Development of self-assembling protein only nanoparticles for targeted therapies.

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ARTICLE 3

Protein-only, antimicrobial peptide-containing recombinant nanoparticles with inherent built-in antibacterial activity.

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Developing cationic antimicrobial peptides (AMPs) as an antibacterial therapy, alternative to the use of antibiotics, is of a special interest because of the increased prevalence of resistance to conventional antibiotics. AMPs have been successfully incorporated into topical drug formulations, but their applicability in systemic therapies remains a challenge due to their low molecular mass and limited stability.

The engineering of low molecular weight AMPs as protein building blocks that self-assemble into NPs would allow their applicability in systemic therapies enhancing solubility, stability and avoiding their metabolic excretion. Moreover, it would represent a new biological platform for the design of antimicrobial nanomaterials, that exhibit higher biocompatibility and wider structural and functional versatility than those based on metals which are currently under intense exploration.

In this work, we have explored the potency of a synthetic cationic AMP to prompt the formation of protein only nanoparticles while keeping its high antimicrobial activity for the systemic treatment of bacterial infections **(objective 4)**.

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Short communication

Protein-only, antimicrobial peptide-containing recombinant nanoparticles with inherent built-in antibacterial activity

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ABSTRACT

The emergence of bacterial antibiotic resistances is a serious concern in human and animal health. In this context, naturally occurring cationic antimicrobial peptides (AMPs) might play a main role in a next generation of drugs against bacterial infections. Taking an innovative approach to design self-organizing functional proteins, we have generated here protein-only nanoparticles with intrinsic AMP microbicide activity. Using a recombinant version of the GWH1 antimicrobial peptide as building block, these materials show a wide antibacterial activity spectrum in absence of detectable toxicity on mammalian cells. The GWH1-based nanoparticles combine clinically appealing properties of nanoscale materials with full biocompatibility, structural and functional plasticity and biological efficacy exhibited by proteins. Because of the largely implemented biological fabrication of recombinant protein drugs, the protein-based platform presented here represents a novel and scalable strategy in antimicrobial drug design, that by solving some of the limitations of AMPs offers a promising alternative to conventional antibiotics.

Statement of Significance

The low molecular weight antimicrobial peptide GWH1 has been engineered to oligomerize as self-assembling protein-only nanoparticles of around 50 nm. In this form, the peptide exhibits potent and broad antibacterial activities against both Gram-positive and Gram-negative bacteria, without any harmful effect over mammalian cells. As a solid proof-of-concept, this finding strongly supports the design and biofabrication of nanoscale antimicrobial materials with in-built functionalities. The protein-based homogeneous composition offer advantages over alternative materials explored as antimicrobial agents, regarding biocompatibility, biodegradability and environmental suitability. Beyond the described prototype, this transversal engineering concept has wide applicability in the design of novel nanomedicines for advanced treatments of bacterial infections.

1. Introduction

The efficacy of antibiotics has been hampered by the increasing incidence of multi-resistant bacterial infections [1]. In this context, naturally occurring cationic antimicrobial peptides (AMPs) are of a special interest because of their potential as alternatives to conventional antibiotics [2–4]. Being an important functional arm of the innate immune system they act as a first line of mucosal defence against a broad spectrum of microorganisms [5]. AMPs are rather short, with a total molecular mass between 2 and 9 kDa. The cell lytic activity of AMPs is associated to pore formation in cell membranes [6–8]. This occurs by the selective peptide binding to negatively charged cell surfaces and the subsequent membrane permeabilisation [9]. For some AMPs, alternative internal targets have been also identified, which might show multiple modes of action apart from the main lytic activity [6,10,11]. Although AMPs have been successfully incorporated into topical

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drug formulations, their applicability in systemic therapies remains a challenge due to their low solubility, limited stability and a rapid metabolic excretion favoured by their low molecular mass.

In an attempt to increase AMP performance, protein engineering has allowed generating new or more effective AMP variants with improved biological activity and higher clinical opportuneness [12–15]. Among them, GWH1 is particularly interesting. Folding as an amphipathic helix, it shows high antimicrobial activity and low haemolytic potential [16]. In addition, GWH1 also exhibits antitumor activities both *in vitro,* over cultured cancer cells, and *in vivo,* in animal cancer models [17]. We wondered if the cationic nature of GWH1 could be exploited to prompt the formation of protein-only nanoparticles while keeping its membrane lytic activities. Cationic peptides, when fused to the amino terminus of His-tagged proteins, promote symmetric cross-protein interactions and the formation of regular oligomers ranging between 12 and 80 nm [18]. These nanoparticles are fully stable *in vivo* upon systemic administration and useful as vehicles for targeted drug delivery and imaging [18–20]. So far, GWH1 has been solely produced by chemical synthesis. However, obtaining this peptide as recombinant self-assembling proteins for nanoparticle formation might largely expand its functional versatility and biological applicability. In addition, this would represent a new biological platform for the design of antimicrobial nanomaterials, that solely based on proteins are to be environmentally friendly, and exhibit higher biocompatibility and wider structural and functional versatility than those based on metals [21], currently under intense exploration [22–24]. Investigating this possibility, protein-only nanoparticles empowered by GWH1 have been generated and successfully characterized as functional nanoscale materials with built-in antibacterial properties.

2. Materials and methods

2.1. Protein design, production and purification

Recombinant pET22b-derivatives encoding proteins GWH1-GFP-H6, T22-GWH1-GFP-H6 and their parental GFP-H6 (Fig. 1A) were designed in-house and produced by GeneArt. T22-GFP-H6, used as control, has been described in detail elsewhere [20]. *Escherichia coli Origami B* (BL21, OmpT$^-$, Lon$^-$, TrxB$^-$, Gor$^-$, Novagen) cells were transformed by heat-shock and cultured in 2 l shaker flasks with 500 ml of LB medium [25] containing 100 μg/ml ampicillin, 15 μg/m kanamycin, and 12.5 μg/ml tetracycline at 37 °C. The induction of recombinant gene expression was done at an OD between 0.5 and 0.7 by the addition of 0.1 mM isopropyl-β-thio galactopyronaside (IPTG). Then, bacterial cells were kept growing overnight at 20 °C. After sedimentation at 5000 g (4 °C, 15 min), the cell pellet was resuspended in Wash buffer (Tris 20 mM, pH 8.0, NaCl 500 mM, imidazole 10 mM) with 0.5% Triton X-100 (Roche Diagnostics GmbH) and ethylenediamine tetra-acetic acid-free protease-inhibitor (Complete EDTA-Free, Roche) for T22-GWH1-GFP-H6 and in Wash buffer with complete EDTA-Free for GWH1-GFP-H6. Cells were then disrupted by sonication (2 rounds of 10 min at 10% amplitude and 1 round of 10 min at 15% of amplitude) and soluble fractions separated by centrifugation for 45 min (15,000*g* at 4 °C). Protein purification was carried out with a His tag affinity chromatography using HiTrap Chelatin HP 1 ml column (GE Healthcare) in an AKTA purifier FPLC (GE Healthcare).

After filtering the soluble fraction, samples were loaded onto the column and washed to remove Triton X-100 with 60 column volumes of Wash buffer for T22-GWH1-GFP-H6 and 10 column volumes for GWH1-GFP-H6. Elution was achieved by a linear gradient of 20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 8.0. The purified fractions were collected and analyzed by TGX gel chemistry and Western Blotting with anti-His monoclonal antibody (Santa Cruz Biotechnology Inc). Proteins were finally dialyzed against sodium bicarbonate buffer with salt (166 mM $NaHCO_3$ pH 8 + 333 mM NaCl) overnight at 4 °C. Protein purity and integrity were checked by mass spectrometry (MALDI-TOF), and protein amounts were determined by Bradford's assay [26]. Protein production has been partially performed by the ICTS "NANBIOSIS", more specifically by the Protein Production Platform of CIBER-BBN/ IBB (http://www.nanbiosis.es/unit/u1-protein-production-platform-ppp/).

2.2. Nanoparticle Characterization

Volume size distribution of unassembled proteins and resulting nanoparticles was determined by Dynamic Light Scattering (DLS) at 633 nm in a Zetasizer Nano ZS (Malvern). For that, each sample was measured three times in saline sodium bicarbonate buffer. The GFP fluorescence emission of the materials was determined in triplicate in a Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) at 510 nm using an excitation wavelength of 488 nm. For that, protein samples were diluted in the corresponding storage buffer to 1 mg/ml, in a final volume of 100 μl.

To characterize protein nanoparticles, a drop of 3 μl of GWH1-GFP-H6 was directly deposited on silicon wafers (Ted Pella Inc) for 5 min, excess blotted with Whatman filter paper number 1 (GE Healthcare), air dried, and observed without coating with a high resolution in-lens secondary electron detector in a field emission scanning electron microscope (FESEM) Zeiss Merlin (Zeiss) operating at 0.5 kV. A qualitative approach of size and shape at ultrastructural level of GWH1-GFP-H6 nanoparticles was evaluated at this nearly native state.

2.3. Ultrastructural analysis

To determine the ultrastructural effects of nanoparticles over bacterial cells, 1 ml of cultures of each *E. coli* and *S. aureus* growing in two media (LB and MHB) were incubated 24 h alone or with protein (2 μM) in 24-well plates with a coverglass. Coverglasses and medium were processed for scanning electron microscopy (SEM) and transmission electron microscopy (TEM), respectively, by adjusting standard procedures developed for bacterial analysis [27–30]. Briefly, coverglasses with each sample were fixed in 2.5% (v/v) glutaraldehyde (Merck) in 0.1 M PB for 12 h at 4 °C, post-fixed in 1% (w/v) osmium tetraoxide (TAAB Lab) containing 0.8% (w/v) potassium hexocyanoferrate (Sigma-Aldrich) in PB, and, then, dehydrated in graded series of ethanol, dried with CO_2 in a Bal-Tec CPD030 critical-point dryer (Balzers), mounted in stubs, and observed in a FESEM Zeiss Merlin as previously described for nanoparticles. Supernatants of each sample were fixed, post-fixed, and dehydrated as for SEM, embedded in Spurr resin (Sigma-Aldrich), and polymerized at 60 °C for 48 h. Ultrathin sections (70 nm) of selected areas of semi-thin sections (1 μm) were obtained with Leica ultracut UCT microtome (Leica Microsystems, Germany), placed on 200 mesh copper grids, and contrasted with conventional uranyl acetate (30 min) and lead citrate (5 min) solutions. For the qualitative approach, 10 randomly selected areas of each grid were observed with a TEM Jeol JEM-1400 equipped with a CCD Gatan ES1000 W Erlangshen camera.

2.4. Determination of the antibacterial activity

Wild strains of two Gram-positive (*Staphylococcus aureus* ATCC-29737 and *Micrococcus luteus* ATCC-10240) and two Gram-negative bacterial species (*Escherichia coli* ATCC-25922 and *Pseu-*

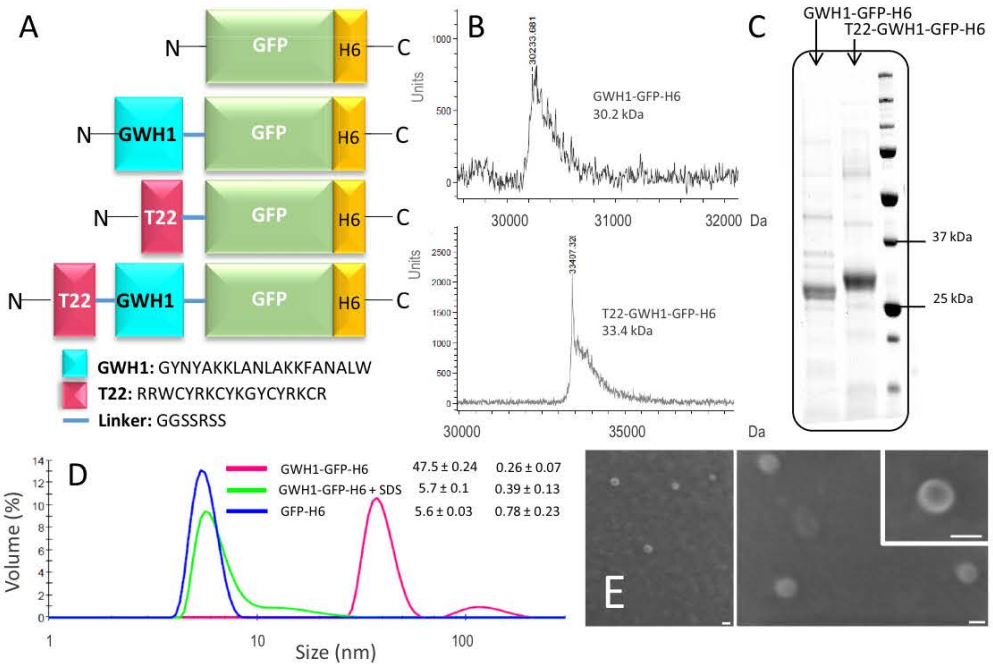

Fig. 1. Characterization of GWH1-based protein nanoparticles. A. Schematic representation of recombinant proteins used in this study. Length of boxes are only representative. T22-GFP-H6 [20] and T22-GWH1-GFP-H6 (Serna et al., submitted) have been fully described elsewhere. B. Mass spectrometry analysis of recombinant GWH1-based proteins, upon affinity chromatography. C Visualization of purified proteins through TGX gel chemistry upon SDS-PAGE. D. Size of GWH1-GFP-H6 nanoparticles compared to the parental GFP-H6. The size of T22-GWH1-GFP-H6 nanoparticles is 24.6 nm. E. FESEM imaging of purified GWH1-GFP-H6 nanoparticles at different magnifications. The bars indicate 50 nm.

domonas aeruginosa ATCC-27853) were cultured at 37 °C (*M. luteus* at 30 °C) and used for the measurement of the antibacterial activity of the fusion proteins. Antimicrobial activity was determined using the Broth micro-dilution method. Various concentrations of 100 μl protein solution (ranging from 0.25 to 2 μM) were added to 96-well plates after 1:10 dilution in sterile Luria broth (LB, Pronadisa), Mueller-Hinton broth (MHB, Oxoid) or Tryptone Soya broth (TSB, Oxoid) medium. Each well was inoculated with a microbial inoculum containing 10^6 cfu/ml prepared in LB, MHB or TSB medium after 1:150 dilution of microbial suspension adjusted to 0.5 McFarland scale. After well-mixing, the inoculated 96-well plates were incubated without agitation under suitable conditions depending on the culture conditions of each microorganism. The number of viable cells was determined in triplicate by CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer's protocol and ATP was quantified using the Multilabel Plater Reader VICTOR3 (PerkinElmer).

2.5. Cell culture and flow cytometry

The CXCR4$^+$ HeLa cell line (ATCC-CCL-2) was cultured in Eagle's Minimum Essential Medium (Gibco) supplemented with 10% foetal calf serum (Gibco), and incubated at 37 °C and 5% CO_2 in a humidified atmosphere. HeLa cells were cultured on 24-well plate at $3{\cdot}10^4$ cells/well for 24 h until reaching 70% confluence. The media was exchanged for serum free Optipro medium (Gibco) prior to the addition of nanoparticles. Cells were incubated for 24 h with different concentration of nanoparticles and analysed in duplicates by measuring fluorescence using FACS-Canto system (Becton Dickinson) with a 488 nm blue laser and using 530/30 nm detector for GFP fluorescence emission.

Specific internalization of nanoparticles was measured using the CXCR4 receptor specific antagonist, AMD3100 (Octohydrochloride hydrate, Sigma). 250 nM of AMD3100 was added 1 h prior to the nanoparticles. T22-GWH1-GFP-H6 and GWH1-GFP-H6 were added subsequently at 25 nM during 1 h. The uptake kinetics were recorded by exposing the cells to nanoparticles at 3 μM for 5 min, 10 min, 20 min, 30 min, 1 h, 2 h, 3 h, 4 h, 5 h, and 24 h. Fluorescence data recorded by cytometry was corrected by the specific fluorescence of each protein, determined by the fluorimeter, to render comparative units in terms of protein amount.

2.6. Cell viability assay

The number of viable HeLa cells was determined in triplicate by CellTiter-Glo® Luminescent Cell Viability Assay (Promega) according to the manufacturer's protocol. Briefly, cells were plated at a density of 3,000 cells/well in DMEM supplemented with 10% FCS in 96-well plates and were allowed to adhere for 24 h. After that, cells were incubated in presence of 10 μM nanoparticles during 24 h at 37 °C. Subsequently, 100 μl of the single reagent (CellTiter-Glo® Reagent) was added directly to cells and the plates were assayed using the Multilabel Plater Reader VICTOR3 (PerkinElmer).

2.7. Statistical analyses

Quantitative data are presented as mean ± SEM. One-way ANOVA followed by Fisher's least significant difference (LSD) method was used for multiple comparisons. Pairwise comparisons were performed using Student-*t* test. Statistical differences were assumed at $p<0.01$. Microsoft Excel was used for all statistical analyses.

3. Results

GWH1-GFP-H6 (Fig. 1A) was successfully produced in recombinant *E. coli* without signs of toxicity. Its single-step purification by His affinity chromatography rendered a protein species with the expected molecular mass of 30.2 kDa (Fig. 1B, C). Since the GWH1 peptide is highly cationic and the combination of end terminal cationic peptides plus polyhistidines promotes protein self-assembling [18], we tested the potential of this protein to form oligomers. Indeed, the spontaneous formation of nanoparticles was observed by DLS in pure preparations of the protein (Fig. 1D), indicative of the good performance of GWH1 as a nanoscale architectonic tag. Those nanoparticles, peaking at 47 nm, were fully disassembled by 0.1% SDS, rendering building blocks of 5 nm, that matched the size of the parental unassembled GFP-H6 (Fig. 1D). The formation of regular GWH1-GFP-H6 nanoparticles was fully assessed by FESEM (Fig. 1E).

To test if GWH1-GFP-H6 would keep the antimicrobial activity upon assembly as protein nanoparticles we exposed cultures of several bacterial species to this material in different media. As observed (Fig. 2A), the antimicrobial activity of GWH1-GFP-H6 in both Gram-negative and Gram-positive species was influenced by the type of medium employed, showing a clear antibiotic activity in LB and in MHB. Because of the enhanced cell death in this media, LB was used for further explorations. More importantly, GWH1-GFP-H6 displayed a potent antibiotic activity over three out of four species, that was clearly dose-dependent (Fig. 2B). The activity of the protein over *P. aeruginosa* was still evident but milder than in the rest of targets. Bacterial death was in all cases clearly linked to cell lysis (Figs. 2D, 3 and Supplementary Fig. 1), strongly suggesting that the antimicrobial activity was reached by the conventional membrane activity of GWH1. Although the effects of GWH1 nanoparticles in baccilli and coccoid cells started with reduction of cytoplasmic electrodensity and end in membrane and wall lysis (Fig. 3), few ultrastructural differences between species were observed (Supplementary Fig. 1). The minimum inhibitory concentration (MIC) of the free GWH1 peptide has been described to be 7.0 μM in *S. aureus* (in TSB) and 3.5 μM in *E. coli* (in NB) [16], whereas GWH1-GFP-H6 exhibited higher bactericidal activity than GWH1, with a MIC of 1 and 0.5 μM respectively (both in LB, not shown).

Since it has not been previously described if a free AMP amino terminus is actually required for such activity (what happens in the case of other AMPs, [31,32]), and envisaging a future potential design of more complex GWH1-based recombinant constructs, we also tested T22-GWH1-GFP-H6 nanoparticles in the same

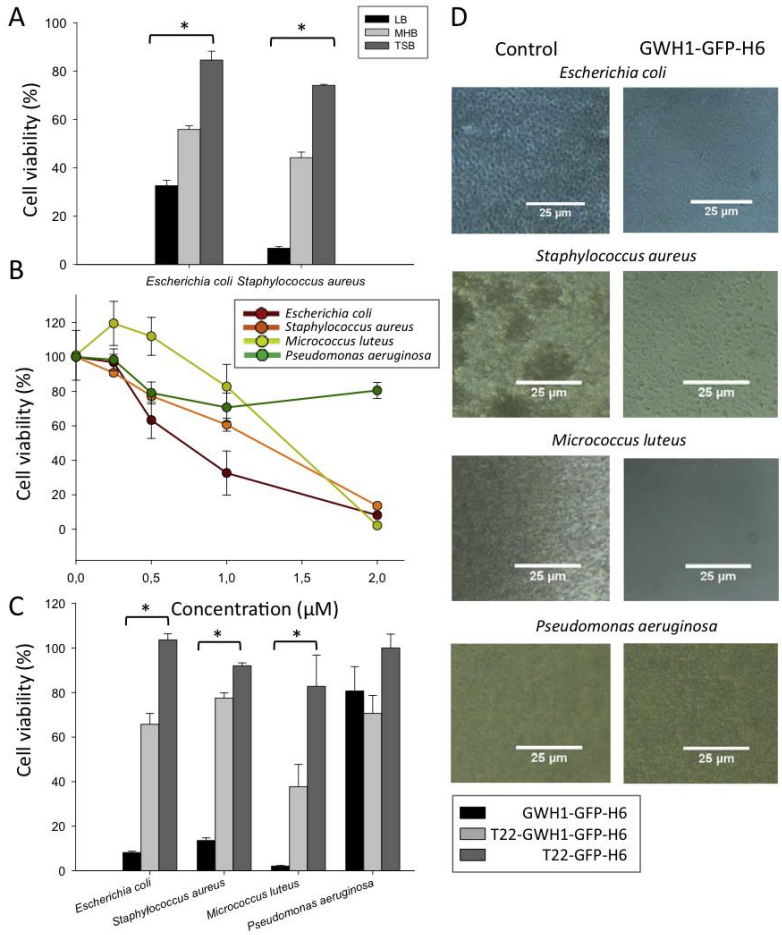

Fig. 2. Antibacterial activities of GWH1-based protein nanoparticles. A. Antibacterial activity of 1 μM GWH1-GFP-H6 upon incubation for 24 h in different media. B. Dose-dependent antibacterial activity of GWH1-GFP-H6 upon incubation for 24 h (in all experiments, 48 h for *M. luteus*) in LB medium. C. Cell viability of different bacterial species exposed to 2 μM GWH1-based protein nanoparticles for 24 h. T22-GFP-H6 is included as negative control. D. Bacterial cell lysis upon incubation with 2 μM GWH1-GFP-H6 nanoparticles for 24 h (48 h for *M. luteus*) monitored by light microscopy.

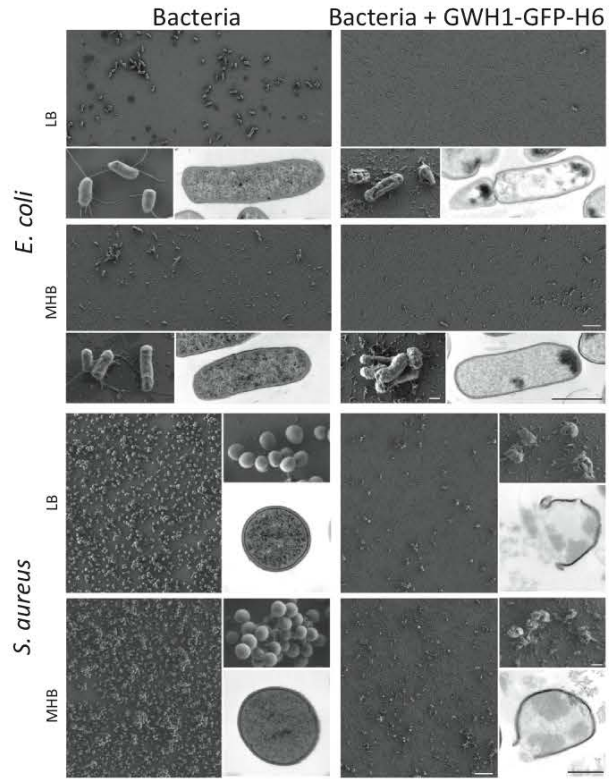

Fig. 3. Representative FESEM and TEM images (general view and detail) of untreated *E. coli* and *S. aureus* cells growing in different culture media, and of culture replicas upon exposure to GWH1-GFP-H6 nanoparticles. Note the abnormal cell morphologies (altered, or death bacteria) and the abundant cell debris and empty membranes or cell walls, in bacterial cultures exposed to nanoparticles. Bars size: 5 μm (general view) and 0.5 μm (detail).

assay. T22 is a cationic ligand of the cytokine receptor CXCR4, that might be clinically relevant to HIV infection (since this protein is a co-receptor of the virus, [33]) and to several human cancers such as pancreatic cancer, metastatic melanoma or osteosarcoma that overexpress this receptor [34–38]. As observed, T22-GWH1-GFP-H6 nanoparticles were still moderately active over two of the target bacterial species (namely *E. coli* and *M. luteus*), although with a milder efficiency than that of GWH1-GFP-H6 (Fig. 2C). The antimicrobial properties of these materials were not provided by T22, as the related oligomeric construct T22-GFP-H6 did not show any biological effect (Fig. 2C).

Because of the previously reported cytotoxic activities of GWH1 over cancer cells, we were interested in knowing if GWH1-GFP-H6 nanoparticles would also show some cytotoxic potential. This might be of relevance since any residual anti-cellular activity of GWH1 nanoparticles would preclude their potential use as antimicrobials. Since GWH1-GFP-H6 exhibited a specific fluorescence that represented approximately 50% of that shown by a His-tagged GFP (not shown), we were able to monitor potential internalization in cultured human cells. As observed, GWH1-GFP-H6 did not internalize HeLa cells, while the related constructs carrying the CXCR4 ligand T22 were both able to penetrate these cells in culture (Fig.4A). This is expected to occur through the known interaction between T22 and the cell surface receptor CXCR4 and the subsequent endosomal uptake of the cargo material [39]. The increase in the positive net charge provided by GWH1 seemed to enhance the penetrability of the construct (in form of T22-GWH1-GFP-H6) probably by favouring T22-mediated protein-cell interactions, which are partially based on the cationic nature of the peptide. GWH1-GFP-H6 nanoparticles were equally inefficient in promoting HeLa cell death, as it also occurred with the control T22-GFP-H6 (Fig. 4B, C). However, cytotoxicity of the protein material was apparent when cells were exposed to T22-GWH1-GFP-H6 nanoparticles (Fig. 4B), that was followed by intensive cell detachment and lysis (Fig. 4C). This fact indicated that the combination of an intracellular targeting agent (T22) and the AMP was highly efficient in cell killing. This observation was in agreement of a suggested antitumoral mechanism for naked GWH1 based on its effective interaction with the mitochondrial but not with the cytoplasmic membrane after receptor-mediated internalization into eukaryotic cells [17]. More importantly, the need of a cell-targeting agents for the cytotoxic properties of GWH1 on eukaryotic cells also allowed to discard, a priori, potential side effects on higher organisms upon the systemic administration of GWH1-based nanomaterials.

4. Discussion

We have produced a recombinant version of the AMP GWH1 (GWH1-GFP-H6) in *Escherichia coli* (Fig. 1A–C), in form of a GFP fusion, that keeps the potent antibacterial functionalities of GWH1 (Fig. 2). The format of the modular protein in which GWH1 has been inserted allows the spontaneous formation of regular and fluorescent nanoparticles of around 50 nm, in which GWH1 simultaneously acts as an architectonic tag and as an antimicrobial agent. The permanence of AMP functions indicates a solvent exposure of the cationic amino terminus in the toroid material, in agreement with what has been demonstrated earlier in the case of other cationic peptides with nanoarchitectonic potential, namely A5, T22 and FN I/II/V [39,40]. Cyclic protein materials are of particular interest as they allow the multiple display of functional stretches [41–43], that in the case of membrane active peptides can cooperatively stimulate membrane binding and cell lysis [44]. The exposure of a free amino terminus of GWH1 has been proved here as highly convenient but not critical for the AMP functionalities of the material (Fig. 2), a fact that allows an easy design of protein nanoscale materials based on this peptide. Importantly, the resulting nanoparticles show, apart from their self-assembling properties mediated by both cationic and the poly-histidine ends, antimicrobial properties, fluorescence and the possibility to one-step purification by the His-based affinity chromatography. These materials, being chemically homogeneous, recruit then multiple functions in the line of the emerging interest for biocompatible nanoscale materials based on technologically simple and cost-effective biofabrication [45]. In this form of nanoparticles GWH1 does not show cytotoxic activities, as it is not able to penetrate cultured cells (Fig. 4). However, by adding to the building block a potent intracellular ligand of a cell surface receptor (T22, which binds CXCR4), the cytotoxic activities of the AMP are apparent, proving its intrinsic antitumoral potential even upon forced oligomerization and the subsequent non-conventional presentation.

GWH1-based nanoparticles have been proved to be active against Gram-positive and Gram-negative pathogenic bacteria (Fig. 2) by a conventional pore-based cell lytic process mechanism in both baccilli and coccoid bacteria (Fig. 3, Supplementary Fig. 1), influenced by the culture media (Fig. 2). Such AMPs action is due to a drastic reduction of the osmoregulation capability of bacterial cell membrane and the pore formation [8], producing a cytoplasmic lost and cell death, as clearly demonstrated by our ultrastructural data in *E. coli* and *S. aureus*. Since the biological activities of AMPs have been determined by diverse methods (mainly serial dilution method and broth micro-dilution) and in different culture

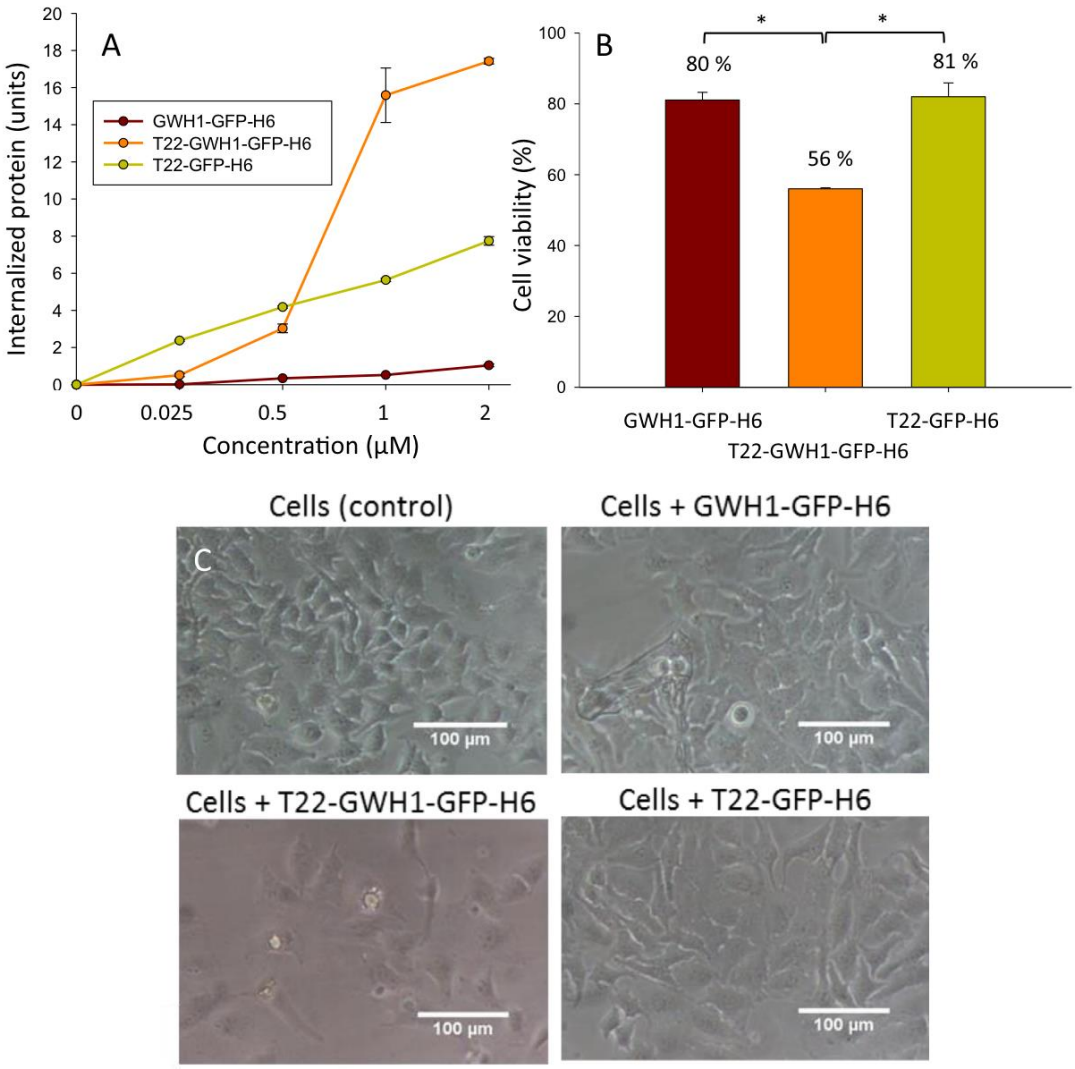

Fig. 4. Cytotoxic activities of GWH1-based protein nanoparticles. A. Protein internalization monitored through intracellular GFP fluorescence 24 h after exposure to nanoparticles. Data has been corrected by specific fluorescence values to allow comparison in a molar basis. B. HeLa cell viability upon exposure to 10 μM of protein nanoparticles for 24 h. C. Light microscopy of cultured Hela cells exposed to protein nanoparticles upon conditions of panel B.

media (including NB, TSB and LB), a precise transversal comparison of potencies between GWH1-based nanoparticles and the free 2.28 KDa GWH1 peptide is not feasible. However, the MIC determined here for the protein materials in LB are higher than those previously described for *E. coli* (0.5 versus 3.5 μM) and for *S. aureus* (1.0 versus 7.0 μM). Even considering the higher potency that AMPs seem to show in rich media (Fig. 2A), these values are essentially within the same order of magnitude (if not higher) than those described for the synthetic peptide. Although the present study is intended to describe a transversal platform to generate nanoscale-organized AMPs rather than to propose a specific product, the biological activity of GWH1-GFP-H6 strongly supports the generic principle, that might be applied to a larger number of peptide candidates. Interestingly, the lytic properties of GWH1 are not eclipsed or precluded by its oligomeric version in form of fusion proteins acting as building blocks (Fig. 3).

Because of the emergence of antibiotic multi-resistances, nanomaterials, and especially silver nanoparticles [22,24,46], show promises for a unique or combined mode of antibacterial therapy alternative to plain antibiotic administration. However, a generalized use of nanoparticles in general and metal nanoparticles in particular, intended as antimicrobials in human therapies, raise severe concerns regarding safety at both patient and environmental levels [47,48]. We have demonstrated here the possibility of the biofabrication of protein-only nanoparticles, formed by AMP-based self-assembling building blocks with in-build antibacterial activities. These kind of cyclic protein materials, being fully compatible, self-organize by electrostatic interactions between building blocks and are fully stable *in vivo* upon systemic administration, showing escape from renal filtration and other properties, such as tissue penetrability, usually associated to nanoscale materials [19]. Furthermore, in absence of cell targeting agents such as ligands of cell surface receptors GWH1 did not show any inherent cytotoxicity over mammalian cells, while keeping a broad spectrum antibacterial potential.

5. Conclusions

In summary, we demonstrate here, for the first time, the possibility to produce by single step biofabrication fully biocompatible,

AMP-based protein-only nanoparticles exclusively active over bacterial cell membranes, with potencies within the same order of magnitude than those exhibited by synthetic short peptide versions. This innovative approach combines the functional and structural versatility, biodegradability and biosafety of protein-only nanoparticles, the structural and functional versatility of recombinant DNA procedures and the appealing potential of AMPs as unconventional but promising antibacterial drugs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2017.07.027.

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REVIEW 1

Protein-Based Therapeutic Killing for Cancer Therapies.

Naroa Serna, Laura Sánchez-García, Ugutz Unzueta, Raquel Díaz, Esther Vázquez, Ramón Mangues and Antonio Villaverde.

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Proteins with potent cytotoxic activities are natural drugs of high pharmacological interest that can be fine-tuned as therapeutic agents. For instance, proapoptotic proteins, antimicrobial peptides, toxins and venoms components have been engineered as potent therapeutic drugs that maintain (and in some cases enhance) product safety and efficacy.

In this review, we have summarized the different class of cytotoxic proteins that along with nanobiotechnological principles may result in a highly efficient antitumor drugs. We highlighted the approaches and emerging trends in protein drug development for the generation of new antitumor drugs that might be competitive in the biopharma market for safer, highly efficient, and more precise cancer therapies.

This is the result of thorough bibliographic research which has allowed us to choose the optimum therapeutic domains, such as proapoptotic proteins, to be incorporated as building blocks into nanoparticles.

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Review

Protein-Based Therapeutic Killing for Cancer Therapies

Naroa Serna,[1,2,3] Laura Sánchez-García,[1,2,3] Ugutz Unzueta,[3,4] Raquel Díaz,[1,2,3] Esther Vázquez,[1,2,3] Ramón Mangues,[3,4,*] and Antonio Villaverde[1,2,3,*]

The treatment of some high-incidence human diseases is based on therapeutic cell killing. In cancer this is mainly achieved by chemical drugs that are systemically administered to reach effective toxic doses. As an innovative alternative, cytotoxic proteins identified in nature can be adapted as precise therapeutic agents. For example, individual toxins and venom components, proapoptotic factors, and antimicrobial peptides from bacteria, animals, plants, and humans have been engineered as highly potent drugs. In addition to the intrinsic cytotoxic activities of these constructs, their biological fabrication by DNA recombination allows the recruitment, in single pharmacological entities, of diverse functions of clinical interest such as specific cell-surface receptor binding, self-activation, and self-assembling as nanoparticulate materials, with wide applicability in cell-targeted oncotherapy and theragnosis.

Highlights

Targeting cytotoxic drugs in oncology is essential because side toxicities limit reaching effective local doses.

Functionalization of nanoscale drug vehicles has so far achieved a moderate targeting effect. The nanoscale size of drug preparations favors enhanced permeability and retention (EPR) and reduces renal filtration.

Proteins are used as inert nanoscale carriers and as functional targeting agents in the form of antibodies or ligands that bind to tumor cell-surface markers.

Many protein species exhibit potent cytotoxic activities that have been exploited to develop new antitumor drugs.

Protein engineering and recombinant DNA technologies allow cytotoxic proteins to be empowered with accessory domains for oligomerization, targeting, endosomal escape, and self-activation. Therefore, the production of self-assembling, self-delivered protein drugs for oncology is becoming feasible.

Antitumor Drugs: Molecular Size, Circulation, and Specificity

Regenerative medicine aims at favoring cell adhesion, viability, and spread under adverse physiological conditions. By contrast, therapies of cancer and of inflammatory or autoimmune diseases (such as Crohn's disease, lupus erythematosus, and multiple sclerosis) are based on effective cell killing. In oncotherapy, the destruction of differentiated cancer cells decelerates tumor growth, while efficient killing of **cancer stem cells** (CSCs, see Glossary; still to be fully accomplished in a clinical context) is expected to control recurrence and metastasis, the primary causes of patient death [1]. Conventional cancer treatments are based on a wide spectrum of systemically administered small molecular weight chemicals (alkylating agents, anthracyclines, microtubule inhibitors, antimetabolites, platinum-based agents, topoisomerase inhibitors, tyrosine kinase inhibitors, and histone deacetylase inhibitors, among others). In the absence of targeting, hepatic and renal damage, and undesired toxicity over other healthy organs, results in numerous life-threatening side effects (Figure 1) including bone marrow toxicity (anemia, thrombocytopenia, and neutropenia), nausea, vomiting, cardiotoxicity, and immunosuppression leading to enhanced susceptibility to infectious diseases. Because systemic toxicity restricts the doses to be administered, drugs do not reach the local concentration necessary for fully effective activity [2]. Insufficient therapeutic effect is also related to the small molecular size of antitumor drugs. Drugs that are below the renal filtration cut-off (estimated to be between 5 and 7 nm [3,4]) are cleared by the kidneys, minimizing their amount in blood and their circulation time (Figure 1). Conjugation to **polyethylene glycol** (PEG) increases drug hydrophilicity, impairs uptake by reticuloendothelial cells, minimizes clearance by neutralizing antibodies, and reduces renal filtration, globally enhancing the therapeutic effect [5]. However, because PEGylation does not add any targeting ability, it does not represent a significant improvement regarding side toxicities. Moreover, reduced circulation time and the absence of selective cell killing in conventional chemotherapeutics have pushed the field towards exploring

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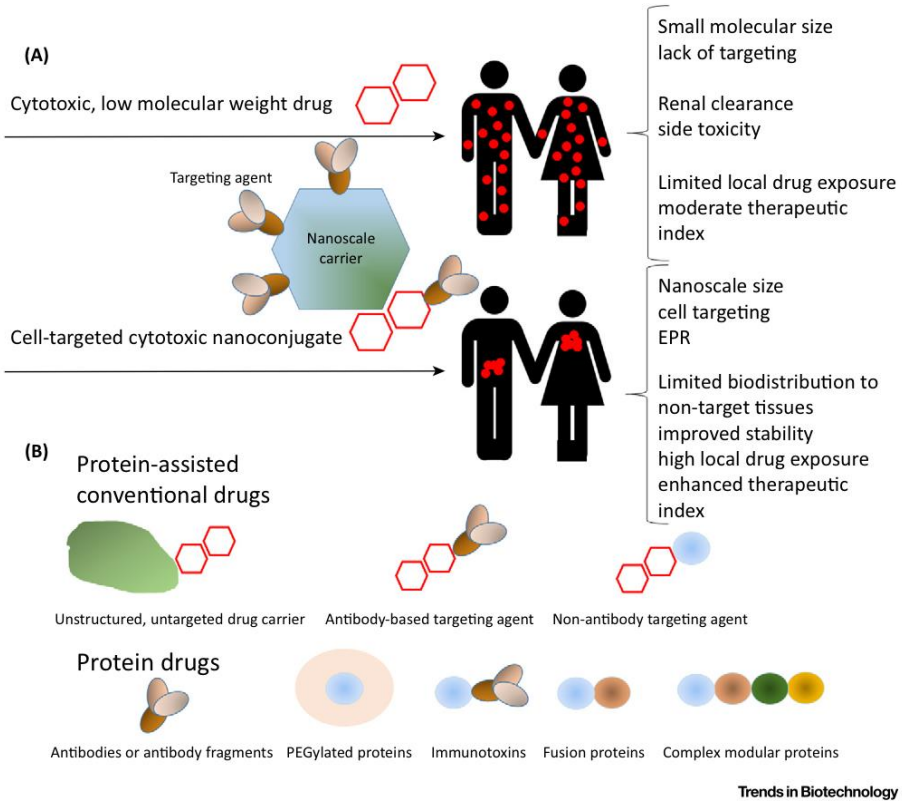

Figure 1. Spectrum of Current Antitumoral Drugs and their Relevant Features. (A) The chemotherapy of cancer is commonly approached by the use of low molecular weight chemicals (red symbols and dots) that display generic cytotoxicity to both tumor and healthy cells. Their low molecular size (usually <5 nm, permitting renal clearance) and lack of selectivity confer them with an undesirable biodistribution. This is associated with severe side effects and suboptimal drug concentrations in tumor tissues. Pharmacological linkage of these chemicals to nanoscale carriers (bottom, blue) and their functionalization with targeting agents (purple) can minimize renal clearance of the nanoconjugates and increase local drug levels. Connecting the drug to carrier nanoparticles or to targeting agents are mechanistically independent strategies which do not need to be necessarily coupled. As an example, antibody–drug conjugates (ADCs; Box 2) consist of chemical drugs that are directly linked to antibodies against tumor cell-surface targets. (B) Diverse roles of proteins in oncotherapy formulations, either as drug-assisting agents (providing nanoscale size and stability or targeting) or as drugs themselves with intrinsic cytotoxic activities. Depending on the designed functionalities, the protein drugs can be presented in alternative constructions or formulations. Abbreviations: EPR, enhanced permeability and retention; PEGylation, linkage to polyethylene glycol.

nanoscale drug carriers [6], which are nanosized particles to which the drug is associated to form a drug nanoconjugate [7,8]. These vehicles, because of their size scale [9], are thought to play a dual role in (i) allowing the effective anchoring of sufficient ligands of tumor surface markers for cell targeting, and (ii) enlarging the size of the conjugate to over the renal cut-off value, thereby minimizing renal filtration [10] (Figure 1).

Cell-Targeted and Untargeted Nanocarriers

Regarding cell-targeted drug delivery, different types of targeting moieties induce selective accumulation in target tissues by exploiting cell-surface molecules that are overexpressed in some cancer cell lineages (Box 1). Binding to these molecules usually promotes receptor-mediated endosomal uptake of the ligands and linked payloads. Internalization is favored by

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Box 1. Cell-Surface Molecular Targets in Cancer

Tumor cells overexpress on their surface different types of molecules (membrane receptors or markers), mainly proteins, that can serve as targets for drug anchorage and specific cell penetration through functionalization with specific ligands [13]. Earlier attempts to target cytotoxic drugs to cancer cells were aimed to fast-dividing tumor cells, leaving tumor-initiating cells unattended. This might result in consequent relapse a few months later because these therapies increase the percentage of CSCs that repopulate the tumor mass and that also account for metastases and resistance to treatment. Therefore, current research on cancer surface markers is mainly focused on CSCs. CSCs are defined by a combination of membrane markers or receptors that are common to different tumors, such as CD44, CD133, CD24, ESA, CXCR4, $\alpha_2\beta_1$, and the multidrug resistance MDR1 and ABCE2 [101,102]. Some of them are particularly associated with specific types of cancer in rapidly expanding catalogues that include CD44, CD24, and ALDH1 with breast cancer [103], CXCR4, LGR5, CLDN1, LY6G6D/F, and TLR4 with colorectal cancer [104–106], CD151 with ovarian cancer [107] and Sox2, Oct4, and CD90 with lung cancer [108]. Because these markers are also expressed by progenitor non-tumor cells [109], the potential risk of side effects is not completely excluded. Therefore, it is a challenge to identify truly selective CSC markers that are sufficiently overexpressed versus progenitor cells to allow safe expansion of the therapeutic window [106]. The development of multispecific of multiparatopic drugs or nanoconjugates should pave the way for more specific delivery into tumor CSCs.

multivalent display of ligands on nanoscale entities, that promotes multiple cell anchorage and favors endosome formation [11]. Aptamers, **monoclonal antibodies** (mAbs), antibody derivatives or mimetics, and receptor specific peptidic ligands [12] have been explored as targeting agents [13]. Avidity (the strength with which a non-covalent attachment to a target molecule occurs) and selectivity (the ability to recognize a very specific target cell or receptor among other cell types or receptor molecules) can be further enhanced by the use of multiparatopic [14] or multispecific [15] agents that bind to different epitopes of a given cell-surface marker or to several markers, respectively, by the recruitment of diverse ligands in the conjugate.

When drugs are required to be relatively large [9], incorporating molecular carriers that are too big might lead to aggregation in the lung and undesired clearance by macrophages of the mononuclear phagocyte system acting in the liver (Kupffer cells) or spleen. This can be avoided by keeping the conjugate size above 7 nm but below 100 nm (in the size range of most viruses) [11]. The nanoscale character of drug–carrier nanoconjugates offers additional advantages such as **enhanced permeability and retention** (EPR) and improved drug stability *in vivo* [10]. The transcellular pores and fenestrae in the tumor vasculature are estimated to measure up to 500 nm [16], allowing the passage of materials up to this size. Targeting agents are usually attached to the carrier (Figure 1). Of course, targeting can be directly conferred to the drug without any carrier by direct chemical coupling between the chemical and a cell-surface receptor ligand. The chemical linker must remain stable during the extracellular phases of the delivery process [17], keep the drug functional, and maintain the proper biodistribution conferred by the targeting agent [18]. **Antibody–drug conjugates** (ADCs, Box 2), using mAbs as drivers, are the best representatives of this category of complexes. The antibody counterpart passively confers a nanoscale size (mostly <10 nm), but usually only monovalent or divalent binding to the target cell.

Many categories of materials (dendrimers, metals, polymers, carbon nanotubes, and proteins, among others) are being explored as partners in drug nanoconjugates. Because most are highly stable and poorly biocompatible, there are reasonable concerns about their intrinsic toxicity, challenging both patient and environment safety [6]. In this context, proteins, as biocompatible macromolecular materials, are especially appealing as drug partners. Protein production in cell factories is undertaken by fully scalable, environmentally friendly, and reliably tested procedures. Since the approval of insulin by the US **Food and Drug Administration** (**FDA**) in the early 1980s, recombinant DNA technologies for protein engineering and production have been extensively developed [19]. Most protein–drug conjugation methods are based

Glossary

Anticancer peptides (ACPs): AMPs that bind to negatively charged molecules on the cancer cell membranes and selectively induce tumor apoptosis or necrosis.
Anti-drug antibodies (ADAs): these are generated during the immune response against an antigen present in a protein therapeutic after its administration to an organism.
Antibody–drug conjugate (ACD): a chemically coupled complex between a drug and a targeting antibody that offers cell selectivity in the delivery process.
Antimicrobial peptide (AMP): often referred as host defense peptides, they are important players in the innate immune response.
Cancer stem cell (CSC): cancer cells with capacity for self-renewal and differentiation into diverse cell types occurring in tumors. The subset of CSCs differ from more differentiated tumor cells in their unique capacity to initiate and repopulate a tumor.
Diphtheria toxin (DT): an exotoxin secreted by the pathogenic bacterium *Corynebacterium diphtheriae*, the etiological agent of diphtheria.
Enhanced permeability and retention (EPR): local drug retention resulting from the highly permeable tumor vasculature combined with poor lymphatic drainage.
Epidermal growth factor receptor (EGFR): a transmembrane protein that acts as a receptor for specific ligands, such as EGF and transforming growth factor-β, that bind to the receptor to activate cell signaling.
Fab fragment: the antigen-binding fragment of an antibody.
Food and Drug Administration (FDA): the US federal agency responsible for protecting public health by ensuring the safety and efficacy of drugs and biopharmaceuticals.
Fv fragment: the variable region of an antibody; comprises the variable loops of the light and heavy chains that are responsible for antigen binding.
Major histocompatibility complex (MHC): a set of proteins displayed on the surface of cells that recognize foreign antigens to trigger their

Box 2. The Antibody–Drug Conjugate Concept – Successes and Limitations

ADCs represent the earliest and simplest strategy to increase drug aggressivity and selectivity against tumor cells. The first approved ADCs were gemtuzumab ozogamicin (Mylotarg) in 2000, indicated for acute myeloid leukemia, and ibritumomab tiuxetan (Zevalin) and tositumomab (Bexxar) in 2002 and 2003, respectively, both indicated for non-Hodgkin lymphoma. In ADCs, mAbs directed against cell-surface markers (Box 1) are used as delivery agents for targeted systemic transport of chemically coupled cytotoxic drugs, ideally inactive in the linked state. Microtubule inhibitors including maytansinoids (DM1/DM4) and auristatins (in form of **monomethyl auristatin E/F**: MMAE, MMAF) rapidly kill proliferating cells and are the most commonly used drugs in ADCs. Cytotoxicity is achieved by receptor-mediated internalization and drug release from lysosomal compartments. Several generations of new ADCs have been developed with increasing efficacies and clinical successes. Humanizing the mAb [110], improving linkers for maximal extracellular stability and intracellular drug release [111], and maximizing the molar ratio between drug and mAb [112] have resulted in improved immunoconjugates. However, they only marginally meet the expected clinical standards regarding efficiency and lack of side toxicity. Frequent life-threatening toxicities are reported for ADCs [113], mainly due to highly potent payload drugs (required because only <1% of the injected ADC dose reaches the tumor [114,115]). The most common adverse effects of ADCs include MMAE-mediated bone marrow suppression leading to neutropenia, infections, and sepsis, and DM4-induced ocular toxicity. MMAF-based conjugates induce thrombocytopenia and ocular toxicity whereas DM1 causes gastrointestinal toxicity, thrombocytopenia, and neutropenia [113]. More than 70 ADCs are currently in clinical development, whereas 20 have been discontinued. As a paradigm of ADC development, gemtuzumab ozogamicin delivers calicheamicin γ1 (one of the most cytotoxic antitumor drugs so far identified) to CD33-expressing cells through a humanized mAb to which the drug is linked by cleavable bonds. The use of gemtuzumab was discontinued in 2010 because of a lack of improved efficacy regarding free drug and significant side effects including severe myelosuppression, type III hypersensitivity, vein occlusion, and death. Only two ADCs are currently on the market, Adcetris® (brentuximab vedotin, targeting monomethyl auristatin E to $CD30^+$ cells and indicated for anaplastic large cell lymphoma and Hodgkin lymphoma) and Kadcyla® (trastuzumab emtansine, targeting emtansine to $HER2^+$ cells and indicated for breast cancer). Both are under strict pharmacovigilance. Slightly differently from ADCs, immunocytokine conjugates do not internalize into cells but instead localize their antitumor effect by stimulating the immune system. This is an active area of research with many new compounds entering clinical trials, such as A-dmDT390-bisFv(UCHT1), moxetumumab pasudotox, LMB-2 [anti-Tac(Fv)-P38], and RG7787 [SS1(dsFv)-PE38]. Taking a fully different perspective, mAbs have been also explored for tumor delivery of more complex antitumor entities. Among them, the CD20-targeted delivery of *Salmonella* bacterial cells expressing prodrug-converting enzymes [116] is particularly interesting in the context of prodrug technologies that pursue the enzyme-mediated local (cell-targeted) activation of the drug cytotoxicity [117].

on lysine-amine and cysteine-thiol coupling by amine-activated ester/carboxylic acid and thiol-maleimide chemistries, respectively. The use of non-natural amino acids (oxime ligation, azide-alkyne cyclization) or enzyme-assisted ligation (sortase A, transglutaminase, glycan remodeling) [20,21] is also common. A paradigm of how proteins are incorporated as partners of small molecular drugs to enhance size and stability is abraxane (Nab-paclitaxel) that was first FDA-approved for breast cancer in 2005. Abraxane is a nanostructured complex (sized 130 nm [22]) formed by non-covalent hydrophobic interaction and high-pressure homogenization of human albumin and paclitaxel. This results in a nanoparticle colloidal suspension [23] for use in metastatic breast, pancreatic, and non-small lung cancers. Similar approaches are represented by Nab-rapamycin, that incorporates rapamycin to albumin and is undergoing clinical trails for refractory bladder cancer, and by xyotax (paclitaxel-polyglumex), a nanometric polymer of polyglutamate conjugated to paclitaxel, in clinical trials for the treatment of ovarian or head and neck carcinomas and glioblastoma.

Cytotoxic Proteins

Many proteins from diverse natural sources exhibit potent cytotoxic activities toward mammalian cells, through deleterious enzymatic activities or by precise interventions in the cell cycle. Snakes are a rich source of cytotoxic proteins for oncology and cardiovascular disorders [24]: marine snails, of ion channel blockers [25]; scorpions, of neurotoxins, antitumor agents, and ion channel blockers [26]; and spiders for painkillers, inflammation, and cardiovascular disorders [27]. Furthermore, plants [28] and bacteria [29] have provided a diversity of protein-based antitumor agents. Botox (Allergan), the *Clostridium botulinum* neurotoxin A (also marketed as

processing and the activation of an immune response. They are classified into class I and II. MHC class II proteins are expressed on dendritic cells, macrophages, and B cells.

Monoclonal antibody (mAb): an antibody produced by the controlled culture of a clone of antibody-producing immune cells.

Monomethyl auristatin E (MMAE): a synthetic derivative of dolastatin, a peptapeptide inhibitor of tubulin polymerization with potent antimitotic activity that was isolated from a species of sea hare.

Monomethyl auristatin F (MMAF): a synthetic derivative of dolastatin, an inhibitor of tubulin polymerization with lower antitumor activity than MMAE.

Platelet-derived growth factor receptor (PDGR): a cell surface receptor that binds to and is activated for cell signaling by the family of PDGF polypeptides.

Polyethylene glycol (PEG): a polymer of ethylene oxide that, once bound to nanoparticles, inhibits their clearance by the immune system.

***Pseudomonas aeruginosa* exotoxin A (PE):** a bacterial secreted protein that inhibits the elongation factor-2 in protein synthesis.

Ribosome-inactivating protein (RIP): a bacterial or a plant protein toxin that arrests protein synthesis in eukaryotic cells by acting on the ribosome.

scFv fragment: single-chain variable region fragment; a fusion between the variable regions of the heavy and light chains of an antibody that is produced as a recombinant protein.

Therapeutic index (TI): an indicator of drug toxicity versus therapeutic efficacy. TI is determined in animal models as the lethal drug dose for 50% of the treated individuals (LD_{50}) divided by the minimum effective dose, also for 50% of the individuals (ED_{50}).

Vascular endothelial growth factor (VEGF): a hypoxia-induced secreted protein that stimulates the formation of blood vessels in normal tissues and in tumors.

Vascular endothelial growth factor receptor (VEGFR): a cell-surface receptor that is bound and

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activated by its ligand VEGF for cell signaling.

Dysport, Ipsen; and Xeomin, Merz Pharma) blocks the neuronal release of acetylcholine, resulting in muscular paralysis [30]. As a paradigm of the wide applicability of toxic proteins, the FDA has approved this bacterial toxin to treat chronic migraines, abnormally intense sweating, strabismus, overactive bladder, and muscle spasms, among other therapeutic applications (apart from the better-known cosmetic use in wrinkle reduction). In this context, venom components and toxins, **antimicrobial peptides** (AMPs), and proapoptotic factors emerge as powerful therapeutic candidates. In addition, antibodies directed to particular cell-surface targets, apart from being used as tools for selective delivery, might initiate themselves deadly signaling cascades by acting as indirect cytotoxic drugs. Many natural or modified forms of these proteins are in clinical trials or are already FDA-approved for oncotherapy (Table 1). Furthermore, the flexibility of proteins as tunable macromolecules allows their functional and structural tuning to reach the desired nanoscale size and targeting [31], that might be achieved in modular, multidomain proteins by the appropriate combination of functional stretches [32,33].

Venoms

Venoms are complex combinations of toxins which are highly bioactive (cytotoxic) molecules that are generally peptides and proteins [34]. They act on exposed cells by diverse mechanisms that include cell-cycle alterations, induction of apoptosis and necrosis [35], cell membrane depolarization [26], cell growth inhibition, cellular membrane disruption, or JAK2/STAT3 downregulation [36]. Numerous venom protein toxins have been produced in recombinant forms (Table 2), revealing a similar modular architecture [37] that offers additional versatility in the engineering of these agents as multifunctional drugs (Figure 2).

Plant Toxins

Individual toxins are found in plants, amphibians, and microorganisms. Plant toxins are extremely potent molecules. Many of them (such as ricin, saporin, abrin, trichosanthin, bouganin, and gelonin) fall into the category of **ribosome-inactivating proteins** (RIPs), *N*-glycosidases that depurinate a single adenine residue in the 23S/25S/28S rRNA stem-loop, blocking protein translation and leading to cell death. Some RIP plant toxins such as trichosantin exhibit an inherent preferential activity for cancer cells that blocks the PKC/MAPK signaling pathway and induces apoptosis [38]. Trichosanthin and related toxins are particularly interesting because they also inhibit HIV-1 multiplication owing to their capacity to cleave supercoiled double-stranded DNA into linear and nicked circular DNA [39,40].

Microbial and Animal Toxins

Microbial toxins have been also adapted as drugs. Denileukin diftitox (Ontak®) is an engineered, FDA-approved drug based on the *Corynebacterium diphtheriae* toxin (**diphtheria toxin**, DT) fused to interleukin-2 that targets the toxin to leukemia and lymphoma cells that display IL-2 receptors [41]. ***Pseudomonas aeruginosa* exotoxin A** (PE) has been also produced through recombinant methodologies in different versions which are in clinical trials to treat mesothelioma and leukemia [42,43]. Among animal toxins, melittin, a 26 amino acid peptide, is the main component of bee (*Apis mellifera*) venom and shows high membranolytic activity [35]. Chlorotoxin is a scorpion peptide (from *Leiurus quinquestriatus*) that can bind selectively to cancer cells via matrix metalloproteinase 2 (MMP-2) and annexin 2 expressed by several malignancies [44].

Antimicrobial Peptides

AMPs are short polypeptides (2–9 kDa) that, in the innate immune system of higher organisms, act as a first line of defense against microbial infections. AMPs show avidity for negatively charged cell membranes and promote cell lysis through pore formation [45]. Some AMPs,

Table 1. Representative Examples of Cytotoxic Antitumor Drugs Involving Proteins and Their Main Side Effects[a]

Drug	Marketed/in trials	Structure/target molecule	Pharmacological indication	Adverse effects	Refs
Chemical drugs					
Protein-stabilized nanoparticles					
Paclitaxel polyglumex	Xyotax	Paclitaxel–poly-L-glutamic acid macromolecular nanoparticle conjugate	Advanced non-small cell lung cancer, recurrent ovarian or colorectal cancer	Neurological toxicity (severe neuropathy), hematological toxicity	[81]
Nab-paclitaxel	Abraxane	Albumin-bound paclitaxel nanoparticle formulation	Metastatic breast cancer, advanced non-small cell lung cancer, pancreatic carcinoma	Electrocardiogram abnormality, peripheral sensory neuropathy, dehydration, nausea	[82]
Protein drugs					
mAbs					
Trastuzumab	Herceptin	Binds to the extracellular domain of HER2 to inhibit the growth of $HER2^+$ tumors	Metastatic $HER2^+$ breast cancer, metastatic $HER2^+$ gastric cancer	Cardiomyopathy, heart failure, infusion reactions (dyspnoea, hypoxia, interstitial pneumonitis), nephrotic syndrome	www.uptodate.com/online/
Cetuximab	Erbitux	Binds to EGFR, HER1, and c-ErbB-1, inhibiting EGF binding, leading to tumor cell apoptosis and inhibition of tumor growth	K-Ras wild-type metastatic colorectal cancer, head and neck cancer, squamous cell carcinoma	Cardiopulmonary arrest, acneiform rash, hypomagnesemia Infusion reactions, hypotension, loss of consciousness, shock, myocardial infarction, interstitial lung disease	www.uptodate.com/online/
Bevacizumab	Avastin	Binds to VEGF-A, preventing its association with endothelial receptors Flt-1 and KDR to block endothelial proliferation, inhibiting angiogenesis and tumor growth	Metastatic cervical, colorectal, or renal cell carcinomas, glioblastoma, non-small cell lung cancer, epithelial ovarian cancer	Gastrointestinal fistula and perforation, heart failure, hemorrhage hypertension, infusion reactions, necrotizing fasciitis	www.uptodate.com/online/
Olaratumab	Lartruvo	Binds to PDGFR-α, preventing PDGF-AA, PDGF-BB, and PDGF-CC binding to block growth and angiogenesis in sarcomas	Soft tissue sarcoma	Nausea, vomiting, diarrhea, hematopoietic toxicity, infusion reaction, hypotension, anaphylactic shock, cardiac arrest	www.uptodate.com/online/
Ipilimumab	Yervoy	Binds to CTLA-4 on cytotoxic T cells, enhancing T cell immune responses against tumors	Unresectable or metastatic melanoma, adjuvant treatment of cutaneous melanoma	Life-threatening immune-mediated dermatitis, colitis and neuropathies, endocrine disorders,	www.uptodate.com/online/

Table 1. (continued)

Drug	Marketed/in trials	Structure/target molecule	Pharmacological indication	Adverse effects	Refs
				hepatotoxicity, ophthalmic toxicity	
Nivolumab	Opdivo	Binds to the PD-1 receptor, blocking PD-L1 and PD-L2 binding and restoring antitumor T cell immune response	Metastatic colorectal, head and neck, squamous, non-small cell lung, renal cell, and urothelial carcinomas, Hodgkin lymphoma, and metastatic melanoma	Adrenal insufficiency, immune-mediated rash, type 1 diabetes, encephalitis, colitis, thyroiditis, nephritis, hepatitis, pneumonitis, hypophysitis, infusion reactions	www.uptodate.com/online/
Multispecific antibodies					
Catumoxomab	Removab	Trifunctional bispecific (EpCAM and CD3) mAb binding tumor, T cells, and Fc region to activate immunity	Malignant ascites due to epithelial carcinomas	Lymphopenia, abdominal pain, nausea, vomiting, diarrhea, pyrexia, fatigue, chills, pain	[83]
Blinatumomab	Blincyto	Bispecific mAb that binds to CD19 on B cells and CD3 on T cells	Relapsed or refractory B cell precursor acute lymphoblastic leukemia	Cytokine release syndrome, neurological toxicity	[84]
Cergutuzumab amunaleukin	In clinical trials	IL-2 variant (IL2v) moiety: bivalent carcinoembryonic antigen (CEA) mAb	Locally advanced and/or metastatic carcinoembryonic antigen-positive solid tumors	Fever, chills, flu-like symptoms, nausea, diarrhea, hypotension	[85]
PEGylated proteins					
Pegaspargase	Oncaspar	PEGylated bacterial asparaginase	Acute lymphoblastic leukemia, extranodal natural killer/T cell lymphoma	Delayed hypersensitivity reactions, neurotoxicity, hepatotoxicity	[86]
Peginterferon	Pegintron	PEGylated Interferon	Melanoma	Neuropsychiatric disorders, bone marrow suppression, autoimmune disease, acute hypersensitivity	NIH database https://clinicaltrials.gov/ NCT00238329
Immunotoxins					
A-dmDT390-bisFv(UCHT1)	In clinical trials	Anti-CD3-gamma-epsilon Fv fragments–modified form of DT	Cutaneous T cell lymphoma	Fever, chills, edema, hypoalbuminemia, hypotension, transaminasemia	[63]
Moxetumumab pasudotox	In clinical trials	Anti-CD22 mAb–modified PE fragment	Relapsed and refractory hairy cell leukemia, acute lymphoblastic leukemia	Hypoalbuminemia, aminotransferase elevations, edema, headache, hypotension, nausea, fatigue	[60]

Table 1. (continued)

Drug	Marketed/in trials	Structure/target molecule	Pharmacological indication	Adverse effects	Refs
LMB-2 [anti-Tac(Fv)-P38]	In clinical trials	Anti-α subunit IL-2R (CD25) mAb–modified PE fragment	Hairy cell leukemia, cutaneous T cell lymphoma, chronic lymphocytic leukemia	Reversible cardiomyopathy, transaminase elevations, fever	https://clinicaltrials.gov/ NCT00321555
RG7787 SS1(dsFv)-PE38	In clinical trials	Mesothelin-binding SS1 Ab–modified PE fragment	Mesothelioma, triple-negative breast cancer, gastric cancer	Edema, hypoalbuminemia, fatigue, vascular leak syndrome	https://clinicaltrials.gov/ NCT00024687
Fusion proteins					
Aflibercept	Zaltrap	VEGFR1 and 2 fragments–Fc human IgG1 fusion protein	Metastatic colorectal cancer	Hemorrhage, gastrointestinal perforation, hypertension, infection	[87]
Etanercept	Enbrel	Tumor necrosis factor receptor–Fc human IgG1 fusion protein	Lymphoma and other malignancies	Tuberculosis, fungal or viral infections, injection site reaction	https://clinicaltrials.gov/ NCT00201682
EphB4-HSA	In clinical trials	EphB4 extracellular domain fused to human serum albumin acting as decoy receptor	Advanced urothelial, head and neck, non-small cell lung carcinomas and melanoma	Steven–Johnson syndrome, toxic epidermal necrolysis, peripheral edema, hematotoxicity	https://clinicaltrials.gov/ NCT01642342 and NCT0271756
Denileukin diftitox	Ontak	Interleukin 2–DT fragments A and B fusion protein	Cutaneous T cell lymphoma	Infusion reactions, hepatotoxicity, visual loss, vascular leak syndrome	[88]
OXS-1550 (DT2219ARL)	In clinical trials	Bispecific scFv anti-CD19 and anti-CD22 mAbs–modified form of DT fusion protein	Relapsed/refractory B cell lymphoma or leukemia	Peripheral edema and hypoalbuminemia	https://clinicaltrials.gov/ NCT02370160

[a]The list is not exhaustive but includes the most explored/used agents.

Table 2. Representative Examples of Main Cytotoxic Proteins Explored as Antitumor Drugs Produced as Recombinant Versions in Bacterial Cell Factories

Protein	Source	Mechanism of action	Therapeutic application	Recombinant protein (producing organism)	Cancer tested	Refs
Proapoptotic						
BID	*Homo sapiens*	Activator: interacts with high affinity to all antiapoptotic proteins and directly activates BAX and BAD	Proapoptotic	*E. coli* RosettaBlue (DE3), *E. coli* M15, *E. coli* BL21 (DE3)	Breast, ovarian, and prostate cancer	[65]
PUMA	*Homo sapiens*	Activator: interacts with high affinity to all anantiapoptotic proteins and directly activates BAX and BAK	Proapoptotic	*E. coli* BL21 and *E. coli* origami B	Colon cancer	[80]
BAD	*Homo sapiens*	Sensitizer: interacts with antiapoptotic proteins High affinity for BCL-2 and BCL-XL	Proapoptotic	*E. coli* BL21	Glioma, leukemia, and gastrointestinal carcinoma	[69]
BIK	*Homo sapiens*	Sensitizer: interacts with antiapoptotic proteins High affinity for BCL-W and BCL-XL	Proapoptotic	*E. coli* BL21 and *E. coli* DH5α	Colon adenocarcinoma	[68]
BAKBH3	*Homo sapiens*	Antagonizes antiapoptotic protein function	Proapoptotic	*E. coli* origami B	Cervical and colon cancer	[80]
Toxin or venom component						
Diphtheria toxin (DT)	*Corynebacterium diphtheriae* (bacterium)	Inhibition of EF-2 and therefore protein synthesis	Proapoptotic	*E. coli* BL21(DE3)	Neuroblastoma, breast cancer, and colon cancer	[89]
Exotoxin A (PE)	*Pseudomonas aeruginosa* (bacterium)	Inhibition of EF-2 and therefore protein synthesis	Proapoptotic	*E. coli* BL21(DE3)	Burkitt's lymphoma	[90]
Chlorotoxin	*Leiurus quinquestriatus* (scorpion)	Chloride channel blocker	Targeting and slightly apoptotic	*E. coli* BL21 Star™ (DE3)	Glioma	[91]
Melittin	*Apis mellifera* (bee)	Surfactant activity	Cytotoxicity	*E. coli* Rosetta	Glioma	[92]
Gomesin	*Acanthoscurria gomesiana* (spider)	Pore formation	Proapoptotic	*E. coli* BL21(DE3)	Epidermoid carcinoma, cervical adenocarcinoma, and breast adenocarcinoma	[93]
Agkihpin	*Gloydius halys* Pallas (snake)	induces apoptosis or necrosis but the mechanism remains to be explored	Anti-metastasis	*E. coli* BL21 (DE3) and DH5α	Liver cancer	[94]

Table 2. (continued)

Protein	Source	Mechanism of action	Therapeutic application	Recombinant protein (producing organism)	Cancer tested	Refs
Colombistatins 2, 3, and 4	*Bothrops colombiensis* (snake)	Inhibit ristocetin, ADP, collagen	Potent anti-platelet aggregation activity	*E. coli* BL21 star	Human skin melanoma	[95]
Ricin	*Ricinus communis* (plant)	Protein synthesis inhibition by the cleavage of a single adenine residue in 28S ribosomal RNA	Anti-proliferative activity	*E. coli* strain MV1190	Leukemia and lymphoma	[96]
Abrin	*Abrus precatorius* (plant)	Protein synthesis inhibition by the cleavage of a single adenine residue in the 28S ribosomal RNA	Cell growth inhibition	*E. coli* BL21(DE3) and Rosetta strains	Melanoma and colon cancer	[97]
Gelonin	*Gelonium multiflorum* (plant)	Protein synthesis inhibition by the cleavage of a single adenine residue in the 28S ribosomal RNA	Anti-proliferative activity	*E. coli* BL21 (DE3) and TOP10 strains	Leukemia, glioblastoma, cervical, prostate, and ovarian cancer	[98]

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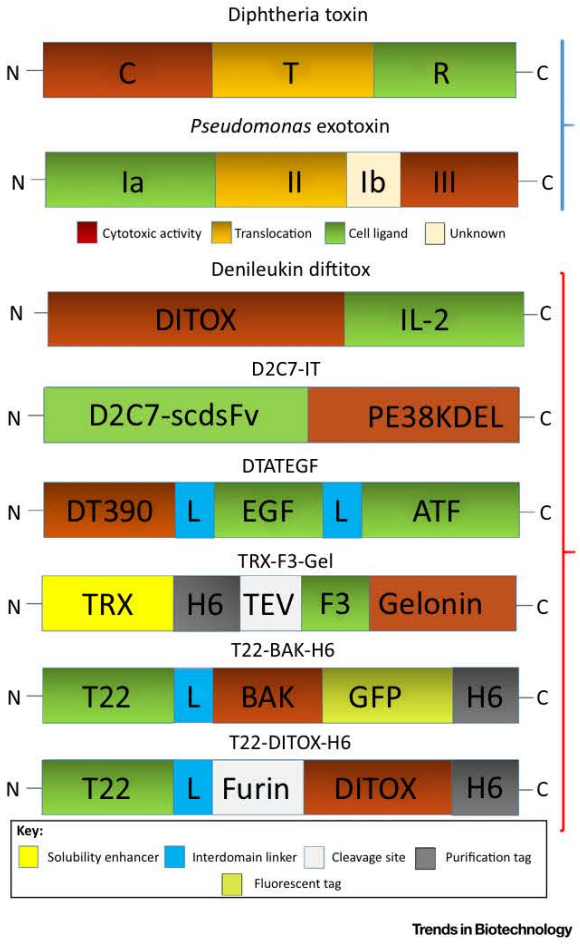

Figure 2. Modular Organization of Natural and Representative Engineered Toxins. Natural toxins (blue set) usually show a modular architecture, illustrated here by diphtheria toxin (DT) and *P. aeruginosa* exotoxin A (PE). Engineered versions (red set) have been adapted by modular protein engineering for functional recruitment as antitumor drugs. Denileukin diftitox is an immunotoxin that delivers DT (lacking the receptor-binding domain, Ditox) and targets the IL-2 receptor [88]. D2C7-IT is an immunotoxin fusion consisting of single-chain variable-region antibody fragments (**scFv fragments**) of the mAb D2C7 (D2C7–scdsFv). It targets both the wild-type form (EGFRwt) and the in-frame deletion mutant form (EGFRvIII) of epidermal growth factor receptor (EGFR), and is fused to domains II and III of PE (PE38KDEL) [D2C7-(scdsFv)-PE38KDEL] [99]. DTATEGF is a bispecific immunotoxin based on a DT version (DT390) that binds to both the EGF receptor (EGFR) and the urokinase-type plasminogen activator receptor (uPAR) [100]. In TRX-F3-Gel the active N-terminal segment of the plant toxin gelonin is targeted by F3, a ligand of nucleolin that is overexpressed by several tumor cell lineages. The thioredoxin (TRX) H6 segment, that is used to enhance solubility and for purification upon recombinant production, is removed *in vitro* by Tev protease [98]. In T22-BAK-H6 the human proapoptotic BAK is targeted by T22, a ligand of the cell-surface tumor marker CXCR4. The construct self-assembles as toroid nanoparticles through the combined presence of T22 and H6 (H6 also acts as a purification tag, and GFP allows visualization of the material) [80]. In T22-DITOX-H6 the C and T domains of DT are presented in a similar way. The inserted furin cleavage site complements the internal site located between the C and T domains. In the endosome, the minimal cytotoxic segment of the construct, namely the C domain, is released upon endosomal acidification. Box sizes are merely illustrative and do not reflect actual proportions.

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called **anticancer peptides** (ACPs), selectively bind to cancer cells, inducing tumor apoptosis or necrosis [46,47]. Some ACPs also inhibit tumor angiogenesis [48] and show immunomodulatory activities [49]. Most ACPs are of human or animal origin, but others have been isolated from peptide libraries or have been generated by *de novo* design.

Proapoptotic Proteins

The apoptotic cell death program serves as a natural barrier to tumor development through the extrinsic apoptosis pathway, that is activated by extracellular proapoptotic stimuli, and via the intrinsic pathway, that is mainly controlled by the BCL-2 family of proteins consisting of antiapoptotic and proapoptotic members [50]. Proapoptotic proteins can be categorized into BH3-only proteins (BIM, BID, PUMA, NOXA, BAD, BIK, and HRK) that contain only one BCL-2 homology (BH) domain (BH3), and into multidomain proteins (BAX and BAK) with four BH regions (BH1, BH2, BH3, and BH4) [51]. BH3-only proteins are divided into activators and sensitizers [52]. Activators convert inactive BAX/BAK monomers into pore-forming proteins that assemble into oligomeric complexes in the mitochondrial outer membrane. Sensitizers displace activator BH3 proteins from binding to antiapoptotic members, leaving them free to bind to and activate BAX/BAK [53]. The clinical value of proapoptotic proteins (and many AMPs as well) as drugs in oncology is enriched because of the human origin of these proteins, which administration would not promote the immunotoxicity that is usually associated with heterologous protein drugs.

mAbs

mAbs are not only used as drivers in targeted drug delivery but they can also induce antitumor effects by direct interaction with the target protein [54]. Therefore, they represent the largest group of approved therapeutic proteins in oncology [55]. Most inhibit target receptors involved in tumor epithelial cell growth [such as HER2, **epidermal growth factor receptor** (EGFR) and **platelet-derived growth factor receptor α** (PDGFR-α)], but others inhibit tumor growth indirectly by targeting ligands or receptors involved in tumor angiogenesis, such as **vascular endothelial growth factor** A (VEGF-A) or **vascular endothelial growth factor receptor** 2 (VEGFR-2). In addition, the fastest-developing mAb drugs target cancer and immune cell (e.g., T cell) molecules (CTLA-4, PD-1, PD-L1) to reactivate antitumor immune cell function (Table 1). In comparison to untargeted chemotherapy, mAbs display a longer half-life, increased selectivity, and reduced off-target effects. However, their limited extravasation and tumor access promote the development of tumor resistance and dose-limiting toxicities [56].

Engineering Cytotoxic Proteins as Drugs

Most cytotoxic proteins that are approved or under clinical development are not natural but are modified versions with improved functionalities. Toxins and mAbs of non-human origin are generically immunotoxic and require deimmunization-oriented engineering. By contrast, nanoscale organization through multimeric self-assembling, ideally conferring multivalent cell targeting (necessary for non-antibody protein drugs), requires functional recruitment by the fusion of additional protein stretches to the active drug domain (Figure 2). Therefore, protein-based cytotoxic drugs usually have a modular architecture, a concept clearly illustrated by immunotoxins that are simple modular fusions of a toxin (for cytotoxicity) and an antibody or antibody fragment (**Fab fragment**) (for cell targeting).

Deimmunization

Drugs based on non-human proteins contain antigenic peptides that are presented by **major histocompatibility complex** (MHC) II molecules on antigen-presenting cells in a process that activates T cells and stimulates B cells to generate **anti-drug antibodies** (ADAs). In addition, B

cells can be directly activated by multivalent ligands and B cell receptor crosslinking by foreign epitopes [57], which leads to ADA-mediated immune responses during drug treatment upon re-exposure. This event, occasionally inconsequential, may instead neutralize drug effectiveness or cause serious clinical adverse effects which may terminate drug development or lead it to be withdrawn from the market. In this context, hypersensitivity reactions have been reported [58], including acute infusion reactions occurring shortly upon re-exposure (e.g., denileukin difitox, brentuximab vedotin, trastuzumab emtansine), hypersensitivity to unrelated allergens, or the development of autoimmune diseases and flu-like reactions (cergutuzumab, amunaleukin, blinatumomab) associated with cytokine release (Table 1). Less often, therapeutic proteins may be immunosuppressive, leading to frequent and often severe adverse effects such as relapsed bacterial, viral, or fungal infections (e.g., Y90-ibritumomab tiuxetan, etanercept, aflibercept, sitimagene cerdenovec, and talimogene laherparepvec) and complications such as virus-induced neoplasias.

Early immunotoxins (i.e., immune-targeted toxins, see below) lacked a sufficient therapeutic window because of dose-limiting toxicity that induced the life-threatening vascular leak syndrome (edema, weight gain, hypoalbuminemia, and orthostatic hypotension) [59]. Precise protein engineering has been applied to reduce the immunogenicity of PE and DT catalytic fragments to be incorporated to immunotoxins. The portions of these toxins that are not essential for cytotoxic activity or processing have been deleted from the sequence, reducing the molecular weight of the cytotoxic drug component [58]. Moreover, immunotoxicity has been minimized by eliminating antigenic T and B cell epitopes, which limits the immunogenicity of the toxin and reduces the off-target effects that prevent repeated treatment cycles. Deimmunization of a PE fragment (PE38) was achieved by introducing mutations in B or T cell epitopes without compromising antitumor potency, and by deletion of the PE domain II which prevented the induction of vascular leak syndrome [60]. A truncated DT (DT390) has also been deimmunized by point mutations of surface-exposed highly hydrophilic amino acids (R, K, D, E, and Q) to eliminate B cell epitopes without losing antitumor activity [61]. Third-generation immunotoxins consisting of a humanized targeting moiety (e.g., a mAb, **Fv fragment**, or Fab) fused to a deimmunized cytotoxic domain of the toxin are currently entering clinical trials. mAbs tend to offer a higher **therapeutic index** (TI) than small-molecule drugs, namely a wider margin between effective and toxic doses. However, their protein nature and relatively large size may stimulate the immune system, leading to various adverse effects (Table 1). Murine mAbs induce the formation of human anti-mouse antibodies in patients, but protein engineering efforts to humanize them have significantly reduced their immunogenicity [58].

Simple Fusion Technologies

Immunotoxins (Table 1) are composed of catalytic fragments of highly cytotoxic plant or bacterial toxins bound to highly selective targeting mAbs, Fv, or Fab fragments. They kill dividing and non-dividing cells by inhibition of protein synthesis, a unique mechanism of action that is synergistic in combination with genotoxic chemotherapy provided that they show non-overlapping toxicities [62].

An immunotoxin containing the DT A and B fragments fused to human IL-2 was marketed in 2001 as denileukin diftitox. It showed activity against several hematological malignancies, particularly cutaneous T cell lymphoma (CTCL). However, the induction of vascular leak syndrome has limited its use. Two additional immunotoxins are currently in clinical assays. A-dmDT390-bisFv(UCHT1) is a fusion protein of DT bound to the Fv fragment of CD3 that targets T cells and is active against CTCL [63], and DT2219ARL consists of a DT fragment bound to Fv fragments of CD19 and CD22 that are active against B lineage leukemia or

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lymphoma. In addition, an immunotoxin consisting of PE38 fused to an anti-Tac subunit of IL-2R [LMB-2; anti-Tac(Fv)-PE38] is currently in clinical trials, and shows activity in several hematological neoplasias. RG7787 is composed of an Fab version of the SS1 antibody bound to a modified and less-immunogenic PE fragment. Because it is active in animal models of mesotheliomas without significant adverse effects, it is expected to enter clinical trials soon. Moxetumomab pasudotox is an anti-CD22 Fv fused to PE38 that is being evaluated for the treatment of $CD22^+$ B cell malignancies (e.g., hairy cell leukemia, acute lymphoblastic leukemia) which show high response rates [60]. Antibody and antibody fragments have been also used for the targeting of non-toxin cytotoxic proteins such as proapoptotic factors. An example is e23sFv-TD-tBID, which exploits a single-chain anti-HER2 antibody fragment to target the proapoptotic BCL-2 family member BID [64].

From a different approach, simple fusion technologies facilitate selective binding and/or cellular penetration of protein drugs by non-antibody protein agents such as cell-penetrating peptides (CPPs). Proapoptotic peptides fused to the transactivator of transcription (TAT) of human immunodeficiency virus (TAT–BID) [65], Antennapedia homeoprotein (Ant–BAKBH3) [66], or the receptor-binding domain of DT (Bad–BTTR) [67] immediately activate untargeted apoptosis. Other driving peptides used as fusions are gonadotropin-releasing hormone (GnRH, in the form of GnRH–BIK, GnRH–BAK, and GnRH–BAX) [68] and the human granulocyte-macrophage colony-stimulating factor (as hGM-CSF–BAD) [69]. Similar approaches applied to AMPs promote their internalization and mitochondrion-dependent apoptosis in the micromolar range. For example, the natural magainin II (MG2) fused to the CPP penetratin shows an IC_{50} in the micromolar range [70]. Even more appealingly, MG2 linked to bombesin recognizes a variety of human cancer cells and it shows specific and higher cytolytic effects compared to magainin alone in mice bearing MCF-7 breast tumor grafts [71]. Moreover, the *de novo* designed antimicrobial peptide KLAKLAK fused to a protein transduction domain (PTD) specifically kills endothelial cells [72], and the same peptide fused to HER2-targeting/neutralizing domain targets specifically HER2-overexpressing cells *in vitro* and *in vivo* [73].

More sophisticated versions of fusion technologies generate modular recombinant proteins with diverse functionalities through domains collected from different origins (Figure 2). Functional recruitment enhances the precision in the protein drug delivery process, enabling the polypeptide to perform accurate extra- and intracellular activities. Most of these constructions are produced in very simple microbial cell factories (Table 2) according to generic protein production technologies.

Modular Design of Smart Cytotoxic Proteins

Innovative antitumor drugs still show severe side effects despite these engineering efforts (Table 1), and have therefore driven further drug development based on safer principles. The two-partner fusion strategies discussed above (and also most of the modular approaches) enhance specificity but with still inappropriate nanoscale size and usually with mono- or divalent presentation of the targeting agent. Conventional nanoscale carriers used in nanomedicine, however, impose an undesirable burden of potentially toxic bulk material that prompts urgent exploration of vehicle-free nanostructured drugs able to self-assemble [10]. In this emerging concept, self-assembling protein domains [74] can be used in modular constructs that self-organize as vehicle-free multifunctional protein drugs. For instance, some cationic peptides that are potent ligands of tumor markers promote oligomerization of fusion proteins when combined with polyhistidines. As a paradigmatic example, the peptide T22, a ligand of CXCR4 (overexpressed in >20 human cancers), has been incorporated to histidine-tagged GFP constructions, and makes them self-organize into regular nanoparticles of 12–60 nm that feature

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multivalent display of this peptide [75,76]. Upon injection, these materials accumulate in tumor tissues in absence of renal filtration [3]. The same principle has been applied to protein-only blood–brain barrier (BBB)-crossing nanoparticles [77] and to CD44-targeted nanoparticles for imaging or drug delivery in breast cancer [78]. The modular architecture of these fusions allows the incorporation of additional functional domains such as fusogenic peptides for enhanced endosomal escape [79]. By exploiting this principle, proapoptotic peptides, AMPs, and microbial toxins have been instructed to self-assemble as cell-targeted nanoparticles ([80] and our unpublished data).

These strategies, together with the accumulated information on cytotoxic proteins, targeting agents, recombinant antibodies, and other functional domains discussed above, should allow fast emergence of truly vehicle-free [10] and cell-targeted cytotoxic nanomedicines that, based on functional recruitment, would necessarily involve multifunctional proteins as core components.

Concluding Remarks and Future Perspectives

Unquestionably, targeting cytotoxic agents in cancer therapies is urgently needed. A plethora of approaches in this regard, using nanotechnological principles, have so far offered improved but still only moderately effective drugs mainly because of associated side toxicities. Empirical observations but also emerging bioengineering concepts point to the design of protein-based cytotoxic drugs as promising alternatives. Proteins are extremely versatile macromolecules produced in recombinant cell factories by cost-effective and fully scalable methods based on recombinant DNA technologies that have been developed and optimized for almost 40 years. In contrast to other biological macromolecules, nanostructured materials, and chemicals, proteins can simultaneously execute, in single-chain polypeptides, all the functions required in oncotherapy (see Outstanding Questions). These activities include efficient cell targeting, potent cytotoxicity, self-assembly to achieve the optimal nanoscale size, and regular oligomerization for multiple and ordered display of cell ligands. The incorporation of functional cassettes by simple fusion approaches allows affinity tags to be recruited for one-step purification from cell factories, endosomolytic agents, protease target sites, and intracellular trafficking domains, among others. Anticipated bottlenecks in the use of these biopharmaceuticals have been already observed and minimized during the development of the >400 protein drugs that are approved for human use. Protein engineering offers valuable approaches for significant deimmunization or ablation of residual drug interactivity with non-target organs (that might lead, for instance, to hepatic toxicity). In this context, an increasing number of protein-only prototypes have already confirmed the possibility of recruiting high functional complexity in simple and safe biological entities. This is in contrast to chemically heterogeneous nanoconjugates in which these functions are provided by the conjugation of different types of molecules, mostly produced by non-biological processes. The expanding catalogues of functional modules (venoms, toxins, proaoptotic factors, AMPs, and others) and cancer-relevant ligands, together with emerging nanobiotechnological principles, are expected to result in a new generation of antitumor drugs that – solely formed from recombinant proteins – might be competitive in the biopharma market for safer, highly efficient, and more precise cancer therapies.

Outstanding Questions

Can cytotoxic proteins be engineered to fully eliminate their side toxicities through precise protein engineering or humanization?

Are ligands of CSC-specific markers sufficiently potent to allow a significant local accumulation of associated drugs in cancer tissues?

Would protein engineering provide satisfactory tools for competitive large-scale recombinant production of effective protein-only cytotoxic drugs?

Would self-assembling, cell-targeted, and self-delivered protein drugs be a realistic alternative or a synergistic complement to current cancer therapies based on untargeted chemical drugs?

Disclaimer Statement

A.V., E.V., N.S., L.S-G., U.U., and R.M. are coinventors of a patent covering the use of self-assembling, nanostructured cytotoxic proteins.

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Appendix A Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.tibtech.2017.11.007.

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The following papers have been placed in the **annex** of this PhD thesis as they are not part of the main body but will be referred to the discussion section:

- CXCR4-targeted toxin-based protein only-nanoparticles for oncology:
  - Annex 1, Article 4:
    Self-assembling toxin-based nanoparticles as self-delivered antitumoral drugs.
  - Annex 2, Draft 1:
    Toxin-based nanoparticles are effective tools against human colon cancer stem cells.

- Endosomolytic peptides for enhanced transfection efficiency of protein-only nanoparticles :
  - Annex 3, Article 5: The fusogenic peptide HA2 impairs selectivity of CXCR4-targeted protein nanoparticles.
  - Annex 4, Manuscript 1: Tuning tumor cell targeting and penetrability of self-assembling polypeptides through functional recruitment.

- Annex 5, Manuscript 2: Switching cell penetrating and CXCR4-binding activities of nanoscale-organized arginine-rich peptides.

DISCUSSION

Conventional cytotoxic drugs have a narrow therapeutic window, with high peaks and troughs of plasma concentration. Nanoparticle formulations of cytotoxic drugs can enhance pharmacokinetic characteristics and facilitate passive targeting of drugs to target tissues via the enhanced permeability and retention effect, thus mitigating toxicity[58;64;114]. By enlarging the size of the whole drug conjugate over ~ 5–8 nm up to 100 nm, renal clearance and accumulation in organs are avoided while cell penetrability is preserved and often enhanced. Currently, the new nanoparticle drug formulations in development and undergoing clinical trials utilize active targeting or trigger drug release based on environmental stimuli that ensure more efficient outcomes[61;67;70;93;124].

Nanomaterials under investigation for the generation of the carriers include mostly liposomes, polymeric micelles, protein nanoparticles, and dendrimers. Among them, protein-based nanomaterials such as synthetic protein NPs, antibodies and protein assemblies are the most appealing carriers for drug delivery due to their high biocompatibility and biodegradability[107;111]. However, these protein nanomaterials show some weaknesses that must be discussed. Regarding to synthetic protein NPs, currently available methods for synthesizing nanoparticles can easily promote agglomeration and lead to nanoparticle heterogeneity and instability[244-247]. With respect to antibodies, these targeted carriers show poor penetrability into the tumor tissue and require highly potent payload drugs leading to frequent life-threatening toxicities[171;172;178]. On the other hand, protein assemblies formed endogenously in living organisms such as ferritins, small-heat shock proteins, eukaryotic vaults and bacterial organelles offer poor functional flexibility, tunability and limited controlled-geometry[181;182]. Viruses, other well-studied examples in nature of protein self-assembled cages, have been exploited as nucleic acid-containing delivery agents but transient transgene expression and strong side effect have limited their use[191]. In the line of safest approaches, virus like particles mimic a supramolecular self-assembling behavior of viruses but are non-infectious, non replicative and are therefore safer than viral vectors. However, their lack of flexibility, limited DNA-carrying capacity and poor functionality has strongly limited their general applicability[192-194].

In this context, fully de novo designed self-assembling proteins, generated through genetic engineering to incorporate desired functions, appear as very attractive alternative to conventional protein carriers. The construction of these assemblies relies on the controlled oligomerization of individual protein motifs or polypeptides, which act as building blocks of complex supramolecular arrangements[183;248]. Self-assembling of polypeptides occurs by non-covalent, weak cross-molecular local contacts between monomers including the formation of disulphide bridges, water-mediated hydrogen bonds, electrostatic interactions and van der Waals interactions. Such self-organizing processes can be controlled by extrinsic parameters such as temperature, ionic strength, pH and the nature of the solvent[249].

Many natural (collagen-mimetic peptides, β-sheet peptides and straight α-helices) or in-silico designed peptides (amyloidogenic or amphiphilic) have been recognized as building blocks for the predictable bottom-up design of protein nanostructures including fibrils, tubes, spheres, ribbons, vesicles, micelles, monolayers, bilayers and hydrogels[200;201]. Unfortunately, no regular cages have been so far constructed with these self-assembling peptides as a fully systematic approach due to their moderate functional versatility. Furthermore, when fused as tags to larger proteins and produced in biological systems, these peptides promote the aggregation of the fusion protein into inclusion bodies proving that these agents are not valuable as fine architectonic tags in larger modular protein constructs[202]. Therefore, the development of new engineering oligomerization approaches is needed. Unluckily, only isolated cases of successful de novo protein-only nanoparticles construction and controlled structural modulation have been reported, being mostly derived from unanticipated observations[250;251].

On this background, our research group has developed a new engineering oligomerization approach based on the combined use of non-amyloidogenic architectonic tags which enables to obtain NPs that present functional versatility, high stability and simple and cost effective biological production by recombinant systems[235;237].

Nanocarriers, being in general biologically inert, once nanostructured, must be functionalized to gain cytotoxic activity among other properties necessary for a proper

biodistribution such as targeting ability, cell surface receptor binding, membrane crossing and nuclear penetration. The general approach to provide cytotoxicity involve the loading of chemical drugs in nanosized carriers[249;252]. In protein nanoparticles, drugs are usually conjugated by lysine-amine and cysteine-thiol coupling by amine-activated ester/carboxylic acid and thiol-maleimide chemistries, respectively[253;254]. In some cases, chemical linkers can be used to enhance drug conjugation efficacy. However, limited drug loading, instability of the chemical linker during the extracellular phases of the delivery process and drug leakage that leads to possible side effects may limit the use of protein DDS[238]. Furthermore, NP drug loading may affect the therapeutic efficacy and pharmacokinetics of the DDS as demonstrated in different studies[255].

In this context, protein fusion technologies allow the employment of many cytotoxic proteins as building blocks of supramolecular entities being an alternative approach to conventional drug protein nanoconjugates avoiding sequential, biologically unfriendly and chemically complex functionalization processes[216;241]. For instance, toxins and venom components, proapoptotic factors, and antimicrobial peptides have been already engineered as highly potent drugs. Immunotoxins are the best representative example of these protein complexes but they usually show lack of sufficient therapeutic window because of dose-limiting toxicity, possible immunogenicity, poor tissue penetrability and only a mono- or divalent presentation of the targeting agent[243]. From a different approach, protein drugs have been also fused to peptides that facilitate selective binding and/or cellular penetration of protein drugs such as cell-penetrating peptides (CPPs)[256]. However, this fusion strategy still shows inappropriate nanoscale size and inefficient therapeutic index.

Thus, we think that the fusion of cytotoxic proteins in a de novo designed self-assembling protein platform that contain all the modules required for a proper biodistribution is a very attractive approach that could allow the generation of "all-in-one" multifuntional protein-only NPs produced in one step by recombinant procedures with an applicability in a variety of diseases.

In this study, we have provided new insights regarding the engineering of peptides that together with polyhistidine tag can act as oligomerization peptides being valuable as

fine architectonic tags in larger modular protein constructs. This engineering principle permits the generation of still functional protein-based carriers with predictable size. Generally, formulating therapeutic proteins as functional nanoparticles allows proteins to correctly biodistribute and to efficiently internalize into target cells upon systemic administration enabling their broad applicability in nanomedicines such as cancer and infectious diseases. However, our results prompt to carefully evaluate the convenience of using protein nanoparticulate materials for BBB-crossing therapies for the treatment of CNS diseases and highlight the need to develop new alternative therapies that may not be focused in nanotechnology approach.

On the other hand, we have generated here for the first time self-assembling and self-delivered protein-only nanoparticles with intrinsic therapeutic activity produced in a single step by recombinant procedures that show proper biodistribution and therapeutic effect being potentially attractive for human pathologies such as cancer and infectious disesases. These protein asemblies are presented as alternatives to conventional carriers in terms of high biocompatibility, biodegradability and functional versatility as well as preventing side effects related to drug leakage and avoiding further biologically unfriendly functionalization steps.

1) Evaluation of a new engineering principle to produce self-assembling protein-only nanoparticles of suitable and predictable size for their broad applicability in biomedicine.

Protein materials are rapidly gaining interest in nanomedicine because of their intrinsic biocompatibility and full biodegradability[111;145]. Additionally, biological and environmental-friendly fabrication of proteins in recombinant organisms and the possibility to modulate their structure and function through genetic engineering allow the generation of multifunctional materials, with a plasticity unreachable by metals, polymers or ceramics. In this context, structurally unrelated functional protein domains can be incorporated in individual polypeptides by simple genetic engineering to generate multifunctional proteins that mimic viral properties such as self-assembling, cell surface receptor binding, internalization and proper intracelular trafficking[216;218].

Recently, we have proposed an oligomerization approach in which a cationic peptide and a polyhistidine tail added at the amino terminus and carboxy terminus of multifunctional proteins respectively promote their self-assembling by electrostatic interactions, forming nanoparticles ranging in size between 10 and 100 nm[235]. We have already proved that the architectonic ability of the self-assembling inducing peptide at the amino terminus is determined by its cationic nature and not by its structure or origin and also, we determined that the size of the generated NP is significantly influenced by the charge of this peptide. In this context, empirical data obtained from a set of different modular proteins have allowed to set a relationship between the number of cationic residues at the amino terminus and the size of the engineered particles. However, this numerical model had been never tested as a rational tool for the engineering of protein self-assembling so far.

On this background, we wanted to explore the possibility to effectively modulate the architectonic properties of non-cationic homing peptides in order to produce

nanoparticles of suitable and predictable size for their biomedical application.

Angiopep-2-GFP-H6 and Seq-1-GFP-H6 are modular proteins displaying low density lipoprotein receptor (LDLR) ligands which are able to cross the BBB[157;158;167]. Angiopep-2 and Seq-1 are unable to promote the self-assembling in higher ordered nanoparticles, probably due to their low cationic amino acid content[257]. Indeed, it has been shown that these modular proteins are able to reach the brain upon systemic administration but with important renal uptake due to their small molecular weight[258]. In an attempt to promote the self-assembling of Angiopep-2-GFP-H6 and Seq-1-GFP-H6 for nanoparticle construction, cationic stretches containing arginine and lysine amino acids were introduced in both proteins, between the ligand and the scaffold protein GFP **(Article 1, Table 1)**. Two different versions of each protein were designed to provide a total of 7 and 8 positively charged residues in the amino terminal region. According to previous numerical modeling, these numbers of cationic residues should enable the proteins to self-assemble as nanoparticles of around 30 nm, which is the optimal nanoparticle size for ligand/receptor-mediated nanoparticle internalization in brain[25].

The new versions of Angiopep-2-GFP-H6 and Seq-1-GFP-H6 were successfully produced in *Escherichia coli* expression system and further characterized. All of them showed expected molecular masses and retained the GFP fluorescence emissions **(Article 1, Table 1)**. However, in the case of Seq-1-derivatives, the fluorescence intensity notably decreased, probably because there were taking place some conformational changes in the protein that might affect the conformation of the chromophore. Indeed, among the four different constructs, only Seq-1-8-GFP-H6 was able to self-assemble and generate nanoparticles of about 30 nm as expected **(Article 1, Figure 1A)**. Note that the size obtained is in full agreement with the numerical model described before[235].

The stability of nanoparticles was demonstrated under different salt contents and ionic strengths **(Article 1, Figure 1B)** and by measuring fluorescence emission after incubation in human plasma for 24 h **(Article 1, Figure 2A)**. Moreover, regular

nanoparticle size and morphology was confirmed by TEM and FESEM analysis **(Article 1, Figure 1C-E)**.

These results indicated that in this platform, protein's self-assembling process is determined on one hand, by a minimum number of positive charges in the amino terminus peptide, since just one additional cationic residue in Seq-1-8-GFP-H6 comparing with Seq-1-7-GFP-H6 allowed promoting self-assembly process. On the other hand, we found that Angiopep-2-8-GFP-H6, having the same amount of positive charges than Seq-1-8-GFP-H6, failed triggering self–assembly, suggesting that the oligomerization process is also influenced by the distribution of the charges in the cationic peptide. In fact, it appears that the charges have to be distributed together in the sequence of the cationic peptide, as in Seq-1-8-GFP-H6, to result in a higher polar splitting of electrostatic charges that supports spontaneous self-organization as nanoparticulate materials through electrostatic interactions.

This engineering principle permits the generation of still functional protein-based nanoparticles with predictable size by the precise sequence manipulation of multifunctional proteins, based on controlled cationic load of the homing peptide at amino terminal region. Note that nanoparticle size and multivalent presentation of the homing peptide allow proteins to correctly biodistribute and to efficiently internalize into target cells upon systemic administration, as it has been demonstrated in some diseases such as cancer and infections[16;114]. Therefore, formulating therapeutic proteins as functional nanoparticles allows, by this engineering principle, their broad applicability in nanomedicine.

2. Analysis of the applicability of protein-only nanoparticles in neurotropic therapy.

Drug nanoconjugates, as well as exhibiting powerful targeting properties, should overcome the biological barriers faced before reaching the right cell compartment in the target organ. This is a requisite when targeting the central nervous system (CNS) that is protected by the blood–brain barrier (BBB), which controls the access of molecules and drugs to the brain[149;150]. The invasivennes that supposes local administration of therapeutics into brain, imposses the necessity of empowering drugs with BBB crossing abilities after systemic administration[259].

It has been previously discussed that the nanoparticulated organization of materials used for systemic drug delivery and their functionalization with the appropiate ligand allow their correct biodistribution and accumulation in target organs. However, the ideal architecture to promote the delivery of drugs into the brain remains to be elucidated. Being paracellular penetration of drugs especially difficult to cross the BBB, their functionalization to ligands with transcytosis capacity seems to be the best approach[259]. However, whether the ligand would be more effective crossing the BBB when is presented in a nanocarrier than when applied in plain ligand–drug complexes needs further investigation. In fact, recent data suggest that presentation of proteins as nanoparticles could not favor their accumation in the brain[257].

To asses BBB-crossing properties of monomeric and nanoparticle protein versions we have compared the unassembled modular version of Seq-1 BBB crossing peptide (Seq1-7-GFP-H6) and its nanoparticle version (Seq1-8-GFP-H6) obtained by the engineering principle explained before.

Nanoparticles showed higher internalization into LDLR HeLa cells **(Article 1, Figure 2B and Figure 3A-B)** and better transcytic properties in a CaCo-2 based test **(Article 1, Table 2)** when compared with unassembled version. This higher penetrability of nanoparticles is probably related with a favoured endosomal uptake of the fusion protein due to the amplification of the material size and multivalent presentation of the Seq1 ligand in the nanoparticle, rather than an enhanced inespecific internalization

of the fusion protein promoted by the addition of positive charged amino acids. Indeed, it has been already demonstrated that the addition of cationic peptides to pre-existing protein nanoparticles did not alter cell penetrability[260;261]. Moreover, the specificity in cell binding and internalization of Seq1 has been already tested in LDLR-HUVEC that was shown to be negligible when comparing with $LDLR^+$ HeLa cells, suggesting a receptor mediated internalization pathway of the fusion protein. We confirmed the endosomal localization of Seq1-8-GFP-H6 by the strong merging signals of the membraneous system and GFP upon exposure to $LDLR^+$ HeLa cells, observed by confocal reconstructions **(Article 1, Figure 3C)**. Although the penetrability *in vitro* is improved by the nanoparticle form, the BBB crossing ability of the nanoparticle and unassembled form *in vivo* was really similar **(Article 1, Figure 4)**.

One reason that explains the obtained results might be that multivalent presentation of the homing peptide in the nanoparticle has not favoured the BBB permeability and accumulation in brain of the fusion protein and that the enhanced penetrability *in vitro* could be due to the different transcytosis activities between the non-cerebral cell lines such as epithelial CaCo-2 cell line and brain endothelial cells[262]. This is in agreement with the poor BBB crossing abilities showed before by protein nanoparticles empowering ApoB ligand[257].

Another reason for this fact could be that Seq1-8-GFP-H6 nanoparticles are more inestable than we expected and disassemble once in the blood stream, as described for other protein nanoparticles such as nab-paclitaxel. Indeed, both Seq1-8-GFP-H6 and Seq1-7-GFP-H6 were found in kidney (but not in other organs) as in the case of GFP-H6 monomer. Moreover, Seq1-8-GFP-H6 nanoparticles progressively disassembled under incubation in human serum and monomers appeared after 5 h of incubation, suggesting nanoparticle inestability in presence of heterologous proteins, which might be related with the nanomaterial destabilization by protein corona formation upon systemic administration[24].

Interestingly, we found that Seq1-8-GFP-H6 protein remained for a longer time in kidney **(Article 1, Figure 5)**. One explanation is that nanoparticulated Seq1 could be

interacting with LDLR family cell surface receptors, found highly expressed in renal cells[263;264], showing enhanced Seq1-8-GFP-H6 protein penetration due to the multivalent presentation of Seq 1 into protein nanoparticles. In this context, the abundance of LDLR in kidney might limit the use of LDLR ligands in BBB-targeted drug delivery systems.

Another explanation might be related with the disassembling process of Seq1 nanoparticles, which implies that the renal clearance of Seq1-8-GFP-H6 occurs more slowly when compared with unassembled versions that are filtered faster. This difference in clearance rate might correspond with the fact that the nanoparticles are progressively disassembled, and as they have to go through this process, the renal clearance is slower.

This study reveals that 1) the stability of nanoparticles obtained by the engineering tool described before should be carefully and further studied in other models due to a probable inestability and dissasembling of nanoentities in the blood stream. 2) high cellular penetrability of protein nanoparticles in cultured cells *in vitro* does not guarantee efficient BBB crossing and brain targeting by transcytosis-associated receptors. This might be indicative of different principles governing the biodistribution of protein nanoparticles displaying BBB-homing peptides when comparing with those that display tumor homing peptides. **This result prompts to carefully evaluate the convenience of using protein nanoparticulated materials for BBB-crossing therapies for the treatment of CNS diseases.**

3. Generation of CXCR4$^+$ targeted protein-only nanoparticles with intrinsic cytotoxic activities for the treatment of solid tumors.

PROOF OF CONCEPT.

Many proteins such as proapoptotic proteins, antimicrobial peptides, toxins or venoms among others are efficient drugs that can be enginereed as potent therapeutic agents usable in oncology **(Review 1)**. As we have already explained in the introduction part of this thesis, converting cytotoxic proteins in building blocks of self-assembling protein-only nanoparticles is an innovative approach that would allow avoiding the risk of drug leakage during circulation and excluding the need of further activation and drug conjugation. These proteins, if empowered with cell-targeting peptides, would act simultaneously as carriers, drugs and targeting agents that could be biologically produced in a single step.

Venoms are complex mixtures, composed mainly by proteins and peptides[265]. The most known and studied poisonous terrestrial animals are snakes, scorpions and spiders. Among marine animals, these are jellyfishes, anemones and cone snails. These peptides act on exposed cells by diverse mechanisms leading to cell death. Individual toxins are also produced by bacteria and plants that are usually involved in enzymatic protein synthesis inhibition[266]. These toxins include the plant toxins such as ricin and saporin and the bacterial toxins such as Diphtheria toxin (DT) and Pseudomonas aeruginosa exotoxin A (PE). Venom components and toxins are extremely potent molecules and reveal a modular architecture that offers additional versatility in the engineering of these peptides as therapeutic agents. However, drugs based on non-human proteins contain antigenic peptides that lead to immune responses during treatment upon re-exposure[267].

Antimicrobial peptides (AMPs) belong to the innate immune system and host defence mechanism of a wide range of living organisms[268]. AMPs are small pore-forming proteins that show avidity for negatively charged cell membranes inducing apoptosis or necrosis and some of them also inhibiting tumor angiogenesis and showing

immunomodulatory activities. Most AMPs are of human or animal origin, but others have been isolated from peptide libraries or have been generated by de novo design.

Proapoptotic proteins have also been engineered as drugs to induce apoptosis in tumour cells[269]. The clinical value of proapoptotic proteins and some AMPs as protein drugs in oncology is enriched because of their human origin which administration would decrease or avoid immunogenicity that is usually associated with heterologous protein drugs[270]. Therefore, we firstly aimed to engineer proapoptotic proteins as building blocks of self-assembling protein-only nanoparticles.

The Bcl-2 family is the best characterized protein family involved in the regulation of apoptotic cell death, consisting of anti-apoptotic and pro-apoptotic members[269;271]. The anti-apoptotic proteins include Bcl-2, Bcl-xL and Bcl-w which share sequence homology within four regions, BH (Bcl-2 homology) 1 to BH4. Proapoptotic proteins can be categorized into BH3-only proteins (BIM, BID, PUMA, NOXA, BAD, BIK, and HRK) that contain only one BH region (BH3) and multidomain proteins (BAX and BAK), that share a similar globular structure with four BCL-2 homology regions (BH1, BH2, BH3 and BH4).

The more advanced drugs focused on Bcl-2 family members of proteins are BH3 mimetics. BH3-mimetics are chemical molecules capable of mimicking BH3-only proteins[272;273]. Great effort has been made to develop suitable BH3 mimetics but unfortunately, their application is currently limited by its major dose-limiting toxicity; Indeed, the most clinically advanced BCL-2 family inhibitors ABT-263 (inhibits BCL-2, BCL-XL and BCL-W) cause thrombocytopenia[274], and Obatoclax which inhibits MCL-1 as well as BCL-2, BCL-XL and BCL-W leads to a unexpected central nervous system toxicity[275].

Nowadays, the engineering of recombinant human pro-apoptotic proteins offers a new therapeutic approach. Human pro-apoptotic proteins fused to peptides that increase their cellular uptake *in vitro* such the transactivator of transcription (TAT) of human immunodeficiency virus (TAT-Bid)[276] or Antennapedia homeoprotein (Ant-BAKBH3)[277]

show direct activation of apoptosis. More interestingly, pro-apoptotic peptides fused to receptor specific peptides or antibody derivatives that recognize tumor markers are specifically delivered into cancer cells promoting apoptosis. Examples of these are pro-apoptotic proteins fused to gonadotropin releasing hormone (GnRH-Bik, GnRH-BAK and GnRH-Bax)[278], to human granulocyte-macrophage colony-stimulating factor (hGM-CSF-Bad)[279] or to specific single-chain anti-HER2 antibody fragment (e23sFv-TD-tBID)[280]. However, these therapeutics achieved by simple fusion technologies do not show appropriate size and multivalency for an optimal therapeutic treatment of systemically administered drugs.

In this context, we aimed to engineer the functional BH3 domain of the proapoptotic Bcl-2 homologous antagonist killer (BAK) protein as building blocks of self-assembling protein-only nanoparticles. As the full length BAK has highly hydrophobic nature due to the transmembrane region[281], we decided to recombinantly produce the functional BH3 domain (BAK BH3) that it has been already demonstrated to show proapoptotic activites[277;282]. We fused the cationic T22 peptide, as oligomerization tag and as targeting agent of $CXCR4^{+}$ tumors, the BAK BH3 domain as therapeutic domain, the green fluorescent protein (GFP) as scaffold protein that allows to monitor the localization of the fusion protein and finally, the histidine tag as oligomerization and purification tag **(Article 2, Figure 1A)**. This fusion platform shows potency for theragnosis as contains both, diagnostic and therapeutic functions in one integrated system.

T22-BAK-GFP-H6 nanoparticles were succesfully produced in *Escherichia coli* and purified in a single step by conventional procedures **(Article 2, Figure 1B)**. Generated nanoparticles, being 13.5nm in diameter **(Article 2, Figure 1C-D)**, showed appropiate fluorescence emission for quantitative imaging and were stable in complex physiological media such as Optipro medium (results not shown).

In relation to their capacity to bind and internalize into CXCR4-positive cells, we firstly demonstrated *in vitro* efficient internalization and CXCR4 dependent uptake of

nanoparticles into CXCR4$^+$ HeLa and SW1417 cells **(Article 2, Figure 2)**. Indeed, the kinetics of accumulation was compatible with a receptor mediated endocytosis, nanoparticle internalization was significantly reduced in both cell lines using AMD3100 (an inhibitor of T22-CXCR4 interaction), and the fusion proteins lacking the T22 peptide failed to enter the cells.

Due to its high CXCR4 linked penetrability *in vitro*, we moved to explore nanoparticle biodistribution in a CXCR4$^+$ colorectal cancer mouse model. NPs specifically accumulated in tumor cells upon systemic administration with absence of renal accumulation, aggregation in lungs or toxicity **(Article 2, Figure 3),** which indicates their high stability in plasma. Surprisingly, the non-targeted and monomeric protein BAK-GFP-H6 did not filtered in kidneys as expected for unassembled versions such as GFP, and reached the tumor in the first two hours. This is probably because the 6.45 nm BAK-GFP-H6 shows a higher size than the 5.1 nm GFP-H6 **(Article 2, Figure 1C)** and thus, this protein may be in the limit or even above the renal filtration threshold (generally determined at 6 nm)[11;30] and it is able to avoid the renal filtration and reach the tumor by the EPR effect. This is also indicative of the high stability of this plain fusion protein in plasma. However, note that targeted nanoparticles remained longer in the tumor compared with the non targeted BAK-GFP-H6 **(Article 2, Figure 3A-C)**.

Regarding the cytotoxic activity, BAK is a multidomain pro-apoptotic protein belonging to the Bcl2 protein family that controls the intrinsic apoptotic pathway[283]. BAK shares a similar globular structure with four BCL-2 homology regions (BH1, BH2, BH3 and BH4) and in contrast to BAX, BAK is constitutively inserted into the mitochondrial outer membrane in healthy cells through its transmembrane domain. Upon activation, 1) it interacts through its BH3 domain with antiapoptotic proteins and inactivates them and 2) it assembles into oligomeric complexes in the mitochondrial outer membrane (MOM), triggering caspase-dependent apoptotic pathway through the mitochondrial membrane permeabilization and cytochrome C release (Figure 14). Contrary to the full lenght BAK, BAK BH3 lacking the transmembrane region is not able to insert in MOM and create pores[277]. Therefore, the apoptotic effect is related with caspase activation

without loss of mitochondrial membrane potential or detectable translocation of cytochrome c from mitocondria to cytosol. This caspase activation is triggered by its interaction with antiapoptotic proteins in the cytosol and their inhibition. In detail, it has been described a predominant interaction between BAK BH3 and the antiapoptotic protein Bcl-x_L. Reportedly, Bcl-x_L interacts with the apoptotic protease activating factor 1 (Apaf-1) and inhibits the ability of Apaf-1 to promote the activation of caspase-9. Thus, the BAK BH3 peptide may antagonize Bcl-x_L and promote apoptosis by preventing Bcl-x_L/Apaf-1 heterodimerization, leaving Apaf-1 free to participate in the fast activation of caspases[277] (Figure 14).

We demonstrated that T22-BAK-GFP-H6 compared to T22-GFP-H6 was able to induce cell death in target cells *in vivo* associated with caspase-3 activation, proteolysis of poly(ADP-ribose) polymerase (PARP) catalyzed by caspase-3, ocurrence of apoptotic bodies, increase of necrotic areas in tumor tissues and decrease of mitotic figures **(Article 2, Figure 4)**. As expected, tumor cell apoptosis peaked at short times, 5 h **(Article 2, Figure 4B-D)** that may be related with a fast caspase activation and necrosis, which was significantly observed at longer times, 24 h and 48 h **(Article 2, Figure 4E)**. Moreover, we confirmed the absence of cytochrome C activation (not shown) as it was expected, due to the inability of BAK BH3 to perform mitochondrial pores.

Importangly, non-target organs remained intact **(Article 2, Figure 3E)**. Likewise, untargeted BAK-GFP-H6 triggered only a tenuous induction of apoptosis that was not significant comparing with the apoptosis in buffer treated tumors **(Article 2, Figure 4F),** suggesting an early accumulation of this protein in tumor tissue due to EPR effect but unable to internalize into cancer cells if lacking the targeting peptide.

Based on this data we have demonstrated the possibility to engineer a proapoptotic protein to self-assemble as nanoparticles that show intrinsic apoptotic activies and in which BAK BH3 domains are fully functional.

TRANSLATIONAL APPLICATION.

We wanted to explore the generic applicability of this platform using any recombinant protein drug to generate intrinsically functional protein nanoparticles, which having a dual role as drug and carrier, are designed to be administered without the assistance of heterologous vehicles. In this context, we succesfully generated nanoparticles based on the human p53-upregulated modulator of apoptosis PUMA[284;285] **(Article 2, Figure 5)**, the synthetic anticancer peptide GWH1[286;287] **(Article 2, Figure 5)** and toxins[288] **(Annex 1, Article 4)**. All nanostructured drugs were able to accumulate in tumor tissues upon systemic administration, which is again indicative of the stability of oligomers in plasma as materials over 6 nm in size.

PUMA, similarly to BAK BH3, is a BH3-only proapoptotic protein that interacts with antiapoptotic Bcl-2 family members, inhibiting their interaction with the proapoptotic molecules and inducing fast caspase activation[285]. Moreover, this protein also directly activates BAX and BAK leading to mitochondrial disfunction and cell death (Figure 13). We saw that T22-PUMA-GFP-H6 *in vivo* induced fast cell death at 5h after its systemic administration **(Article 2, Figure 5E)**. Note that the therapeutic value of proapoptotic peptides such as BAK BH3 and PUMA is that they are from human origin so they should not be immunogenic when administered in patients.

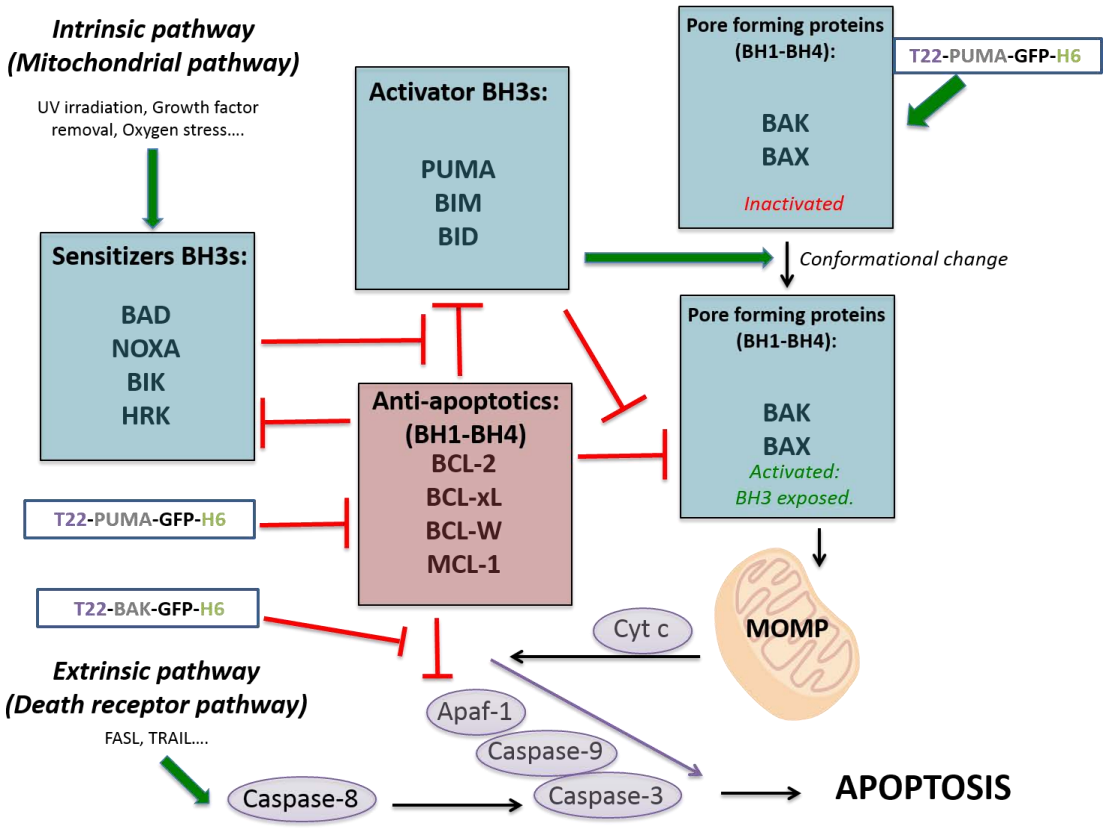

Figure 14: *Proposed caspase activation pathways of T22-BAK-GFP-H6 and T22-PUMA-GFP-H6 proteins. BAK BH3 directly binds to anti-apoptotic proteins that interact with the apoptotic protease activating factor 1 (Apaf-1), thus leaving Apaf-1 free and trigeering a fast caspase activation. PUMA BH3* directly *binds to the prosurvival Bcl-2-like proteins and also activates Bax and Bak. Once activated, Bax and Bak oligomerize to form pores in the mitochondrial outer membrane that release cytochrome* c.

It is known that in the absence of a good endosomolytic agent, the endosomal uptake of fusion protein drugs drives the engulfed material to a lysosomal pathway limiting their cytotoxicity[83]. Unfortunately, we demonstrated that the proton sponge effect expected by His-rich peptides in T22-GFP-H6 was innefficient promoting the release of fusion protein drugs into the cytosol, and around 90 % of the protein was proteolyzed **(Annex 4, Manuscript 1, Figure 2B)** being similar for T22-BAK-GFP-H6 and T22-PUMA-GFP-H6 (results not shown). Therefore, the exploration of peptides that are identified as strongly endosomolytic, is a priority to improve the design of these prototypes that lack a proper endosomolytic domain. In this regard, we have already incorporated the fusogenic peptide HA2 from the influenza virus hemagglutinin **(Annex 3, Article 5)** and the pore forming cationic peptide GWH1 **(Annex 4, Manuscript 1)** to the T22-GFP-H6 protein platform. The obtained results show a dramatic enhancement of cell penetrability and a high transfection efficiency of the material into the cytosol, but with a significant reduction in the specificity between the interaction of nanoparticles

and cell receptor. Indeed, T22-GWH1-GFP-H6 when administered *in vivo* shows less accumulation in tumor than T22-GFP-H6 with an occurrence in kidney **(Article 2, Figure 5C)**. Therefore, a further exploration is required to properly incorporate endosomal escape domains in our prototypes. In this context, our group is currently working in the exploration and suitable incorporation of human endosomolytic domains into building blocks.

GWH1 is an antimicrobial cationic peptide (AMP) that exerts its cytolytic activity by folding into an amphipathic helix upon selective binding and insertion into negatively charged cell membranes, leading to cell death[286]. As we have already explained, this activity besides promoting cell death can also be exploted to promote endosomal escape of the fusion protein **(Annex 4, Manuscript 1)**. Firstly we demonstrated that GWH1 was able to form α-helical structures when entered in contact with membranes **(Annex 4, Manuscript 1, Figure 1B)**, remaining funcional in nanoparticulated form. Regarding to the cytotoxic activity, AMPs, as effective self-defence tools, exhibit a threshold concentration for their membrane activity below which no effect is observed[289]. This threshold concentration is lower on procaryotic cells than on eukaryotic cells, providing a security level that avoid harming own body cells. In cultured cancer cells, it has been previously reported a threshold concentration ranging from 20 µM to 250 µM for the free GWH1. We firstly determined *in vitro* the threshold concentration of T22-GWH1-GFP-H6 over $CXCR4^+$ HeLa cells that was defined at 8uM **(Annex 4, Manuscript 1, Figure 1C)**. This was significantly lower than the one predicted for the GWH1 peptide alone, probably because of an enhanced penetration of nanoparticles due to the multivalent presentation of T22 into protein nanoparticles **(Annex 4, Manuscript 1, Figure 2A)**. When we analyzed this cell killing ability *in vivo* at 5h after administration, we saw a milder effect than proapoptotic proteins **(Article 2, Figure 5F),** probably because this peptide acts by two sequential events and requires longer time interval to induce noticeable cell death, such as 24-48h, as we have seen in cultured cells **(Annex 4, Manuscript 1, Figure 1C)**. Thus, it is recommendable to study the cell killing of this peptide *in vivo* at longer times and, if possible, with a repeated administration regimen.

The therapeutic value of AMPs relies on their low propensity to elicit development of resistance by tumor cells and because, as proapoptotic proteins, they can also be from human origin.

Diphtheria toxin and *Pseudomonas aeruginosa* exotoxin are microbial toxins that perform ADP-ribosylation of the elongation factor 2 (EF-2) resulting in the inhibition of protein synthesis and cell death[290]. Interestingly, the evolutionary analysis has revealed a modular architecture of many toxins that offers additional versatility in the engineering of these agents as multifunctional drugs. We designed and produced T22-DITOX-H6 and T22-PE24-H6 nanoparticles **(Annex 1, Article 4, Figure 1-2)**. DITOX contains the catalytic and translocation domains of the diphtheria toxin from *Corynebacterium diphtheriae*. PE24 is based in the de-immunized catalytic domain and furin clevage site of the translocation domain of *Pseudomonas aeruginosa* exotoxin A in which point mutations that disrupt B and T cell epitopes have been incorporated[291]. Moreover, it has been added a KDEL sequence in the C-terminus of T22-PE24-H6, which enables the binding to KDEL receptors more efficiently at the Golgi apparatus during subsequent intracellular trafficking[292]. The furin cleavage site inserted between the T22 ligand and the functional domain allows the release of the active proteins in the cell cytoplasm, resulting in a high cytotoxic potency[293]. Both, T22 empowered Diphteria toxin (T22-DITOX-H6) and *Pseudomonas aeruginosa* toxin (T22-PE24-H6) were able to promote cell killing in vitro mostly at 24h-48h **(Annex 1, Article 4, Figure 3)** and in vivo after a single dose administration **(Annex 1, Article 4, Figure 4)**. Importantly, toxin-based nanoparticles increased apoptosis in tumor tissue and induced a significant reduction of tumor volume after repeated dose administration with no alteration of mouse body weight **(Annex 1, Article 4, Figure 7)**. In this regard, T22-PE24-H6 and T22-DITOX-H6 appear to have a therapeutic index high enough to consider their potential use for the treatment of human $CXCR4^+$ tumors.

The good results obtained with microbial toxins encouraged us to work also with potent plant toxins such as ricin (results not shown). Major therapeutic values of toxins rely on their ability to kill exposed cells through molecular events that are devoid, in general, of cell type specificity and also, on their incredible killing potency[294].

All the data obtained in these studies represent a totally new concept and validate the wide applicability of the transversal concept supporting the engineering of therapeutic proteins into self-assembling and self-delivered protein-only nanoparticles with intrinsic therapeutic activity (Figure 15).

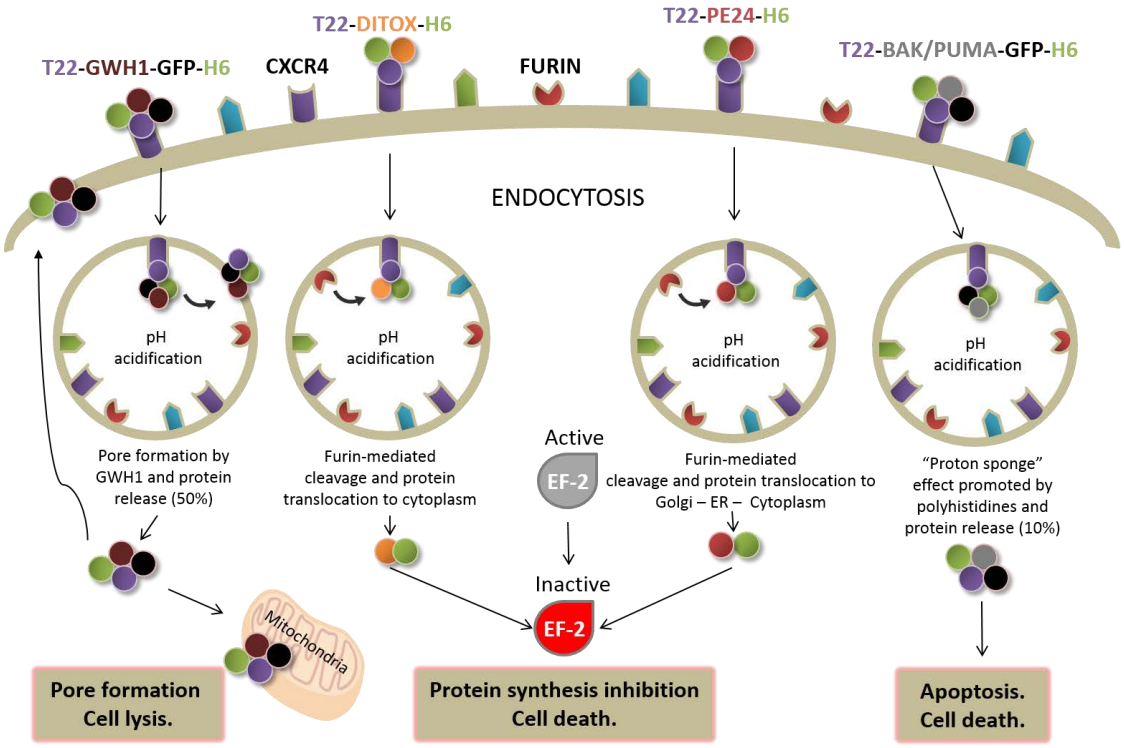

Figure 15: Expected pathway for the cytotoxicity of generated nanoparticles in CXCR4⁺ target cell.

Interestingly, to exploit the clinical applicability of these nanoparticles, we also developed a new methodological approach to easily generate **hybrid nanoparticles** made of different therapeutic domains or displaying different cell-targeting agents in multiparatopic or multi-specific constructs **(Annex 6)**[295].

In this context, we recently analyzed different arginine-rich protein motifs (Rn) as specific ligands of the cell surface chemokine receptor CXCR4. Indeed, polyarginines have been observed as highly potent cell penetrating peptides useful as an internalization tag in cell therapy and drug delivery, but they also show residual specificity for CXCR4[296;297]. We demonstrated that this specificity can be enhanced by a proper presentation of the cationic stretch, specifically by extending the number of arginine residues and, with a milder impact, by its multimeric presentation on the

surface of targeting vehicles **(Annex 5, Manuscript 2)**. These results encourage us to explore the use of R9 peptide in combination with the already mentioned CXCR4 ligand T22 peptide to generate bi-paratopic vehicles, that are extremely appealing for enhanced specificities and cell surface avidity in receptor-mediated drug delivery **(Annex 6)**[295]. In a related context, the combination of different building blocks might allow the administration of synergistically acting therapeutic protein drugs or fluorescent proteins, in a new approach to combined therapies or theragnosis respectively.

All the data obtained here prompted us to apply for an european patent based on the engineering of protein drugs as self-organizing building blocks, that exhibit intrinsic therapeutic activities upon self-assembling as nanoparticles (EP17169722) **(Annex 7)**.

CLINICAL CONSIDERATIONS.

Immunogenicity: The therapeutic value of proapoptotic peptides and some antimicrobial peptides is that they are from human origin. However, being small peptides, GFP has been incorporated in our prototoypes as scaffold and reporter protein. Even GFP is widely used as reporter in fusion proteins, the immunological cytotoxicity elicited by it could limit their therapeutic use through preclinical safety testing and clinical trials[298]. In this context, GFP-free human proteins would be highly desirable as final drugs to avoid possible immunogenicity limitations in repeated administrations. This may be achieved by the use of other human scaffold protein such as albumin. In this regard, our group has started a collaboration with Nanoligent S.L. for the search of human scaffold proteins that could be easily incorporated in our prototypes.

Likewise, toxin based nanoparticles do not use supporting irrelevant proteins such as GFP but special attention must be put on the possible immunogenicity of these non-human domains[294]. Several approaches have been proposed to decrease the immunogenicity of foreign proteins[267]. One involves shielding the protein from the immune system by conjugating high molecular-weight polyethylene glycol (PEG) to the protein. However, the addition of PEG may diminish their cytotoxic activity. Another

approach is to identify and remove epitopes of T or B cells by site directed mutagenesis. Precise protein engineering has been applied to reduce the immunogenicity of PE and DT catalytic fragments to be incorporated as drugs. For instance, many natural or modified forms of toxins are in clinical trials or are already FDA-approved for oncotherapy **(Review 1, table 1)**, so the probability to undergo evaluation in clinical trials is promising for toxin based nanoparticles.

Cancer stem cells and drug resistance: Conventional treatments for colon cancer, such as chemotherapy and radiation, can kill the bulk tumor cells, but fail to induce durable clinical results because they are not effective at eliminating colon cancer stem cells (CSCs)[90;91]. There is an urgent need to develop new drugs that can specifically target and kill colon-CSCs.

The proper therapeutic index showed by the toxin nanoparticles encouraged us to explore their cytotoxic effect on human colon-CSCs. For that, I completed four-month internship in the oncology team of Experimental and Molecular Medicine department at Amsterdam Medical Center (Amsterdam, The Netherlands) led by Prof. J.P. Medema. This group has a great experience in the production of primary colon cancer 3D spheroid cultures directly derived from patients, which represent an amaizing model to perform preclinical studies. We demonstrated that CXCR4-targeted toxin nanoparticles not only target and effectively kill tumor colon CSCs, but also, they are able to overcome the resistance presented by colon-CSCs to traditional therapy *in vitro* **(Annex 2, Draft 1)**. Currently we are running in vivo experiments with colon CSC mice models in colaboration with Dr. Ramon Mangues at Institut de Recerca Biomedica Sant Pau. The preliminary data show that toxin based nanoparticles are valuable tools against colon CSCs and represents a pre-clinical proof of concept for the use of T22-DITOX-H6 and T22-PE24- H6 as potential drug delivery system against $CXCR4^{+}$ tumors.

Sustained release of protein NPs: Immobilized drug delivery platforms capable of a sustained release are an interesting alternative to intravenously administered NPs since they maintain a constant and long lasting NP concentration in the bloodstream[299;300]. In fact, periodical administration of the protein NPs is usually

needed due to the short term stability within the body and fast NP clearance from bloodstream.

Bacterial Inclusion Bodies (IBs) are amyloid like protein particles with sponge like conformation that belong to the insoluble fraction and occur during recombinant biofabrication under physiological conditions[301-303]. IBs are significantly bigger entities than protein NPs, with diameters from 100nm up to 1µm, that contain functional protein. These structures have been shown to act as other functional secretory granules found in the endocrine system and thus, they are able to release funcional protein along time under the appropriate conditions[304].

In this context, we have reported the use of IBs as reservoirs of targeted protein NPs which are able to release fully functional self-assembled protein NPs able to effectively target cells and tissues from the primary implantation point[300]. Therefore, the production of inclusion bodies that contain protein NPs with intrinsic cytotoxic activities would be a very interesting approach to provide a long lasting source of targeted cytotoxic protein only NPs.

4) Generation of protein-only nanoparticles with intrinsic microbicide activities for the treatment of bacterial infections.

Antimicrobial peptides (AMPs) have a good potential as alternatives to conventional antibiotics due to their low propensity for resistance development[115]. They have been successfully incorporated into topical drug formulations but due to their poor stability, low solubility and a rapid metabolic excretion caused by their low molecular mass, their applicability in systemic therapies is limited[114;116].

GWH1 is a synthetic antimicrobial peptide that shows high antimicrobial activity and low haemolytic potential, being a suitable candidate for systemic administration[286;287]. We explote the cationic nature of this peptide to produce biocompatible self-assembling protein only antimicrobial nanoparticles based on technologically simple and cost-effective biofabrication and thus, expanding its biological applicability.

GWH1 acts as an antimicrobial agent and as architectonic tag, that fused to GFP has been successfully produced for the first time in *Escherichia coli* and forms fluorescent NPs of 50nm **(Article 3, Figure 1)**. Interestingly, GWH1 keeps the lytic activity in form of NPs against Gram-positive and Gram-negative pathogenic bacteria **(Article 3, Figure 2-3)** but it is not able to penetrate and kill cultured mammalian cells **(Article 3, Figure 4),** discarding a priory potential side effects upon its systemic administration in patients. Similar to other AMPs that need their amino terminus free to perform their action, in the case of GWH1 it has been proved to be not critical but to highly enhance its activity **(Article 3, Figure 2)**. The antimicrobial activity of GWH1-empowered NPs is based on cell lysis as we demonstrated by ultraestructural data in *Escherichia coli* and S. *aureus* samples, that show abudant cell debris and empty bacteria membranes upon NP treatment **(Article 3, Figure 3)**.

Although we were devoted to describe a protein platform that allows the generation of self-assembling protein nanoparticles with inherent built-in antibacterial activity, we decided to determine the minimum inhibitory concentration (MIC) of these NPs for further caracterization. MIC is a guide to comparative testing of new antimicrobial

agents, to establish the susceptibility of organism to the antimicrobial agent and to aid in treatment decisions[305;306]. The minimum inhibitory concentrations (MICs) of antimicrobial agents is defined as the lowest concentration of an antimicrobial agent that prevents the appearance of visible growth of a microorganism within a defined period of time. Of course, careful control and standardization is required for intra- and interlaboratory reproducibility, as results may be significantly influenced by the method and the medium used. Indeed, the antimicrobial activity of GWH1-GFP-H6 was clearly influenced by the culture media showing a higher activity in Luria Broth medium and Mueller-Hinton broth medium **(Article 3, Figure 2)**.

For MIC determination of GWH1-based NPs and its comparison with free GWH1, we were awared that the reference methods for antimicrobial susceptibility testing are dilution methods such as serial dilution method or broth micro-dilution[306]. For that reason, we decided to measure the antimicrobial activity by Broth micro-dilution method. Moreover, Mueller-Hinton broth is considered the reference medium; however, we were not able to precisely compare the MIC of the free GWH1 peptide since this was previously measured in different culture media including NB, TSB and LB. For a general point of view, the MIC that we determined for NPs in LB for *Escherichia coli* and S. *aureus* was in the same order of magnitude or even higher than those previously described for GWH1 free peptide in different media.

In this context, this oligomerization platform shows promises for the generation of antimicrobial protein-only nanoparticles as antibacterial therapies that might be applied to a larger number of antimicrobial peptide candidates, with special attention to those from human origin.