thomson et al., 1984; Roelofs et al., 1984), probably due to the proximal extent of primary dorsal root ganglia degeneration. Furthermore, the histopathologic features observed are closely related not only to the total cisplatin dose but also to the time from treatment initiation (Gregg et al., 1992). This fact may explain some discrepancies in the findings of necropsic studies. The morphologic changes observed in peripheral nerves show a substantial loss of myelinated fibers and scattered degenerating nerve fibers. The myelin sheath is slightly swollen and transformed into a series of small osmiophilic globules, with occasionally observed focal demyelination phenomena. In all instances, the focal or diffuse myelin sheath abnormities are associated with a disintegrated axon, showing that the primary damage to the nerve is in the axon, and demyelination occurs secondarily. In contrast, the non-myelinated axons are ultrastructurally normal and the Pacinian corpuscles contain normal axons (Thomson et al., 1984; Roelofs et al., 1984; Krarup-Hansen et al., 1993 and 2007). Finally, histopathological studies of oxaliplatin- and carboplatin-treated patients are not available.

Long-term outcome

Peripheral neuropathy usually develops during platinum-drug treatment and it stabilizes and partially improves after treatment discontinuation. However, after cessation of chemotherapy and independently of total dose administered, symptoms and signs may deteriorate (coasting effect). In cisplatin-induced neuropathy, this effect is observed in approximately 30% of patients for 2 to 6 months following treatment discontinuation (Siegal et al., 1990; Cavaletti et al., 1994; von Schlippe et al., 2001). Although the patients with cisplatin-induced neuropathy may show an improvement months after the cessation of therapy, recovery usually remains incomplete. Moreover, almost all quality of life problems due to cisplatin chemotherapy show recovery 9 months after treatment discontinuation; the impact of neuropathic

symptoms on quality of life remains unchanged (Bezjak et al., 2008). Recently, a cross-sectional study of testicular cancer patients, reevaluated between 23 and 33 years after finishing treatment, showed that peripheral neuropathy remains detectable in up to 20% of patients and is symptomatic in 10% (Glendenning et al., 2010). Similar results were found in another study that evaluated cisplatin-treated patients after a median follow-up of 15 years: 38% and 28% patients had asymptomatic and symptomatic neuropathy, respectively, and it was disabling in 6% of patients (Strumberg et al., 2002). Another study with a shorter median follow-up time (5 years) reported transient complaint neuropathic symptoms during a time period of 1 to 36 months, with a mean time duration of 11 months, while 17% of patients at final follow-up had persisting neuropathic symptoms (Bokemeyer et al., 1996). The cumulative dose of cisplatin is the main risk factor associated with persistence of neurotoxicity although cumulative doses under 300 mg/m^2 usually lead to a full resolution of neuropathy (Bokemeyer et al., 1996; von Schlippe et al., 2001). No differences have been observed in combination regimens with vinka alkaloids when analysis is adjusted for cisplatin dose (Bokemeyer et al., 1996). In combinatory regimens with cisplatin and paclitaxel for treatment of ovarian cancer, 41% of patients who developed neuropathy also showed resolution of their symptoms after a median time of 11 months (Sarosy et al., 2010).

Chronic oxaliplatin peripheral neuropathy is partially reversible in about 80% of patients and completely resolved in about 40% of them after 6-8 months from treatment discontinuation (de Gramont et al., 2000; Wilson et al., 2002; Andre et al., 2004), but the coasting effect may also occur with use of this drug, although it has not been well reported. Long-term follow-up patient series focused on neurotoxicity evaluation are scarce, probably due to the relatively recent clinical implementation of oxaliplatin. Two studies have reported persistence of the neuropathy in almost 35% of patients 5-6 years after cessation of oxaliplatin treatment (Pietrangeli et al., 2006; Brouwers et al., 2009). Finally, it has been reported that peripheral neuropathy may be exacerbated by surgery carried out immediately after finishing treatment with oxaliplatin. These patients have usually received high cumulative doses of oxaliplatin (>740 mg/m^2), and the surgery-induced hemolysis is thought to release oxaliplatin accumulated in erythrocytes (Gornet et al., 2002).

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3.4. Vinca alkaloids-induced peripheral neuropathy

Jordi Bruna Escuer
Unit of Neuro-Oncology, Department of Neurology, Hospital Universitari de Bellvitge Hospitalet de Llobregat, and Group of Neuroplasticity and Regeneration (CIBERNED) Institute of Neurosciences, Universitat Autònoma de Barcelona, Spain

Drug history and clinical use

This group of chemotherapeutic agents is a subset of drugs that are derived from the periwinkle plant, *Catharanthus roseus*. It is also commonly called the Madagascar periwinkle or the rose periwinkle. This plant has been used as a folk remedy for centuries; studies undertaken in the 1950s revealed that *C. roseus* contained 70 alkaloids, many of which are biologically active. The first compounds with antineoplastic properties to be identified, vincristine and viblastine, were discovered by serendipity when the plant was being tested for diabetes treatment (Johnson et al., 1963). Vinca alkaloids used in cancer treatment include both natural alkaloids, such as vincristine and vinblastine, and semi-synthetic compounds, such as vindesine, vinorelbine and vinflunine. These drugs have a broad spectrum of indications in the treatment of hematologic and lymphatic malignancies, as well as being used alone or in adjunctive treatment of certain solid tumors (Chu, 2009).

Correspondence/Reprint request: Dr. Jordi Bruna Escuer, Unit of Neuro-Oncology, Department of Neurology Hospital Universitari de Bellvitge, Hospitalet de Llobregat, and Group of Neuroplasticity and Regeneration (CIBERNED), Institute of Neurosciences, Universitat Autònoma de Barcelona, Spain
E-mail:

Vinca alkaloid compounds, although having similar chemical structures, show differing neurotoxicity profiles, with vincristine being the most neurotoxic drug. This old drug, approved for therapeutic use in 1963, is incorporated into almost all frontline combination treatments of hematologic and lymphatic neoplasms, with the dose intensity being an important factor for successful treatment (Carde et al., 1983; Longo et al., 1986; Longo et al., 1991). However, neuropathy remains its main dose-limiting side effect. Moreover, vincristine is also used as an adjunctive treatment of several soft tissue sarcomas and oligoastrocytomas. In an attempt to reduce the toxic profile, a liposomal vincristine formulation has been tested (Sarris et al., 2000; Rodriguez et al., 2009; Gelmon et al., 1999).

Regarding the other vinca alkaloids, vinblastine, accepted for clinical use in 1965, is employed in several combinatory regimens to treat Hodgkin's lymphoma, testicular cancer and Kaposi's sarcoma. Vinorelbine, a semisynthetic vinca alkaloid, was accepted for clinical use in 1994 and is given alone or as adjunctive therapy in several chemotherapeutic lines to treat breast, ovarian and non-small-cell lung cancer. Vindesine, a synthetic derivate of vinblastine, was approved in 1998 and has been used to treat acute

Table 1. Dose range of vinca alkaloids in the main treatment schedules.

VINCRISTINE	
Lymphomas, leukemias and sarcomas:	The doses vary between 0.5-1.4 mg/m^2 iv per cycle. The total individual dose should be limited to 2 mg.
Multiple myeloma:	0.4 mg/day in continous iv infusion for 4 days as a part of doxorubicin and dexametasone regimen.
VINBLASTINE	
Hodgkin's lymphoma:	6 mg/m^2 iv on days 1 and 15, as part of doxorubicin, bleomycin and dacarbazine regimen.
Testicular cancer:	0.15 mg/Kg iv on days 1 and 2, as part of the cisplatin and bleomycin regimen.
Kaposi's sarcoma:	6 mg/m^2 on day 1, as part of doxorubicin and bleomycin regimen.
VINDESINE	
Acute lymphocytic leukemia:	the doses vary between 2-4 mg/m^2 every 1-2 weeks or 1.5 mg/m^2 in continous iv infusion for 7 days every 3-4 weeks.
VINORELBINE	
Non-small cell lung cancer **Breast cancer** **Ovarian cancer**	30 mg/m^2 iv on a weekly schedule either as a single agent or in combination with cisplatin.

iv: intravenous. Vinorelbine can also be administered orally (po): 25 mg po be equal to 60 mg/m^2 iv, and 30 mg po be equal to 80 mg/m^2 iv.

lymphocytic leukemia (Chu, 2009). Finally, vinflunine, the most recent vinca derivate just approved in Europe, has been tested with hopeful results for the treatment of mesothelioma and advanced non-small-cell lung and urothelial cancer (Culine et al., 2006, Bellmunt et al. 2009, Krzakowski et al., 2010). The recommended dose ranges of the vinca alkaloid compounds, summarized in Table 1, are administered in different schedules depending on the primary tumor and the adjunctive therapy.

Clinical aspects of neurotoxicity

Neurotoxicity among the various vinca alkaloids is qualitatively similar but different in severity. Most extensive studies available, related to neurotoxic clinical characteristics of these drugs, are focused on the compounds most frequently used and with greatest incidence of neuropathy: vincristine and vinorelbine.

The earliest manifestation of vincristine-induced peripheral neuropathy is the loss of ankle reflexes and depression of superficial reflexes, usually followed by paresthesias (Sandler et al., 1969; Casey et al., 1973). If treatment continues, generalized depression or loss of deep tendon reflexes appears, followed by muscular weakness when high cumulative doses are reached. Initial depression of reflexes is not accompanied by other symptoms in 60% of the patients (Sandler et al., 1969), and paresthesias, the most common symptom, appear in the hands earlier and more prominently than in the feet (Casey et al., 1973; Verstappen et al., 2005). Objective touch and two-point discrimination sensory loss are usually infrequent or mild and are limited to fingers and toes; however, 70% of asymptomatic patients have increased touch detection thresholds (Postma et al., 1993). In series of elderly patients, vibration detection is rarely more than mildly impaired (Sandler et al., 1969; Casey et al., 1973; DeAngelis et al., 1991) but, when measured by quantitative methods, it is present in 20% of patients in both the fingers and toes, but to a greater extent in the toes (Verstappen et al., 2005). Joint position sense usually remains intact (Sandler et al., 1969; Verstappen et al., 2005). Pain is reported by some patients, usually restricted to the glabrous skin of the fingertips and toes, with elevated sharpness and warm detection thresholds in these areas (DeAngelis et al., 1991; Dougherty et al., 2007). When motor weakness appears, it is most apparent in the ankle dorsiflexors, extensors and everting muscles of the toes (Sandler et al., 1969; Casey et al., 1973; DeAngelis et al., 1991; Verstappen et al., 2005). Sometimes motor symptom limitations reported by patients may seem disproportionate to the weakness determined by motor testing (DeAngelis et al., 1991), probably as a consequence of difficulties in daily life due to an associated significant

sensory impairment. In high-dose intensity treatments muscle cramps of mild severity have often been noted, occurring more frequently in the daytime than at night (Haim et al., 1991; Haim et al., 1994). Symptoms potentially attributable to autonomic dysfunction are colicky abdominal pain and constipation, which can occur even within a few days of drug administration and which precede paresthesias or deep tendon reflex depression without other signs of autonomic neuropathy (Sandler et al., 1969, Haim et al., 1994). Other dysautonomic symptoms such as chronic gastrointestinal toxicity occur less frequently as late complications in patients with severe vincristine-induced neuropathy (Sandler et al., 1969, Casey et al., 1973), while impotence has frequently been reported by 15-24% of patients (Kornblith et al., 1992, Haim et al., 1994). Urinary retention and orthostatic hypotension have been reported anecdotally (Wheeler et al., 1983, Haim et al., 1994).

Vinorelbine treatment usually induces a mild distal axonal sensory-motor neuropathy, involving small and large fibers without evidence of C fiber dysfunction evaluated by sympathetic skin response. Most common symptoms and signs include areflexia or hyporeflexia (94%), paresthesias (50%) and hypopallestesia (9%) without moderate or severe toxicities measured by NCI or neurological scores (Pace et al., 1996).

Incidence

Vinca alkaloid-induced peripheral neuropathy is clearly related to total cumulative dose and to dose-intensity treatment (Sandler et al., 1969, Casey et al., 1973, Gralla et al., 1979, DeAngelis et al., 1991, Pace et al., 1996, Verstappen et al., 2005). However, the fact that these compounds possess differing affinities for tubulin results in different profiles of neuropathy incidence and severity. Regarding neurotoxicity, vincristine is greater than vindesine, vindesine greater than vinblastine, vinblastine greater than vinorelbine and vinorelbine greater than vinflunine (Lobert et al., 1996). All these factors, together with the heterogeneity in chemotherapeutic regimens employed for the treatment of different types of tumors, make it impossible to determine the precise incidence of peripheral neuropathy. Table 2 reports the results of peripheral neuropathy obtained in the most relevant trials using vincristine and vinorelbine.

In relation to vincristine use, it has been observed that all patients receiving at least 4 mg/m^2 have a reduction in or loss of ankle reflexes and most patients who have received total doses of 2-6 mg/m^2 also report mild distal paresthesias. A significant number of patients receiving a total dose of 8 mg/m^2 develop motor weakness or gait impairment (Sandler et al., 1969).

Table 2. Incidence of vinca alkaloids-induced neuropathy. Selection criteria: prospective studies including information about neuropathy and neurological assessment tools.

Author (year)	Patients (n)	Schedule	Dose-intensity Planned (mg/m^2/week)	Neurological Assesment	Neuropathy Incidence
VINCRISTINE					
Sandler (1969)	50	2 mg/m^2 every 14 days	0.5	NCS Neurologic clinical assessment	Depression reflexes: 100% Motor weakness: 34% Sensory disturbances: 46%
Holland (1973)	393	Induction: 75 µg/kg/week for 4 months	NA	Not validated neuropathy related symptoms scale, similar to NCI-CTC	Grade 1-2: 23% Grade 3: 33% Grade 4: 25% Grade 5: 10%
Watkins (1978)	10 13 23 9 5	Mean dose: 0.28 (0.06-0.74) mg/kg 0.23 (0.05-0.75) mg/kg 0.3 (0.05-0.57) mg/kg 0.42 (0.07-1.47) mg/kg 0.23 (0.06-0.51) mg/kg	NA	Neurologic clinical assessment	Patients with PN: 60% 61.5% 4.3% 22% 40%
Thant (1982)	11	1.4 mg/m^2 twice every 21 days for 3 cycles	0.47	NCS Neurologic clinical assessment	Grade 1-2: 63.6% Grade 3-4: 36.4%
Jackson (1984)	25	0.5 mg plus 0.25 mg/m^2/ day during 5 days every 21 days	0.58	Neurologic clinical assessment	Patients with PN: 48% Depression reflexes: 36% Motor weakness: 4% Sensory disturbances: 24%
Powles (1991)	105	1.4 mg/m^2 every 21 days	0.47	WHO	Grade 1:24% Grade 2:14% Grade 3-4:5%
DeAngelis (1991)	27	0.5 mg/m^2/day during 4 days every 7 days for 12 cycles	2	NCS Neurologic clinical assessment	All patients had moderate to severe signs and symptoms of sensory-motor neuropathy.
van Kooten (1992)	15	1.4 mg/m^2 twice every 28 days for 6 cycles	0.35	VPT Neurologic clinical assessment	Patients with PN: 80%

Table 2. Continued.

Broun (1993)	32	1.4 mg/ m^2 was weekly as iv bolus for 4 weeks, then every other week	1.4	NCI-CTC	Grade 2-3: 34%
Haim (1994)	104	1.4 mg/m^2 every cycle	NA	WHO	Sensory: Grade 1: 52.8% Grade 2-3: 11.5% Motor: Grade 2: 17.3% Grade 3: 11.5%
Taylor (1997)	27	1.6 mg/m^2 every 21 days x 6 cycles	0.53	SWOG criteria	Grade 1-2: 22% (mild paresthesias)
Reinders-Messelink (2000)	11	1.5 mg/m^2/week for 8 cycles	1.5	WHO VPT NCS	Grade 1: 9% Grade 2: 73% Grade 3-4: 0% 33% sensory abnormalities 22% VPT abnormalities
Klasa (2002)	44	1.2 mg/m^2 every 21 days	0.4	WHO QLQ-C30	Grade 1: 54% Grade 2: 11% Grade 3-4: 2%
Katsumata (2003)	23	1.2 mg/m^2 every 21 days	0.4	NCI-CTC	Grade 1: 0% Grade 2: 26.1% Grade 3-4: 0%
Verstappen (2005)	47 67	2 mg every 21 days 4 mg every 21 days	0.67 1.33	Neuropathy scales Symptoms scales VPT	Paresthesias 34% Numbness 43% Pain 14% Paresthesias 60% (10%severe) Numbness 70% (4% severe) Pain 62 % (16% severe)
Leighl (2006)	31	1.2 mg/m^2 every 21 days	0.4	NCI-CTC	Grade 3-4: 13%
Rea (2006)	31	2 mg every 7 days for 4 cycles	2	NCI-CTC	Grade 2-3: 45%
Walewski (2010)	24	1.4 mg/m^2 every 21 days for 6-8 cycles	0.47	NCI-CTC	No peripheral neuropathy

Table 2. Continued.

LIPOSOMAL VINCRISTINE					
Sarris (2000)	35	2 mg/m^2 every 14 days for 12 cycles	1	NCI-CTC	Grade 3-4: 31.4%
Rodriguez (2009)	119	2 mg/m^2 every 14 days for12 cycles	1	NCI-CTC	Grade 3-4: 32%
VINBLASTINE					
Druker (1989)	24	6 mg/m^2 on days 1 and 8 every 28 days	3	Neurologic clinical assessment	mild PN: 8%
VINORELBINE					
Pace (1996)	23	25 mg every 7 days for 24 cycles	NA	NSS NCS WHO	WHO>2 : 0% Moderate (5-10): 66.6% Severe (>10): 33%

NA: not available. NCI-CTC: National Cancer Institute-Common Toxicity Criteria. PN: peripheral neuropathy. WHO: World Health Organization. NCS: nerve conduction studies. SWOG: South West Oncology Group. QLQ-C30: European Organization for the Treatment of Cancer quality of life questionnaire-30 items. VPT: Vibration perception threshold. NSS: Neurological Symptom Score.

Neuropathic pain is relatively frequent in high-accumulated dose (10mg/m^2) vincristine-treated patients (Sandler et al., 1969, Dougherty et al., 2007). Liposomal vincristine is a new formulation developed with the aim of improving the efficacy of the pharmacokinetic profile without increasing neurotoxicity. Conflicting results concerning its peripheral neuropathy safety have been reported in phase I and II trials. Given at doses ranging between 2 and 2.8 mg/m^2, sensory-motor neuropathy has been reported in 12-55% of treated patients, and it is severe in 7-34% of them (Gelmon et al., 1999, Sarris et al., 2000, Rodriguez et al., 2009). Moreover, using this formulation a high prevalence of constipation has also been observed. Nevertheless, it should be taken into account that patients in these studies presented higher rates of previous peripheral neuropathy induced by other agents before starting the treatment with liposomal vincristine.

Vindesine, currently less used in standard chemotherapeutic regimens, follows the same peripheral neuropathic pattern as the other vinca alkaloids. Sensory disturbances and depression of deep tendon reflexes begin at 4 mg and reflexes disappear after cumulative doses of 55mg. The earliest electromyographic alterations start after 5-10 mg and these become abnormal in all patients after a cumulative dose of 45 mg (Focan et al., 1981).

Vinblastine produces peripheral neurotoxicity similar to that observed with vincristine; however, hematologic toxicity associated with vinblastine usually precedes neurotoxicity and becomes the main dose-limiting adverse effect (Chu, 2009). Consequently, peripheral neuropathy is less frequently a serious clinical problem in patients treated with vinblastine.

The most recently implemented vinca alkaloid, vinflunine, presents a reduced incidence of peripheral neuropathy in comparison to the other vinca compounds. According to the NCI scale, peripheral neuropathy has been identified in 12% of patients, almost all of them being grade 1 or 2. Of note is the fact that autonomic neuropathy characterized by constipation and abdominal pain is the most frequently reported feature in 20-48% of patients, of whom 8-16% are severe grade (Culine et al., 2006, Bellmunt et al., 2009, Krzakowski et al., 2010).

Cumulative dose and dose intensity are the main risk factors for developing vinca alkaloid-induced neuropathy. To minimize the potential neurotoxic effects of vincristine the usual recommended dose is 1.4 mg/m^2 per single dose with an upper limit of 2 mg on single doses, regardless of body surface area. Nevertheless, neuropathy is still observed at these doses. However, other aspects may influence the emergence of neuropathy.

Combinatory schedules that include other neurotoxic drugs, such as vinorelbine with paclitaxel (Fazeny et al., 1996) and vindesine with cisplatin (Bacon et al., 2003), have been reported to induce higher incidences of peripheral neuropathy.

From case report collections there emerges a possible high-risk situation for severe course and early onset of vincristine peripheral neuropathy, in the form of the unrecognized hereditary peripheral neuropathies, particularly Charcot Marie-Tooth type 1 and 2, and neuropathies with a liability to pressure palsies (Graf et al., 1996, Kalfakis et al., 2002, Trobaugh-Lotrario et al., 2003).

Additionally, a few cases of severe fulminating neuropathy during vincristine treatment, mimicking Guillain-Barré syndrome features, have also been described (Bakshi et al., 1997, González-Pérez et al., 2007, Terenghi et al., 2007). Whether this acute inflammatory neuropathy represents a coincidental classic Guillain-Barré or responds to another circumstance remains an unresolved question.

Finally, patients with hepatic insufficiency are also at increased risk of developing vincristine-induced neuropathy (Sandler et al., 1969), probably because vincristine is metabolized by the liver and excreted through the biliary tract. Age and poor nutritional status have not been well demonstrated as risk factors (Thant et al., 1982).

Neurophysiological results

At neurophysiologic examination, both sensory nerve action potential and compound motor action potential decrease during treatment. The earliest findings during the course of treatment are the decrease in sensory nerve action potentials followed by a decrease in motor action potential amplitudes.

These reduced potentials are more pronounced in the fingers than in proximal segments, particularly in the early stages of the neuropathy. Spontaneous fibrillation associated with a reduction in the interference pattern is found in all distal muscles (Casey et al., 1973). H-reflexes remain present and unchanged when ankle reflexes are absent (Sandler et al., 1969, McLeod et al., 1969), but after 3-5 weeks they disappear in half of the patients. Although sensory symptoms and signs improve when the treatment is withdrawn, the amplitude of sensory nerve action potentials remains altered in most patients (Casey et al., 1973, Ramchandren et al., 2009). Only a slight reduction in nerve conduction velocities is demonstrated in either sensory or motor fibers in the context of severe neuropathy (McLeod et al., 1969, Casey et al., 1973, DeAngelis et al., 1991). Finally, autonomic focus on blood pressure on standing and heart rate variability show contradictory results (Roca et al., 1985, Lahtinen et al., 1989).

Histopathological findings

Necropsic studies of the nervous system in patients treated with vinca alkaloids are scarce, with few analyzed cases, almost all of which are focused on patients that have received vincristine.

Available pathologic knowledge indicates that neuropathy induced by vincristine affects myelinated fibers of all diameters (Gottschalk et al., 1968, McLeod et al., 1969) and, in contrast to animal models, no alterations of unmyelinated axons have been described (Thant et al., 1982). Reduction in fiber density below normal ranges adjusted by age has been observed in the sural nerve. Moreover, morphometric studies have revealed a loss of both large and small diameter fibers (McLeod et al., 1969), although swollen segments of nerve undergoing Wallerian degeneration have also been observed. In teased-fiber preparations, fibers clearly showed various stages of Wallerian degeneration. Furthermore, there was no evidence of segmental demyelinization in patients with neuropathy whose autopsies were performed in the period soon after the administration of vincristine (Gottschalk et al., 1968, McLeod et al., 1969). Schwann cell cytoplasm appears normal (Thant et al., 1982). Cases examined from 5 weeks to 8 months after treatment

discontinuation revealed few fibers, segmental demyelination, marked variability of internodal length without intercalated nodes, and signs of undergoing remyelination (Gottschalk et al., 1968, McLeod et al., 1969). All these findings indicate axonal degeneration of the nerves as the predominant event with secondary demyelination. Another study using frozen sections stained with oil red O revealed numerous deposits of neutral lipid scattered throughout the nerves (Moress et al., 1967). In an anecdotal case of adjunctive treatment with vincristine and etoposide, an electron-opaque granular material was observed within the concentric layers of lamellae or immediately adjacent to the axon (Thant et al., 1982).

In dorsal root ganglia the tissue architecture appears well preserved, although the majority of neurons show an increase in argentophilic fibrillar material with Bodian stain and displacement of the nucleus. Electronic microscopy displays a large increase in neurofilaments, measuring 90-100 Angstroms in diameter and morphologically identical to normal neurofilaments. In more severely affected neurons, these increased numbers of neurofilaments are seen to run parallel to each other in compact bundles. Additionally, a high concentration of lipofucsin granules is seen in many neurons (Shelanski et al., 1969).

In vinblastine treatment, the presence of various stages of Wallerian degeneration were described in the only necropsic study available (Gottschalk et al., 1968).

Long-term outcome

The clinical symptoms of vincristine neuropathy are usually reversible when therapy is discontinued, at least in patients who develop mild to moderate neuropathy. Symptoms of constipation and abdominal pain markedly improve within a few days. However, a coasting effect has been reported in the first month after finishing therapy in up to 30% of patients, more prevalently in high-dose intensity regimens (Verstappen et al., 2005).

Remission of paresthesias is one of the first signs noted during the recovery; this feature is observed 1-2 weeks after finishing treatment (Sandler et al., 1969, Casey et al., 1973, Haim et al., 1994). In patient series followed for an average of 11 months from treatment withdrawal, the median duration of paresthesias and motor weakness after vincristine discontinuation was 3 months, and 5 months for muscle cramps. Remaining sensory symptoms were found in 24% of patients, while impairment of deep tendon reflexes and pinprick sensation was found in 56% and 53%, respectively. Impaired joint position and two-point discrimination were each found in 9% of patients.

Weakness was overcome and disappeared in almost all patients (Haim et al., 1994). Another study, after a median follow-up of 34 months, found 48% and 33% of patients with persisting sensory symptoms and impairment of deep tendon reflexes, respectively (Postma et al., 1993). Although most deep tendon reflexes reappear, ankle reflex recovery is uncommon (Sandler et al., 1969, Casey et al., 1973, Postma et al., 1993).

Symptoms in fully recovered patients persisted maximally between 34 to 40 months from cessation of therapy (Postma et al., 1993, Haim et al., 1994), with slower recovery in patients with grades 2 and 3 of NTCI. Although neuropathy symptoms have a fairly good prognosis after a long follow-up, only 6% remained with grade 3 neuropathies, while those with grade 2 and 3 neuropathies showed complete recovery in only 25% of cases (Haim et al., 1994).

Regarding long-term data concerning other vinca alkaloids, neurotoxicity induced by vinorelbine treatment is reversible after 6 months from discontinuation of the drug (Pace et al., 1996).

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