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 *]

[1] Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain.

[2] Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain.

[3] CIBER de Bioingeniería, Biomateriales y Nanomedicina, 08193 Cerdanyola del Vallès, Spain.

[4] Institut d'Investigacions Biomèdiques Sant Pau and Josep Carreras Research Institute, Hospital de la Santa Creu i Sant Pau, 08025 Barcelona, Spain.

* Corresponding authors:
RM: rmangues@santpau.cat
AV: Antoni.Villaverde@uab.cat

Abstract

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, pro-apoptotic 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 recruiting, in single pharmacological entities, 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.

Keywords

Toxins, recombinant proteins, cell-targeted drug delivery, protein engineering, cancer treatment, nanomedicine

Antitumoral drugs: molecular size, circulation and specificity

Regenerative medicine aims at favouring cell adhesion, viability and spread under adverse physiological conditions. In contrast, therapies of cancer and 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 an efficient killing of cancer stem cells (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 (including alkylating agents, anthracyclines, microtubule inhibitors, antimetabolites, platinum-based agents, topoisomerase inhibitors, tyrosine kinase inhibitors and histone deacetylase inhibitors, among others). In 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 (anaemia, thrombocytopenia, neutropenia), nausea, vomiting, cardiotoxicity and immunosuppression leading to enhanced susceptibility to infectious diseases. As systemic toxicity restricts the doses to be administered, drugs do not reach the required local concentration for a fully effective activity [2]. The insufficient therapeutic effect is also related to the small molecular size of antitumoral 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**, see Glossary) increases drug hydrophilicity, impairs uptake by reticuloendothelial cells, minimizes clearance by neutralizing antibodies and reduces renal filtration, globally enhancing the therapeutic effect [5]. However, not adding any targeting ability, PEGylation 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 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 execute a dual role in (i) allowing the effective anchoring of sufficient ligands of tumoral surface markers for cell targeting and (ii) enlarging the whole conjugate size

over the renal cut-off value, to minimize 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 tissue using cell surface molecules 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 favoured by regular multivalent display of ligands on nanoscale entities, what promotes multiple cell anchorage and favours endosome formation [11]. Aptamers, monoclonal antibodies (**mAb**s), antibody derivatives or mimetics and receptor specific peptidic ligands [12] have been explored as targeting agents [13]. Avidity (the strenght 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 of 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 render aggregation in lung and undesired clearance by macrhophages 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**) effect 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], what allows 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 all 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 (**ADC**s,

Box 2), using mAbs as drivers, are the best representatives of this category of complexes. The antibody counterpart passively confers a nanoscale size (mostly under 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. Most of them, being highly stable and poorly biocompatible, generate 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, and since the approval of insulin by **FDA** in the early 80's, recombinant DNA technologies for protein engineering and production have been extensively developed [19]. Most protein-drug conjugation methods are based on lysine-amine and cysteine-thiol coupling by amine-activated ester/carboxilic acid, and thiol-maleimide chemistries respectively. The use of non-natural amino acids (oxime ligation, azide-alkyne cyclization) or enyme 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), first FDA-approved for breast cancer in 2005. Abraxane is a nanostructured complex (sizing 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] used 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.

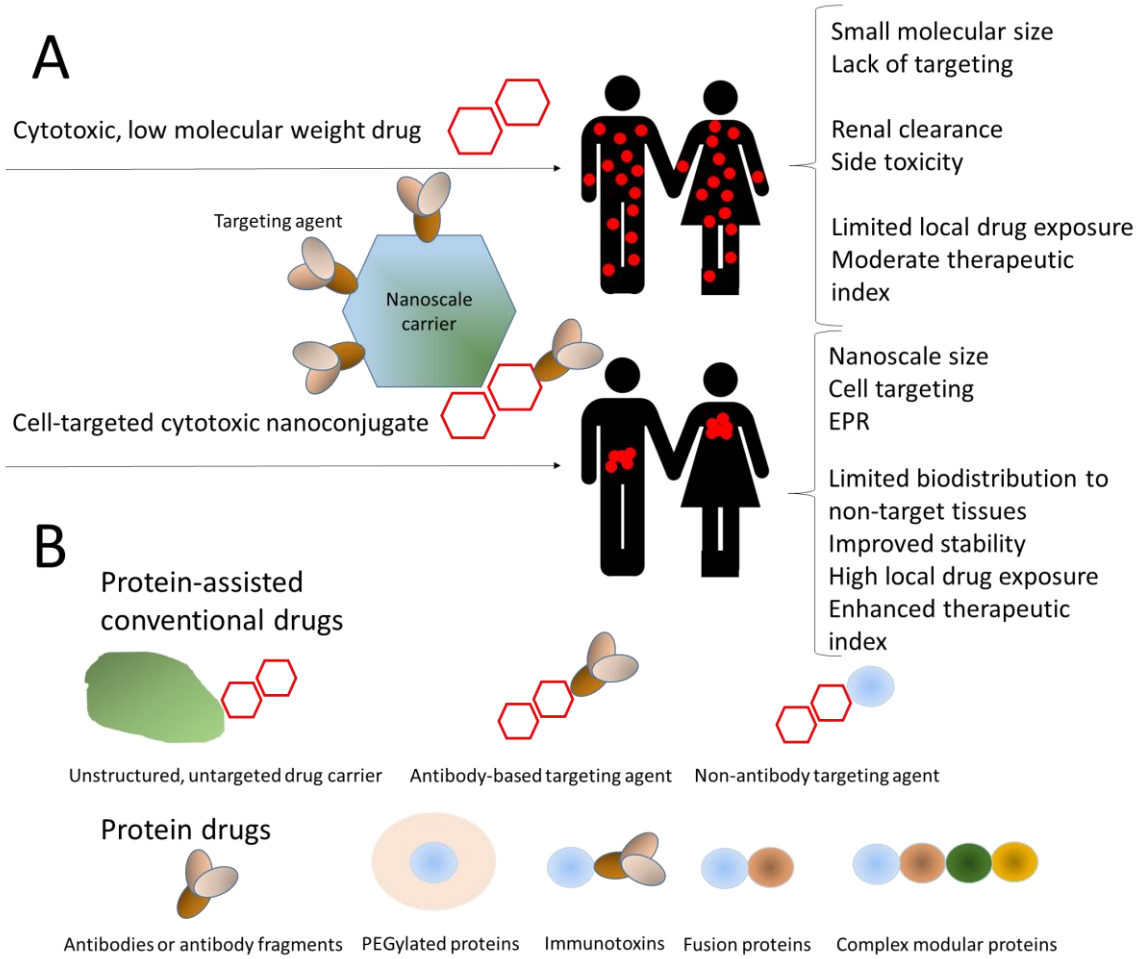

Figure 1. A. The chemotherapy of cancer is commonly approached by the use of low molecular weight chemicals (red symbols and dots), exhibiting generic cytotoxicity over tumoral and healthy cells. Their low molecular size (usually < 5 nm, linked to renal clearance) and lack of selectivity render an undesired biodistribution. This is associated to severe side effects and suboptimal drug concentration in tumoral tissues. The pharmacological linkage of these chemicals to nanoscale carriers (bottom, blue) and their functionalization with targeting agents (purple) 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, ADCs (Box 2) consist of chemical drugs directly coupled to antibodies against tumoral cell surface targets. B. Diverse roles of proteins in oncotherapy formulations, either as drug assistant agents (providing nanoscale size and stability or targeting), or as drugs themselves with intrinsinc cytotoxic activities. According to the designed functionalities the protein drugs are presented in alternative constructions or formulations.

Cytotoxic proteins

Many proteins themselves from diverse natural sources exhibit potent cytotoxic activities toward mammalian cells, through deleterious enzymatic activities or by precise interventions over 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, antitumoral agents and ion channel blockers [26]; and spiders, of painkillers, inflammation and cardiovascular disorders [27]. Furthermore, plants [28] and bacteria [29] have provided a diversity of protein-based antitumoral agents. Botox (Allergan), the *Clostridium botulinum* neurotoxin A (also marketed as 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 uses in wrinkle reduction). In this context, venom components and toxins, antimicrobial peptides and pro-apoptotic 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 signalling cascades, acting as indirect cytotoxic drugs. Many natural or modified forms of these proteins are in clinical trials or already FDA-approved for oncotherapy (Table 1). Furthermore, the flexibility of proteins as tuneable 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 and from which peptides and proteins are the most abundant components [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), thus revealing a very common 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 in the category of ribosome inactivating proteins (**RIP**s), 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 over cancer cells that blocks the PKC/MAPK signalling pathway and induces apoptosis [38]. Trichosanthin and related toxins are particularly interesting since they also inhibit HIV-1 multiplication, due to its capacity to cleave supercoiled double-stranded DNA to linear and nocked 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 fused to interleukin-2, that targets the toxin to leukaemia and lymphoma cells that display IL-2 receptors [41]. The exotoxin A from *Pseudomonas aeruginosa* (**PE**) has been also produced through recombinant methodologies in different versions, which are under 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 a high membranolytic activity [35]. On the other hand, chlorotoxin is a scorpion peptide (from *Leiurus quinquestriatus*) able to bind selectively cancer cells through the matrix metalloproteinase-2 (MMP-2) and annexin-2 expressed in several malignancies [44].

Antimicrobial peptides

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

Pro-apoptotic proteins

The apoptotic cell death program serves as a natural barrier to tumor development through the extrinsic apoptosis pathway, activated by extracellular pro-apoptotic stimuli, and the intrinsic pathway, mainly controlled by the BCL-2 family of proteins consisting of anti-apoptotic and pro-apoptotic members [50]. Pro-apoptotic 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 anti-apoptotic members, leaving them free to bind and activate BAX/BAK [53]. The clinical value of pro-apoptotic proteins (and many AMPs as well) as drugs in oncology is enriched due to the human origin of these proteins, whose administration would not promote immunotoxicity usually associated to heterologous protein drugs.

Monoclonal antibodies

Monoclonal antibodies (mAbs) are used as drivers in targeted drug delivery but furthermore 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 of them inhibit target receptors involved in tumor epithelial cell growth (like Her2, epidermal growth factor receptor-**EGFR-** or **PDGFR**-), but others inhibit tumor growth indirectly, by targeting ligands or receptors involved in tumor angiogenesis (**VEGF**-A, **VEGFR**-2). In addition, the fastest developing mAb drugs target cancer and immune (e.g. T-cell) cell molecules (CTLA-4, PD-1, PD-L1) to reactivate the 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 acces promote fast 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 modified versions with improved functionalities. Toxins and mAbs, of non-human origins, are generically immunotoxic and require deimmunization-oriented engineering. On the other hand, gaining nanoscale organization through multimeric self-assembling and 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). Then, protein-based cytotoxic drugs usually show 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**) (for cell targeting).

Deimmunization

Drugs based on non-human proteins contain antigenic peptides presented by **MHC** II molecules in 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 cross-linking by foreign epitopes [57], which leads to ADA-mediated immune responses during drug treatments 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 (see 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 (that is, immune-targeted toxins, see below) lacked sufficient therapeutic window since presenting dose-limiting toxicity, they 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 *Pseudomonas aeruginosa* and Diphtheria toxin catalytic fragments to be further incorporated to immunotoxins. The portions of these toxins that are not essential for cytotoxic activity or their 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 immunogenicity and reduces the off-target effects that prevent repeated treatment cycles. Deimmunization of a *Pseudomonas aeruginosa* toxin fragment (**PE**38) has been achieved by introducing mutations in B- or T-cell epitopes without compromising antitumor potency, and deletion of the PE domain II which prevented the induction of vascular leak syndrome [60]. A truncated diphtheria toxin (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 lossing antitumor activity [61]. Third generation immunotoxins consisting of a humanized targeting moiety (e.g. a mAb, **Fv** 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, that is, 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 fomation of human anti-mouse antibodies in patients, but protein enginnering efforts to humanize them have significantly reduced their immunogenicity [58].

Simple fusion technologies

Immunotoxins (Table 1) are made 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 as long as they show non-overlapping toxicities [62].

An immunotoxin containing the diphtheria toxin A and B (**DT**) fragments fused to human IL-2 was marketed in 2001 as Denileukin Diftitox. It showed activity against several haematological malignancies, particularly cutaneous T-cell lymphoma (CTCL). However, the induction of vascular leak syndrome has moderated its use. Two additional immunotoxins are currently in clinical assays. A-dmDT390-bisFv(UCHT1) is a fusion protein of DT bound to Fv fragment of CD3 that targets T-cell and is active in CTCL [63] and DT2219ARL consists of a DT fragment bound to Fv fragments of CD19 and CD22 active against B-lineage leukaemia or 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, showing activity in several haematological neoplasias. RG7787 is composed of a Fab version of the SS1 antibody bound to a modified and less immunogenic PE fragment. Being 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 leukaemia, acute lymphoblastic leukaemia) which shows high response rates [60]. Antibody and antibody fragments have been also used for the targeting of non-toxin cytotoxic proteins such as pro-apoptotic factors. An example is e23sFv-TD-tBID, which exploits a single-chain anti-HER2 antibody fragment to target 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). Pro-apoptotic peptides fused to the transactivator of transcription (TAT) of human immunodeficiency virus (TAT-Bid) [65], Antennapedia homeoprotein (Ant-BAKBH3) [66] or receptor binding domain of diphtheria toxin (Bad-BTTR) [67] immediately activate untargeted apoptosis. Other driving peptides used as fusions are the gonadotropin releasing hormone (in 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 mitochondrial-dependent apoptosis in the micromolar range. For example, the natural magainin II (MG2) fused to the CPP penetratin shows an IC50 in the micromolar range [70]. Even more appealing, 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 HER-2-targeting/neutralizing domain targets specifically HER-2 overexpressing cells *in vitro* and *in vivo*[73].

More sophisticated versions of fusion technologies render 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 antitumoral drugs still show severe side effects despite these engineering efforts (Table 1), what pushes towards further drug development based on safer principles. Two-partner fusion strategies discussed above (and most of the modular approaches too) enhance specificity but with still inappropriate nanoscale size and usually 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 to urgently explore 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 tumoral markers promote oligomerization of fusion proteins when combined with polyhistidines. As a paradigmatic example, the peptide T22, a ligand of CXCR4 (overexpressed in more than 20 human cancers), has been incorporated to histidine-tagged GFP constructions, which makes them self-organize into regular nanoparticles of between 12 and 60 nm that feature multivalent display of this peptide [75, 76]. Upon injection, these materials accumulate in tumoral tissues in absence of renal filtration [3]. The same principle has been applied to protein-only blood-brain-barrier crossing nanoparticles [77] or 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, pro-apoptotic peptides, AMPs and microbial toxins have been instructed to self-assemble as cell-targeted nanoparticles ([80] and unpublished data).

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

Concluding remarks and future perspectives

Targeting cytotoxic agents in cancer therapies is unquestionably an urgent demand. A plethora of approaches in this regard, using nanotechnological principles, has so far offered improved but still moderately competent drugs, mainly because of associated side toxicities. Empirical observations but also emerging bioengineering concepts point out 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

388 have been developed and optimized for almost 40 years. Contrarely to other biological
389 macromolecules, nanostructured materials and chemicals, proteins can simultaneously
390 execute, in single chain polypeptides, all the functions required in oncotherapy (see
391 Outstanding Questions Box). These activities include efficient cell-targeting, potent
392 cytotoxicity, self-assembling to reach the optimal nanoscale size and regular
393 oligomerization for a multiple and ordered display of cell ligands. The incorporation of
394 functional casettes by simple fusion approaches allows recruiting affinity tags for one-
395 step purification from cell factories, endosomolytic agents, protease target sites and
396 intracellular trafficking domains, among others. Anticipable bottlenecks in the use of
397 these biopharmaceuticals have been already observed and minimized during the
398 development of the more than 400 protein drugs approved for human use. Protein
399 enginnering offers valuable approaches to significant deimmunization or to the
400 ablation of residual interactivity of a drug with non target organs (that might lead, for
401 instance, to hepatic toxicity). In this context, an increasing number of protein-only
402 prototypes have already confirmed the possibility to recruit high functional complexity
403 in simple and safe biological entities. This is in contast to chemically heterogeneous
404 nanoconjugates in which these functions are provided by the conjugation of different
405 types of molecules, mostly resulting from non-biological fabrication. The expanding
406 catalogues of functional modules (venoms, toxins, proaoptotic factors, AMPs and
407 others) and cancer-relevant ligands together with emerging nanobiotechnological
408 principles are expected to result in a new generation of antitumoral drugs that solely
409 formed by recombinant proteins, might be competitive in the biopharma market for
410 safer, highy efficient and more precise cancer therapies.

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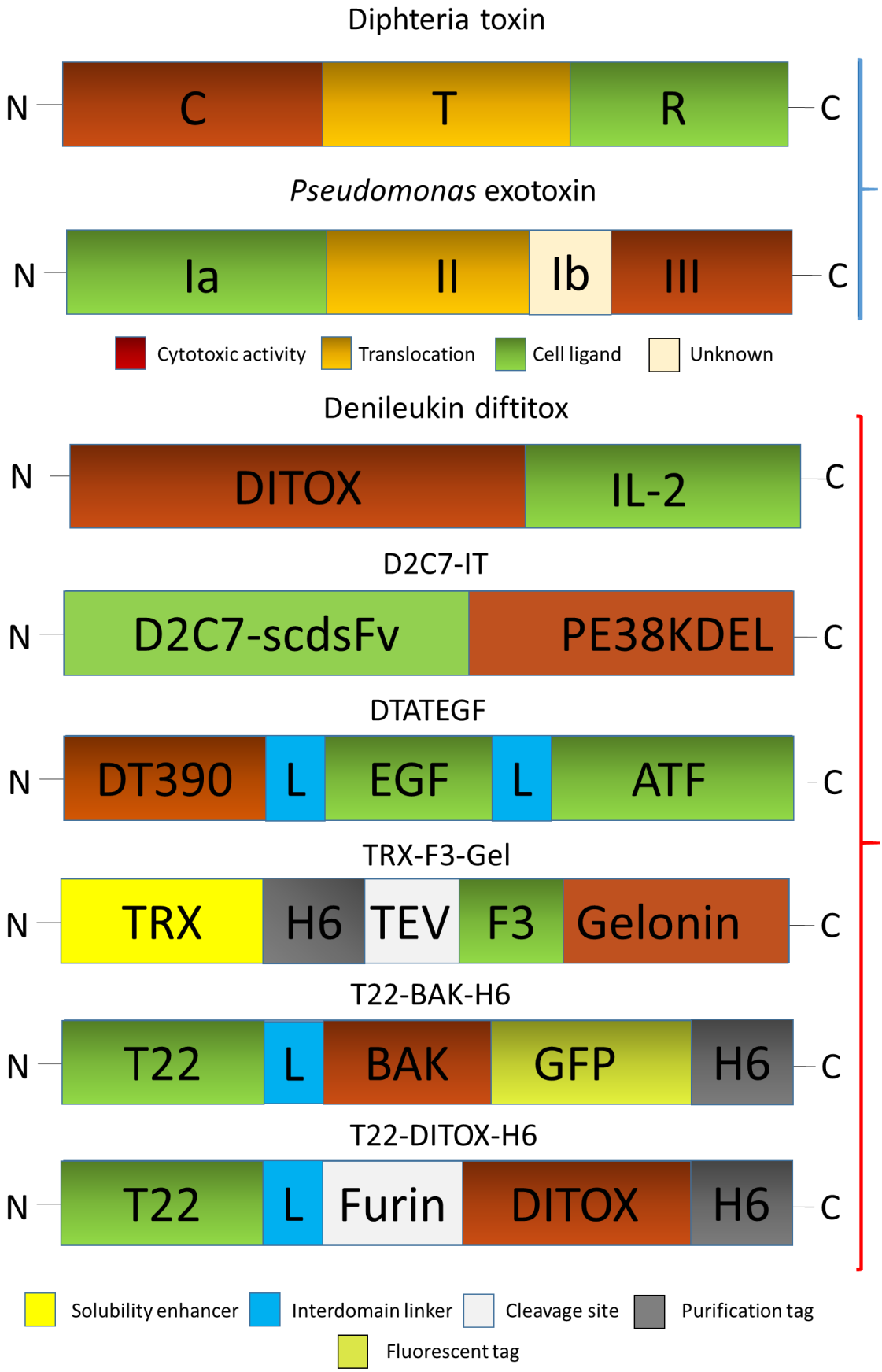
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DITOX
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Solubility enhancer
Interdomain linker
Cleavage site
Purification tag
Fluorescent tag

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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 the diphtheria toxin and by the *P. aeruginosa* exotoxin. Engineered versions (red set) have been adapted by modular protein engineering for functional recruitment as antitumoral drugs. Denileukin diftitox is an immunotoxin that delivers the diphtheria toxin (lacking the receptor binding domain, Ditox), targeting the IL-2 receptor [81]. D2C7-IT is an immunotoxin fusion consisting of single-chain variable-region antibody fragments (**scFv**s) of the monoclonal antibody 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 the *Pseudomonas* exotoxin A (PE38KDEL) (D2C7-(scdsFv)-PE38KDEL [82]. DTATEGF is a bispecific immunotoxin based on a diphtheria toxin version (DT390), that binds both the EGF receptor (EGFR) and the urokinase-type plasminogen activator receptor (uPAR) [83]. In TRX-F3-Gel, the active, N-terminal segment of the plant toxin gelonin is targeted by F3, a ligand of nucleolin overexpressed in several tumor cell lineages. The thioredoxin (TRX)-H6 segment, used to enhance solubility and for purification upon recombinant production, is removed in vitro by the Tev protease [84]. In T22-BAK-H6, the human pro-apoptotic BAK is targeted by T22, a ligand of the cell-surface tumor marker CXCR4. The construct self-assembles as toroid nanoparticles by the combined presence of T22 and H6 (H6 also acting as purification tag. GFP allows the visualization of the material [80]. In T22-DITOX-H6, the C and T domains of the diphtheria toxin are presented in a similar way. The inserted furin cleavage site complements the internal one occurring 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.

Table 1. Representative examples of cytotoxic antitumoral protein drugs involving proteins, and the main side effects.

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

Drug	Marketed / on Trial	Structure / Target molecule	Pharmacological Indication	Adverse Effects	Reference
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), haematological toxicity	[85]
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	[86]
PROTEIN DRUGS					

Monoclonal Antibodies					
Trastuzumab	Herceptin	Binds the extracellular domain of HER-2 inhibiting the growth of Her2+ tumors	Metastatic $Her2^{+}$ Breast cancer, metastatic $HER2^{+}$ Gastric cancer	Cardiomyopathy, heart failure, Infusion reactions (dyspnoea, hypoxia, interstitial pneumonitis), nephrotic syndrome	https://www.uptodate.com/online/
Cetuximab	Erbitux	Binds EGFR, HER1 and c-ErbB-1 inhibiting EGF binding to result in the induction of 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	https://www.uptodate.com/online/
Bevacizumab	Avastin	Binds 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 fístula and perforation, heart failure, haemorrhage hypertension, infusion reactions, necrotizing fasciitis	https://www.uptodate.com/online/
Olaratumab	Lartruvo	Binds 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	https://www.uptodate.com/online/

Ipilimumab	Yervoy	Binds 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, hepatotoxicity, ophthalmic toxicity	https://www.uptodate.com/online/
Nivolumab	Opdivo	Binds 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 and renal cell and urothelial carcinomas, Hodgkin lymphoma, metastatic melanoma	Adrenal insufficiency, immune-mediated rash, type 1 diabetes encephalitis colitis, thyroiditis, nephritis, hepatitis, pneumonitis, hypophysitis, Infusion reactions	https://www.uptodate.com/online/
Multispecific Antibodies					
Catumoxomab	Removab	Trifunctional bispecific (EpCAM & CD3) mAb binding tumor, T cells & Fc region to activate immunity	Malignant ascites due to epithelial carcinomas	Lymphopenia, abdominal pain, nausea, vomiting, diarrhoea, pyrexia, fatigue, chills, pain	[87]
Blinatumomab	Blincyto	Bispecific mAb that binds CD19 on B-cells and CD3 on T cells	Relapsed or refractory B-cell precursor acute lymphoblastic leukaemia	Cytokine release syndrome, Neurological toxicity	[88]
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 diarrhoea, hypotension	[89]

Pegylated Proteins					
Pegaspargase	Oncaspar	Pegylated bacterial asparaginase	Actue lymphoblastic leukaemia, Extranodal natural killer/T-cell lymphoma	Delayed hypersensitivity reactions neurotoxicity, hepatotoxicity	[90]
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 diphtheria toxin	Cutaneous T-cell Lymphoma	Fever, chills, edema, hypoalbuminemia, hypotension, transaminasemia	[63]
Moxetumumab pasudotox	In clinical trials	Anti-CD22 mAb - modified Pseudomonas Exotoxin A fragment	Relapsed and refractory Hairy Cell leukaemia, acute lymphoblastic leukaemia	Hypoalbuminemia, aminotransferase elevations, edema, headache, hypotension, nausea, fatigue	[60]
LMB-2 (anti-Tac(Fv)-P38)	In clinical trials	Anti-alpha subunit IL-2R (CD25) mAb - modified *Pseudomonas* Exotoxin A fragment	Hairy Cell leukemia, cutaneous T-cell lymphoma, chronic lymphocytic leukaemia	Reversible cardiomyopathy, transaminase elevations, fever	NIH database https://clinicaltrials.gov/ NCT00321555

RG7787 SS1(dsFv)-PE38	In clinical trials	Mesothelin-binding SS1 Ab - modified Pseudomonas Exotoxin A fragment	Mesothelioma, Triple negative Breast Cancer, Gastric Cancer	Edema, hypoalbuminemia, fatigue, vascular leak syndrome	NIH database https://clinicaltrials.gov/ NCT00024687
Fusion Proteins					
Aflibercept	Zaltrap	VEGFR1&2 fragments - Fc human IgG1 Fusion Protein	Metastatic colorectal cancer	Haemorrhage, gastrointestinal perforation, hypertension, Infection	[91]
Etanercept	Enbrel	Tumor necrosis factor receptor - Fc human IgG1 Fusion Protein	Lymphoma and other malignancies	Tuberculosis, fungal or viral infections, Injection site reaction	NIH database 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 cancinomas and melanoma	Steven-Johnson syndrome, toxic epidermal necrolysis, peripheral edema, hematotoxicity	NIH database https://clinicaltrials.gov/ NCT01642342 & NCT0271756
Denileukin diftitox	Ontak	Interleukin 2 - Diphtheria toxin fragments A & B fusion protein	Cutaneous T-cell lymphoma	Infusion reactions, hepatotoxicity, visual loss, vascular leak syndrome	[81]
OXS-1550 (DT2219ARL)	In clinical trials	Bispecific scFv anti-CD19 and anti-CD22 mAbs - modified form of diphtheria toxin Fusion Protein	Relapsed/refractory B-cell lymphoma or leukaemia	Peripheral edema and hypoalbuminemia	NIH database https://clinicaltrials.gov/ NCT02370160

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445 **Table 2.** Representative examples of main cytotoxic proteins explored as antitumoral drugs, which are produced as recombinant versions in
446 bacterial cell factories.

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Protein	Source	Mechanism of action	Therapeutic application	Recombinant protein (producing organism)	Cancer tested	References
Pro-apoptotic						
BID	*Homo sapiens*	Activator: Interacts with high affinity to all anti-apoptotic proteins and directly activates Bax and Bak.	Pro-apoptotic	*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 anti-apoptotic proteins and directly activates Bax and Bak.	Pro-apoptotic	*E. coli* BL21 and *E. coli* Origami B	Colon cancer	[80]
BAD	*Homo sapiens*	Sensitizer: Interacts with anti-apoptotic proteins. High affinity to Bcl-2 and Bcl-XL.	Pro-apoptotic	*E. coli* BL21	Glioma, leukemia and gastrointestinal carcinoma	[69]
BIK	*Homo sapiens*	Sensitizer: Interacts with anti-apoptotic proteins. High affinity to Bcl-W and Bcl-XL.	Pro-apoptotic	*E. coli* BL21 and *E. coli* DH5α	Colon adenocarcinoma	[68]
BAKBH3	*Homo sapiens*	Antagonizes anti-apoptotic	Pro-apoptotic	*E. coli* Origami B	Cervical and	[80]

		proteins function.			colon cancer	
Toxin or venom compopnent						
Diphtheria toxin	*Corynebacterium diphtheriae* (bacteria)	Inhibition of EF-2 and therefore protein synthesis	Pro-apoptotic	*E. coli* BL21(DE3)	Neuroblastoma, breast cancer and colon cancer	[92]
Exotoxin A	*Pseudomonas aeruginosa* (bacteria)	Inhibition of EF-2 and therefore protein synthesis	Pro-apoptotic	*E. coli* BL21(DE3)	Burkitt's lymphoma	[93]
Chlorotoxin	*Leiurus quinquestriatus* (scorpion)	Chloride channels blocker	Targeting and slightly apoptotic	*E. coli* BL21 Star™ (DE3)	Glioma	[94]
Melittin	*Apis mellifera* (bee)	Surfactant activity	Cytotoxicity	*E. coli* Rosetta	Glioma	[95]
Gomesin	*Acanthoscurria gomesiana* (spider)	Pore formation	Pro-apoptotic	*E. coli* BL21(DE3)	Epidermoid carcinoma, cervix adenocarcinoma and breast adenocarcinoma	[96]
Agkihpin	*Gloydius halys Pallas* (snake)	induce apoptosis or necrosis but mechanism remains to be explored	Anti-metastasis	*E.coli* BL21 (DE3) and DH5α	Liver cancer	[97]
Colombistatins 2, 3, and 4	*Bothrops colombiensis* (snake)	Inhibit ristocetin, ADP, collagen	Potent anti-platelet aggregation activity	*E. coli* BL21 star	Human skin melanoma	[98]
Ricin	*Ricinus communis*	Protein synthesis inhibition by	Anti-proliferative	*E. coli* strain MV1190	Leukemia, and	[99]

	(plant)	the cleavage of a single adenine residue in the 28S ribosomal RNA.	activity		lymphoma	
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	[100]
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	[84]

Box 1. Cell surface molecular targets in cancer.

Tumoral 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, which leave tumor-initiating cells unattended. This might result in the consequent relapse a few months later, since these therapies increase the percentage of cancer stem cells (**CSC**s), that repopulate the tumor mass and that account also 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 common to different tumors, such as CD44, CD133, CD24, ESA, CXCR4, $\alpha_2\beta_1$, and the multi-drug resistance MDR1 and ABCE2 [101, 102]. Some of them are particularly associated to specific types of cancer, in rapidly expanding catalogues that include CD44, CD24 and ALDH1 to breast cancer [103], CXCR4, LGR5, CLDN1, LY6G6D/F and TLR4 to colorectal cancer [104-106], CD151 to ovarian cancer [107] and Sox2, Oct4 and CD90 to lung cancer [108]. Since 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, sufficiently overexpressed over progenitor cells to allow a safe expansion of the therapeutic window [106]. The development of multispecific of multiparatopic drugs or nanoconjugates should pave the way for a more specific delivery into tumoral CSCs.

Box 2. The Antibody-drug conjugate concept; successes and limitations

ADCs represent the earliest and simplest strategy to increase drug agressivity and selectivity against tumor cells. The first approved ADCs were Gemtuzumab ozogamicin (Mylotarg) in 2000, indicated for acute myeloid leukaemia, and Ibritumomab tiuxetan (Zevalin) and Tositumomab (Bexxar) in 2002 and 2003 respectively, both indicated for non-Hodgkin's 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 or 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. Under these premises, several generations of 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 effect of ADCs includes 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 antitumoral 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 the lack of improved efficacy regarding free drug and the important 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), and 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 the tumoral delivery of more complex antitumoral entities. Among them, the CD20-targted delivery of *Salmonella* bacterials 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].

Glossary

ADAs: anti-drug antibodies. They are generated during the immune response against an antigen present in a protein therapeutic after its administration to an organism

ADC: antibody-drug conjugate. A chemically coupled complex between a drug and a targeting antibody that offers cell selectivity in the delivery process.

ACP: anti-cancer peptide. Antimicrobial peptides that bind negatively charged molecules on the cancer cell membranes and selectively induce tumor apoptosis or necrosis.

AMP: anti-microbial peptide. Often referred as host defense peptides, they are important players in the innate immune response.

CSC: cancer stem cell. Cancer cells with capacity for self-renewal and differentiation into diverse cell types occurring in tumors. The subset of CSCs differs from more differentiated tumor cells in their unique capacity for of initiating and repopulating the tumor.

DT: diphtheria toxin. An exotoxin secreted by the pathogenic bacterium *Corynebacterium diphtheriae*, the ethiological agent of diphtheria.

EGFR: epidermal growth factor receptor. A transmembrane protein acting as a receptor for specific ligands, such as EGF and transforming growth factor-) that bind and activate cell signaling.

EPR: enhanced permeability and retention effect. This is the local drug retention resulting from the highly permeable tumour vasculature combined with a poor lymphatic drainage.

Fab: antigen-binding fragment. The antigen-interacting region of an antibody.

Fv: variable (antibody) fragment. Variable loops of light and heavy chains responsible for antigen binding.

FDA: Food and Drug Administration. A US federal agency responsible for protecting public health by ensuring safety and efficacy of drugs and biopharmaceuticals.

mAb: monoclonal antibody. An antibody produced by the controlled culture of a clone of immune cells.

MHC: major histocompatibility complex (MHC). A set of proteins displayed on the surface of cells that recognize foreign antigens to trigger its processing and the activation of an immune response. They are classified in class I and II. MHC class II proteins are expressed in dendritic cells, macrophages and B cells.

MMAE: monomethyl auristatin E. A synthetic derivative of dolastatin, a peptapeptide inhibitor of tubulin polymerization with potent antimitotic activity isolated from a species of sea hare.

MMAF: monomethyl auristatin F. A synthetic derivative of dolastatin, inhibitor of tubulin polymerization with lower antitumor activity than MMAE.

PDGFR: platelet-derived growt factor receptor. Cell surface receptors that bind and are activated for cell signaling by the family of PDGF ligands.

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

PEG: polyethylene glycol. A polymer of ethylene oxide that once bound to nanoparticles inhibits their clearance by the immune system.

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

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

TI: therapeutic index, an indication of drug toxicity referred to its 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}).

VEGF: vascular endothelial growth factor. A hypoxia-induced secreted protein that stimulates the formation of blood vessels in normal tissues and in tumors.

VEGFR: vascular endothelial growth factor receptor: a cell surface receptor that is bound and activated by its ligand VEGF for cell signaling.

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Competing interests

AV, EV, NS, L S-G, UU and R M are co-inventors of a patent covering the use of self-assembling, nanostructured cytotoxic proteins.

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