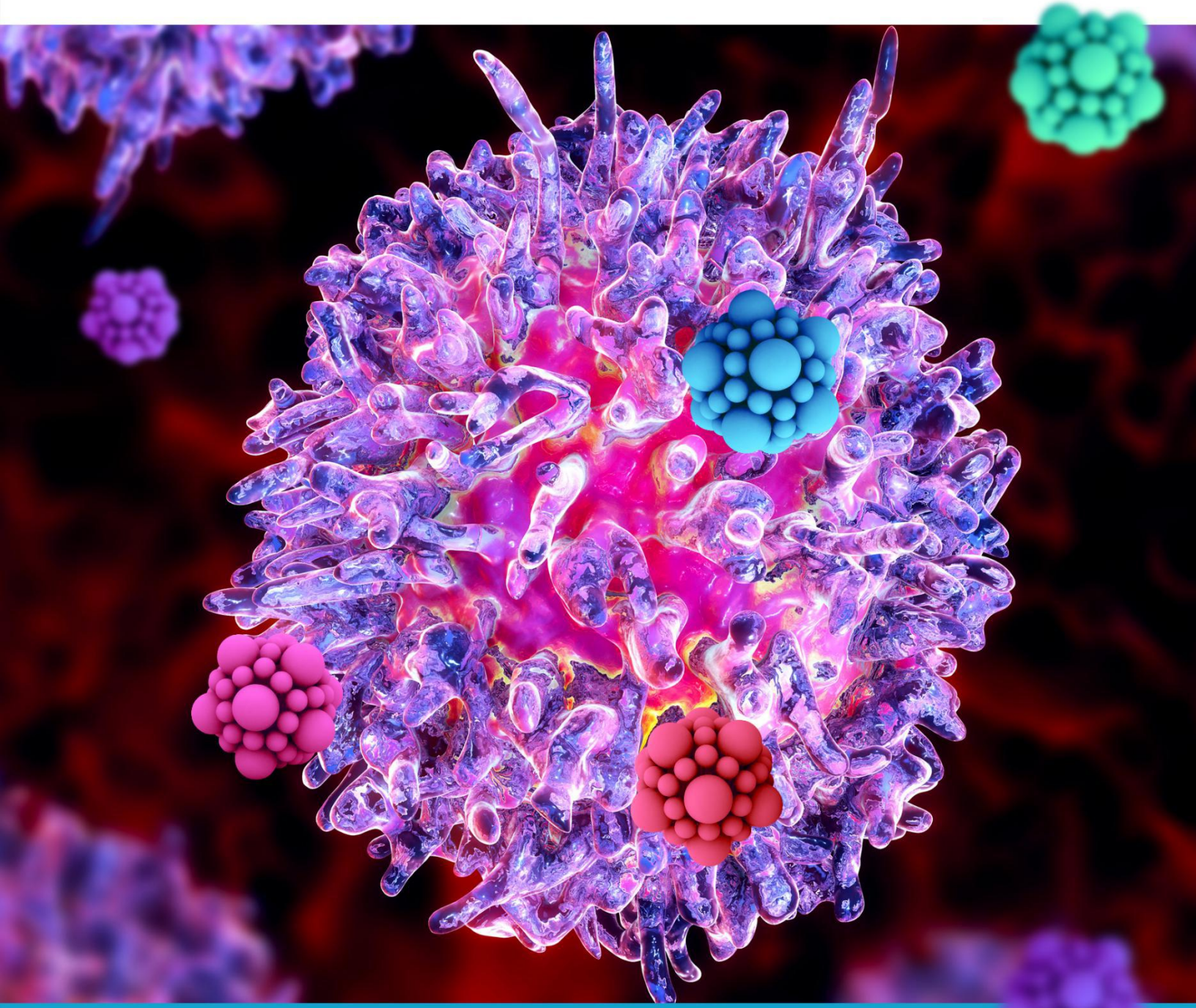
Universitat Autònoma de Barcelona

Recombinant self-assembling nanoparticles for cancer therapy based on toxin and venom compounds

Raquel Díaz Ocaña

Recombinant SELF-ASSEMBLING NANOPARTICLES for CANCER THERAPY

based on toxin and venom compounds



Raquel Díaz Ocaña

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Raquel Díaz Ocaña

Vist i plau dels directors de la tesi:

Antonio Villaverde Corrales Esther Vázquez Gómez Ugutz Unzueta Elorza

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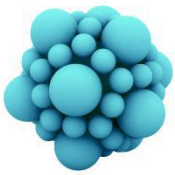
“No siempre podemos hacer grandes cosas, pero sí podemos hacer cosas pequeñas con gran amor”

Madre Teresa de Calcuta



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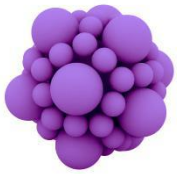

Introduction

Carcinogenesis

Definition and Statistics

A large group of diseases characterized by the growth of abnormal cells beyond their usual boundaries that can then invade adjoining parts of the body and/or spread to other organs is known as cancer, neoplasms or malignant tumors. Cancer is the 2nd leading cause of death globally according to The World Health Organization (WHO)[1]. Cancer burden rises to 18.1 million new cases, as shown in **Figure 1**, and 9.6 million cancer deaths in 2018. The identification followed by the study of molecular biomarkers as prognostic factors in different types of cancer has been very useful to understand how the disease can be faced[2]. Effective nanomedicines against cancer are required, as more than 4,800 new cases each day were projected to be diagnosed in the U.S. during 2019.[3]

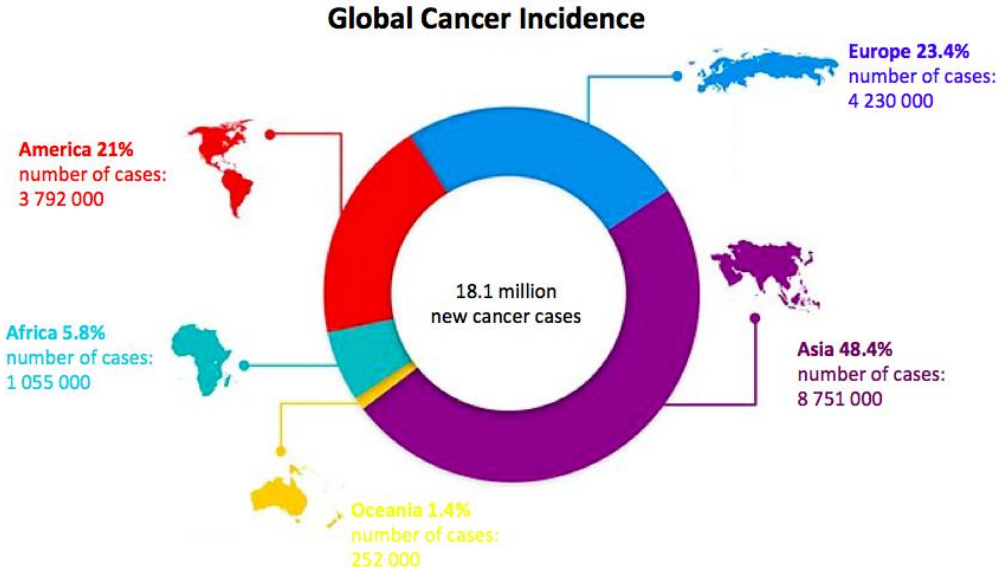

Figure 1. Estimated number of incident cases. Both sexes, all cancer types for all ages worldwide. Modified from[4]

Available Treatments

There are different ways to proceed depending on the cancer type and stage. A treatment combination has shown to be more effective. Surgery represents a conventional procedure where a solid tumor is removed entirely or partially by debulking. The disadvantages involved include anesthesia risks, slow and costly recovery, pain and infection. Radiation therapy, another type of cancer treatment, is used to shrink tumors and kill cancer cells by irreversible damage in the DNA. Unfortunately, it can affect healthy cells leading in difficulties to eat and digest side effects.[5]

When drugs are involved, treatment known as chemotherapy, several limitations are related to the compound cytotoxic side effects on non-directed tissues[6,7]. But also, positive achievements are reached increasingly with innovative drugs, some techniques are mentioned below.

The goal of immunotherapy is to boost the immune system in order to fight against malignancies. For example, checkpoints inhibitors are focused on T-cells and interfere with the ability of cancer cells to avoid the immune system attack[8]. However, even FDA-approved therapeutic drugs are associated with immune-related adverse effects.[9]

The strategy of antigen-selective monoclonal antibodies enhances cancer apoptosis when an effector cell interaction between IgG molecules with the lymphocytes NK cells is applied, by targeting a specific glycoprotein (CD38)[10, 11]. Other kind of antibodies with dual affinity for two epitopes, usually for T cells (CD3) and another cancer-specific antigen are bispecific antibodies (bsAbs). [12]

Cellular therapy involves the usage of T cells, while it is potentially curative at various stages of the disease; it is also limited by significant morbidity and mortality[13]. This therapeutic technique also known as adoptive cell transfer (ACT) increases the availability of immunocompetent effector cells.

Acute Myeloid Leukemia

Acute Myeloid Leukemia (AML) is the most common form of acute leukemia in adults and the 2nd most common type of leukemia diagnosed in children. An estimated of 21450 people of all ages were diagnosed with AML just in the U. S. throughout 2019. [14]

AML, an aberrant expansion of undifferentiated myeloid blasts, is a heterogeneous hematopoietic malignancy. Leukemia stem cells (LSCs) are capable of regenerating AML populations, and present high possibilities of relapse after treatment preventing an effective long-term outcome[15]. LSCs are characterized by their limitless self-renewal capability capable of generating differentiated blast cell lineages comparable to normal hematopoietic stem cells. Conventional chemotherapeutic agents have shown to stop proliferation of leukemic cells but to be ineffective against LSCs[16].

In AML, tumor population is heterogeneous and LSCs can express antigens found in healthy immature myeloid cells, including some common differentiation (CD) markers[17]. Cell markers are expressed depending on the morphological subtype of AMLs and stage of differentiation blocks; even

though some normal cells share cell surface antigens as they are commonly lineage-associated.

AML is known to be an immune-responsive disease but also it is immune suppressive either directly or by different mechanisms that disturb the bone marrow (BM) microenvironment. Patient´s immune effector cells (natural killer cells, macrophages and T cells) that are exposed to cytotoxic chemotherapy could be functionally or numerically impaired. Conventional treatment is based on intensive and consolidation chemotherapy, including demethylating agents, or stem cell transplant. The patient´s response depends on personal characteristics such as comorbidities, genetics and age[18]. Well-tolerated small molecule drugs or some targeted therapies with monoclonal antibodies are commonly used for AML treatment benefiting a subset of patients with moderate overall responses. For relapse prevention post-remission therapy is suggested with the recent form of immunotherapy as the allogeneic hematopoietic cell transplantation (alloHCT). This therapy is successful in patients with morphologic remission, but a low efficiency response has been observed with patients with active disease (marrow blasts higher than 5%). AlloHCT propitiates T cell-based anti-leukemic immunity and allows additional myeloablative chemotherapy boosting healthy hematopoiesis.[19]

AML can be subdivided into three categories based on the cause of the disease: when it is associated with the myelodysplastic syndrome or other myeloid proliferative disorder (MPD), when it is associated to a prior chemotherapy exposure, or when it is a de novo AML[20]. Nevertheless, according to the World Health Organization (WHO), AML is subdivided in seven classifications: AML with recurrent abnormalities and gene mutations,

AML with myelodysplasia-related changes, Therapy related myeloid neoplasms, AML not otherwise specified, Myeloid sarcoma, Myeloid proliferations related to Down syndrome, and Blastic plasmocytoid dendritic cell neoplasm[21]. Approximately 52% of all adult primary AML cytogenetic abnormalities have been disposed as the cause of this disease[22]. Some of the non-random chromosomal alterations such as t(8;21)(q22;q22), t(15;17)(q22;q12) and inv(16)(p13.1;q22), are related to a better treatment response and survival; while abnormalities in chromosomes 5, 7, complex karyotype and 11q23 are identified as determining low remission and short survival opportunities[23]. In the other hand, around 40-50% of all AML patients present normal cytogenetics (CN-AML) with intermediate possibilities of relapse. Molecular screening of AML is important for determining the treatment strategy and classification of the disease.[24]

Anti-leukemic activity of polyclonal T cells is moderate. Immunotherapy kills tumor cells by recognizing tumor antigens through active immune effector cells. Suitable tumor antigens are over expressed specifically on malignant cells. Some examples that are available to be targeted with antibody constructs such as chimeric antigen receptor T cells or bispecific T cell engagers include CXCR4, CD33, FLT3, CD123, CLL1, and CD44v6. Other antigens that can be localized intracellularly could be partially or completely specific to cancerous cells if the mutant peptides could be capable of being presented to T cells in a determined leukocyte antigen molecule (HLA).

The chemokine receptor 4 (CXCR4) in AML is a key mediator of the interaction between AML and BM-microenvironment, as it facilitates leukemia cell trafficking and keeps malignant cells in contact with extracellular matrix and stromal cells, promoting AML growth and resistance. CXCR4 inhibition, by

means of some molecules shown in **Table 1**, entails antileukemia effects. Thus, by blocking CXCR4 and other adhesion molecules in parallel, the interaction of leukemic cells with their BM microenvironment could be avoided. All AML cells express internal CXCR4, even cells without surface CXCR4 expression that may be changed under physiological conditions of hypoxic BM. $CXCR4^{+}$ cells are associated with poor prognosis but at the same time, they offer a huge clinical utility when considered as a target to overcome the disease.

Table 1. Clinical trials of CXCR4 inhibitors in AML. *Relapsed/refractory AML (rrAML)*. Adapted from[25]

CXCR4 inhibitors	Clinical trial Phase	Population type	ClinicalTrials.gov identifier
Plerixafor	2	rrAML	NCT00512252
			NCT00906945
			NCT01435343
		Untreated elderly AML	NCT01160354
BL-8040	1	rrAML	NCT01838395
		AMLin remission	NCT02502968
LY2510924	1	rrAML	NCT02652871
Ulocuplumab	1	rrAML	NCT01120457
		Untreated AML	NCT02305563

Metastasis and Disease Prognosis

Metastasis progression, as an ability of malignant cells to colonize, starts with a concrete local invasion, intravasation, survival in the circulation, extravasation and culminates in a full colonization. [26]

AML mutations define patients' prognosis. The most common mutations found in AML are described below.

- Nucleophosmin1 (NPM1) mutations encompass 25%-30% of all cases being the most frequent molecular abnormality mostly in women diagnosed with AML. An aberrant expression of the NPM1 protein in the cytoplasm rather than the nucleus stimulates myeloid proliferation leading to leukemia development.[23] Nevertheless, this specific mutation is related to favorable overall survival due to a high chemo sensitivity to intense chemotherapy.[27]
- DNA Methyltansferase 3A (DNMT3A) mutations occur around 20% of all AML cases and in 35% of CN-AML. The most common mutations are those that affect the arginine codon 882 (R882-DNMT3A) altering methylation and hematopoiesis[28]. All DNMT3A mutations are described as pre-leukemic adverse mutations showing an early AML evolution and persisting prognostic after remission. The impact of these mutations depends on the age of the patient; younger patients presented shorter disease free survival (DFS) and overall survival (OS). Nevertheless, it was reported that patients treated with the standard-dose of anthracycline induction therapy presented an inferior survival response compared to those treated with high-dose of danorubicin with an improved survival rate when mutations as DNMT3A or NPM1 and MLL translocations.[29]
- Fms-Like Tyrosine Kinase 3 Mutations (FLT3) are overexpressed in hematopoietic stem cells with an impressive role in survival and proliferation. Around 20% of all AML cases present internal tandem duplications (ITD) in the juxta-membrane domain or mutations in the

second tyrosine kinase domain of the FLT3 gene. These mutations boost blasts proliferation presenting an extreme leukocytosis and prominent nuclear invagination. FLT3-ITD mutations are related to an increased risk of relapse[30]. Tyrosine kinase inhibitors (TKI) are being studied in FLT3 mutated AML patients. However when used alone, TKI achieved a partial blasts reduction leading the AML resistance over time.[31]

- Isocitrate Dehydrogenase (IDH) mutations 1 and 2 are oncogenic and cause loss of the physiologic enzyme function and allow enzymes to convert α-ketoglutarate into 2-hydroxyglutarate. These mutations alter the highly conserved arginine residue at codon 132 of IDH1 and codons R140 and R172 of IDH2[32]. Specifically IDH1 mutations are associated with low DFS and OS in CN_AML cases with NPM1 mutations and FLT3.[33]
- Ten-Eleven Translocation 2 (TET2) is found mutated in around 10-23% of AML patients. TET1 function is involved in the DNA demethylation process where 5-methylcytosine (5mC) is converted to 5-hydroxymethylcytosine (5hmC) [34]. Some reports have shown TET2 mutations as an adverse factor for complete response (CR) or overall survival (OS). [35]
- Runt-related Transcription Factor (RUNX1), also known as AML1 or core-binding factor subunit α-2 (*CBFA2*), found at chromosome 21, is necessary for normal hematopoiesis. RUNX1 creates the fusion protein *AML-ETO* or t(8;21)(q22;q22) AML when is commonly translocated with *ETO/MTG8/RUNX1T1* gene located on chromosome 8q22[36],

demonstrating high resistance to standard induction therapy and inferior OS.[32]

- CCAAT Enhancer Binding Protein α (CEBPA) mutations. In hematopoiesis, CEBPA transcription factor controls gene expression while in AML CEBPA involves a combination that includes an N-terminal and bZIO gene mutation. When the mutations are bi allelic, the prognosis supports a higher CR and better OS.[37]
- The function of the Additional Sex Comb-like 1 (ASXL1) protein is believed to be involved in epigenetic regulations[38]. ASXL1 mutations are more common in older patients, over 60 years old, and present an association with t(8;21), mutated *CEBPA*, wild-type *NPM1*, absence of *FLT3-ITD*, and an inferior CR and OS.[39]
- Mixed Lineage Leukemia (MLL) gene at chromosome 11q23 encodes for the protein involved in the histone methyltransferase function that modifies chromatin in the regulatory complex. Translocations affecting the MLL gene characterized by an overexpression of the gene HOX lead to a poor prognosis. [40]
- Tumor protein P53 (TP53) mutations, related to the complex karyotype, entail an adverse prognosis with a high chemioresistance. [41]
- Mutations in the protein KIT, a tyrosine kinase receptor involved in normal hematopoiesis, have shown to confer higher relapse risk and low OS. KIT mutation in the codon D816, specifically in t(8;21)(q22;q22) patients is associated with unfavorable DFS and OS[42]. Desatinb, a tyrosine kinase inhibitor has been combined with chemotherapy in newly core binding factor (CBF-AML) diagnosed patients. Interestingly,

during relapse time there was disappearance of the KIT subclone where desatinb is driven and no survival benefit was achieved. [43]

- Splicing Factor Gene Mutations are associated with pre-leukemic conditions. Some examples are SRSF2, F3B1, U2AF1 or ZRSR2, involved in regulating gene expressions and DNA-loop formations, are pathognomonic of secondary AML developing from precedent myelodysplastic syndrome (MDS). [39]

Receptor CXCR4 and ACKR3

The family of chemokines activates and improves the adhesion and migration of leukocytes during inflammatory reactions or homeostatic processes. Depending on the position of the conserved NH2-terminal cysteine residues chemokines can be classified into homeostatic and inflammatory chemokines. CXCL12 is considered as a homeostatic chemokine but it presents inflammatory activities as well. Modifications in the CXCL12 receptor define diseases progression including cancer, viral infection, ocular and respiratory diseases, autoimmune diseases, neuro and bone inflammatory diseases. The functions of the CXCL12 are exerted by interactions with CXC chemokine receptor 4 (CXCR4), a typical chemokine receptor 3 (ACKR3) and glycosaminoglycans (GAGs) such as heparin, as shown in **Table 2.**

CXCR4 is a rhodopsin - like seven transmembrane domain G protein - coupled receptor (GPCR) that only has CXCL12 as a chemokine ligand. The human immunodeficiency virus (HIV)-1 gp120 protein also binds to CXCR4, which mediates viral entry[44]. There are two splice variants of CXCR4,

i.e. CXCR4-A and CXCR4-B. Interestingly CXCR4-B is more efficiently infected by HIV-1 X4 strains; therefore siRNA treatments that target CXCR4-B reduce viral replications[45]. CXCR4 is widely expressed on hematopoietic cells including HSCs, T cells, B cells, monocytes, macrophages, neutrophils, and eosinophils. Furthermore, the receptor is expressed in the brain, lung, colon, heart, kidney, and liver, as well as endothelial and epithelial cells, microglia, astrocytes and neuronal cells, and progenitor cells including endothelial and smooth muscle progenitors Activation of CXCR4 signaling may affect several major signaling pathways related to cell survival, proliferation, and migration. [25]

ACKR3 is an important factor for tumor development as it binds CXCL12 with high affinity and removes it from the environment by internalization, functioning as a scavenging receptor[46]. ACKR3 is overexpressed in various cancers. Regulatory mechanisms as hypoxia upregulate the expression of ACKR3 and CXCR4 in mesenchymal stem cells, endothelial cells and glioma cells involved in cancer pathology[47]. The role of ACKR3 in CXCL12/CXCR4 - mediated events is complex. Depending on the cancer type, CXCR4 and ACKR3 are expressed on the same or distinct cells[48]. Moreover, co - expression of ACKR3 and CXCR4 on the same cell resulted in enhancing or reducing signal transduction and chemotaxis depending on the cancer cell line used. [49]

CXCR4 is also a stem cell surface marker associated with several human metastatic cancers. Therefore, developing new therapeutic agents targeted to CXCR4 through ligands and peptides that make endocytosis feasible is a recognized priority as well[50]. The potential signal transduction pathways

activated after CXCR4 stimulation with CXCL12 are diverse; G protein-mediated signal transduction occurs by dissociation of the Gβγ and Gα subunits that are bounded to CXCR4. When mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase and phospholipase C are activated, complex signal amplification is entailed and it leads into the target cell migration[51]. Targeting the CXCL12-CXCR4-ACKR3 axis in cancer therapy promotes the regulation of the cancer cell adhesion by integrin inhibition. In the same way, by blocking CXCL12-CXCR4 interactions, cancer cells are released from their protective environment and become more susceptible to chemotherapy using antagonists for CXCR4. [52]

Common antagonists for cancer treatment are AMD3100 for CXCR4 and CCX771 for ACKR3[53]. Notwithstanding T22, the engineered version polyphemusin II peptide from the horseshoe crab, in which three substitutions at residues Tyr5, Lys7, and Tyr12 showed excellent affinity for CXCR4. [50]

Table 2. Role of CXCL12/CXCR4/ACKR3 in cancer. Adapted from[54]

Receptors	Effects
CXCL12	Promotes angiogenesis, invasion and metastasis. It is upregulated by hypoxia.
CXCR4	Antagonists promote chemo sensitization and reduce tumor metastasis, cell migration and angiogenesis. It is upregulated by hypoxia.
ACKR3	Promotes tumor growth and survival. It is upregulated by hypoxia. Antagonists and siRNA reduce tumor size.

Glioblastoma (GBM)

The most common primary malignancy of the central nervous system (CNS) in adults is glioblastoma. The aggressive behavior of the malignancy and the therapeutic resistance reflect an invasiveness seclusion and strong resistance to innate immunoediting strategies. [55]

Histopathology analysis is made prior glioma is diagnosed. The analysis of the mutation status, either by immunohistochemistry or sequencing, of isociatrate degydrogenase (IDH) and *O*6-methylguanine-DNA methyltransferase (MGMT) promoter methylation, is an accurate pathological diagnostic approach. But also the scrutiny of copy number, mutation status, promoter methylation and deletions provides information about the key genes that are involved with a particular GBM subtype like epidermal growth factor receptor (EGFR), ATRX, cyclin-dependent kinase 4 (CDK4), CDKN2A/B, and Tert.

The idea that the brain is an immune-privileged organ has been defeated as increasing evidence demonstrates that the CNS can set immunogenic responses. However, GBM is known to be strongly immunosuppressive joined to a chronic inflammatory microenvironment[56]. Most of the cells present in GBM are microglia and infiltrating macrophages but also lymphocytes, T cells and myeloid-derived suppressor cells (MDSCs) help to drive the tumor-mediated immunosuppression. Some other immune-escape mechanisms include the activation of indoleamine 2,3 dioxygenase, dysregulation of antigen presentation, and myeloid cell suppression driven by transcription factor 3 (STAT3).

The tumor microenvironment in glioma cells is defined by matrix metalloproteinases (MMPs) that are directly involved in tumor progression and metastasis. In glioblastoma MMP-2 and MMP-9 are overexpressed. Both could be targets for inhibition as they are extracellular matrix (ECM) degrading enzymes belonging to the family of gelatinases that promote the angiogenic and invasive potential of glioma cells[57]. By the way, annexin A2 is a calcium-dependent phospholipid-binding protein that is also overexpressed in a wide variety of tumors, but particularly in malignant brain cells. Its functions are related with extracellular matrix degradation, angiogenesis stimulation and promoting fibrinolytic processes. Annexin A2 is implicated in tumorgenesis, cell invasion, proliferation and neovascularization, becoming to be a target not only for inhibition but also for diagnostic and prognostic purposes. [58]

Pathological Classification

The most common diagnosed group of primary brain neoplasms is glioma[59]. By the way, according with the WHO, in order to classify the disease by tumor grades, it stipules that the presence of necrosis, microvascular proliferation and anaplastic features are required to be studied. When referring to slow growing diffusely infiltrating gliomas, it can be summarized as “diffuse gliomas” that can have IDH1 or IDH2 mutation and co-deletion of chromosomal arms 1p and 19q. Four different diffuse gliomas subtypes are classified by histological and genetic characteristics[60], as shown in **Figure 2**. A specific difference between low-grade gliomas from high-grade gliomas relies in the mutation of IDH1, commonly at exon 4 codon 132 (R132H). Considered as a prognostic marker, IDH1 mutation is found in less-aggressive low-grade

gliomas and it is also related to TP53 and ATRX mutations. [61] These concrete mutations correlate to improved prognosis and increased overall survival rate of around 7 years in low-grade gliomas. In this way, IDH1 mutations in GBM present an overall survival of 46 months compared to 13 months of the IDH wild-type tumors.

It is important to note that pediatric GBMs are distinct with common adult GBMs regarding to different molecular characterization and epidemiology. Tumorgenesis in pediatric cases are driven by mutations at K27 and G34 in H3F3A gene, associated with additional mutations in HIST1H3b, ACVR1, TP53 and ATRX[62]. In contrast, GBM in adults present well defined genetic changes that may be linked to poor prognosis. Some examples of these changes are the amplification and mutation of the EGFR locus, which defines the level of invasion and aggression of the disease[63]. It has also been observed that the deletion of the locus encoding CDKN2A and CDNK2B corresponds to low-grade gliomas. Within IDH1 wild-type GBMs, the mean survival for patients with CDK4/MDM2 amplification is around 6.5 months compared to almost 13 months in non-amplified patients.

Mutations detection is not the only concern when treating the disease, but also, in vivo detection of the tumor is crucial when treatment has started. For this reason targeting molecules on matrix as metalloproteinase (MMP)-2, another important marker identified, present on glioma and other tumors but absent in normal brain tissue is a key goal that refers to the tissue invasion capacity of glioma cells. In the same way, Annexin A2, a calcium-dependent phospholipid binding protein on the extracellular side of plasma membrane, play diverse roles in cellular functions as angiogenesis,

apoptosis, migration, proliferation, invasion and cohesion. [64] Similarly, if TERT promoter changes, identified in 40% of GBMs[65], come with EGFR mutations, a shorter overall survival rate has been observed from 13 to 26 months. [59]

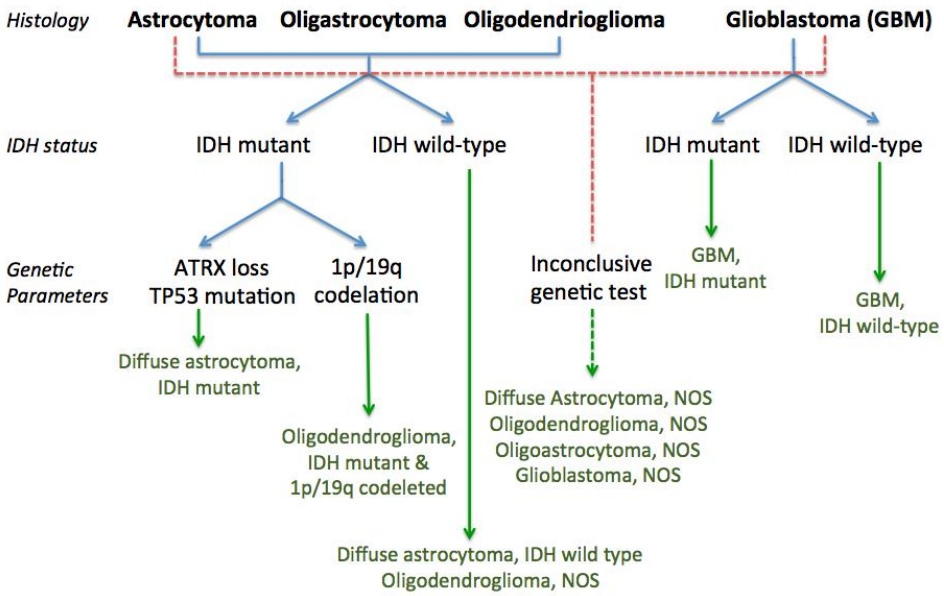

Figure 2. General classification of the diffuse gliomas based on histological and genetic features. Modified from [59]

Nanomedicine

Nanomedicine provides the possibility to overcome the limitations of most conventional drugs while treating particularly difficult cancer types, such as AML or GBM. The use of nanoparticles, as drug delivery vehicles or intrinsically functional nanomedicines, achieves the goal of site-specific targeting and metabolic degradation protection with reduced undesired side toxic effects. The implementation of polymers has also provided biocompatibility and biodegradability effects, combined with tunable properties and site-specific release.

Improvements in personalized therapies based on biomarkers (specific genes expression) can determine specific diseases with an ideal treatment regimen. Chemotherapy is highly toxic, immunogenic and may generate systemic delivery barriers; other limitations are poor solubility and non-specific distribution. [66]

Novel solutions are required to face drug resistance, side effects and inadequate drug delivery; overcoming in this way traditional therapies results. Nanomedicine offers appropriate technologies specifically when using the nanoparticle drug delivery strategy.

Targeting

Cancer therapy requires the previous understanding of the disease pathophysiology. Drug delivery accompanied by either active or passive targeting is fraught with many challenges such as the proper identification and direction of the chemotherapeutic agent to a particular target. [67]

Loading drugs into nanocarriers are expected to circulate in the blood for longer time and accumulate in pathological sites with affected vasculature (tumors and inflammations) via the enhanced permeability and retention (EPR) effect. Passive targeting is based on the longevity of the pharmaceutical carrier in the blood and the accumulation in pathological sites with compromised vasculature due in part to its morphology **(Figure 3).** While active targeting is based on the incorporation of specific ligands to the surface of pharmaceutical carriers able to recognize and attach to pathological cells[68]. Hence, the development of targeted nanocarrier systems loaded with some of the current successful drugs present a more efficient

profile with less toxicity. Some polymeric devices, as hybrid micelles, that depend on stimulis as pH or temperature changes have been effectively used. The limitation of polymeric nanocarriers resides in biocompatibility and biodegradability issues. [69] Liposomes are spherical vesicles nanosystems comprised of phospholipid layers that entrap drug molecules, or nanoparticles. Dendrimers are 3D structures where drugs can be attached chemically. For example, polyester-based dendrimers have shown promising results in CNS disorders, particularly in glioma cell lines[70]. Moreover targeting ligands can be grafted onto the surface. [71]

The tumor microenvironment needs specific strategies based on pH changes, vascular differences, hypoxic environment and metabolic state to overcome the disease effectively. The drug delivery systems require to be specifically directed to the desired biological regions. The formation of new blood vessels involved in tumors development, known as angiogenesis, promotes the oxygen and nutrients supply to the tumor [72, 73]. Blood vessels proliferate rapidly causing aberrant vasculature with regions with high or poor blood pressure or with leakages. Angiogenesis is also altered by other diverse factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, bradykinin and nitric oxide. [74]

Passive Targeting

The goal is to achieve a preferential accumulation of the drug loaded in the nanodelivery system at the target site in order to avoid non-specific distribution effects. Enhanced Permeability and Retention Effect (EPR) allows that 10-100 fold higher concentrations could be achieved in the tumor compared to the conventional intravenously free drug administration[75]. EPR

is a phenomenon mostly due to the high permeability of tumor vasculature thus achieving more accumulation of macromolecular drugs that enter the surrounding tumor tissue passively though leaky endothelial cell junctions.

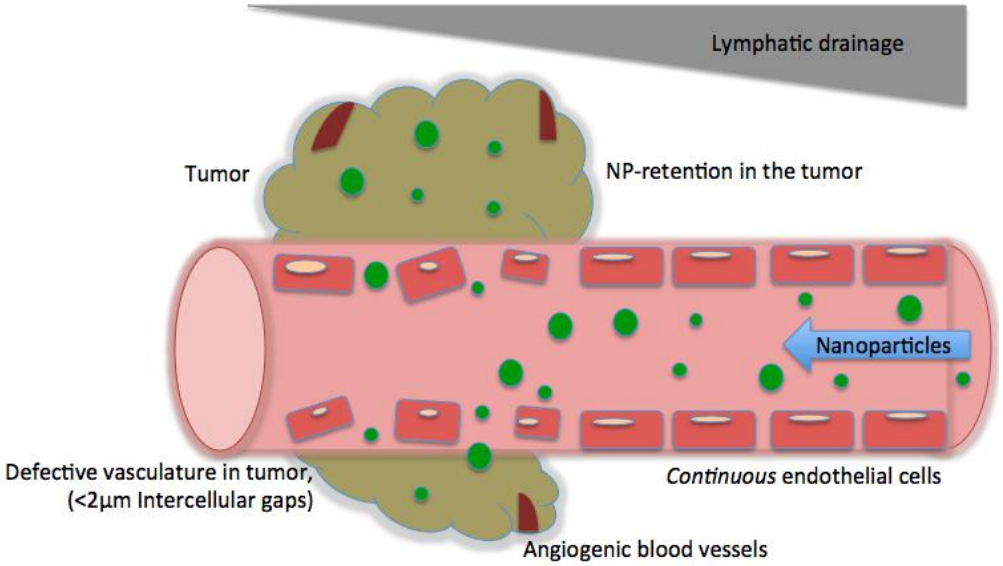

Figure 3. Enhanced permeability retention effect and passive targeting of Nanoparticles (NPs). Modified from [76]

When it comes specifically about nanoparticles, circulation time, capability to overcome barriers and the targeting is very reliant on the shape, size and surface area of the nanosystem. Passive targeting is possible due to diffusion-mediated transport, where size is critically involved in its success. The distribution of particles above 10nm avoid renal clearance while the convenient upper maximum limit is around 400nm[77]. However, particles smaller than 300nm avoid the uptake by the liver or spleen, resulting in a prolonged circulation time. But not only size is related to an affective delivery but also shape and geometry of contact is important for cellular internalization. Larger molecules with different shapes than spherical have

shown only superficial accumulation. However small spherical particles are able to penetrate deeply into the tumor with a more uniform distribution. [78]

Mechanisms of Cellular Uptake

Passive targeting can be regulated by changing size, shape and the surface dimensions of nanoparticles. These modifications also affect the cellular internalization process, directed by either phagocytosis, macropinocytosis, caveolar-mediated endocytosis or clathrin-mediated endocytosis[79]. However, the major drawback of passive targeting is that healthy tissue is not distinguished from the diseased one, as it happens in conventional chemotherapeutic treatments.

The cellular uptake is influenced by different factors as concentration, incubation time, temperature, cell penetrating peptide (CPP) type, or membrane structure. CPPs are short peptide sequences with positive global charge, rich in lysine or arginine residues. They are also known as protein transduction domains, membrane transduction peptides or Trojan peptides. They have advantages like high internalization, ease of sequence modification or low cytotoxicity. CPPs can be classified into cationic, amphipathic and hydrophobic classes, being the cationic ones the most common[80]. The internalization mechanisms have been described as follows.

Direct Penetration

Occurs mostly at high concentration values of the nanoparticle employed[81]. Penetration happens due to the positively charges attributed to the CPP and the negatively charged components of the cell membrane (such as heparin sulfate or a phospholipid barrier), even at low temperatures or in presence of

endocytosis-inhibitors. Previous steps as pore formation and destabilization of the cell membrane precede the complete internalization. The barrel stave model and the toroidal pore model are related to the pore formation process, while the carpet-like model and inverted micelle formation induce membrane destabilization and direct internalization. **Figure 4** shows the hydrophilic part of the CPP as red peptides while the hydrophobic part is represented by blue peptides.

- **Barrel stave model**. In this model, hydrophobic residues of the helix structures face toward the hydrophobic tails of a lipid bilayer, while the hydrophilic residues of the cell penetrating peptide (CPP) form the internal environment of the pore.
- **Toroidal pore model.** It occurs when the pores are formed by peptides that are associated with the polar groups of lipids inside the cell membrane. The hydrophilic core of the toroidal pore is lined with hydrophilic groups of the phospholipid cell membranes. For example, Melittin is a peptide that induces toroidal pore formation. [82]
- **Carpet-like model.** It happens when peptides are in parallel orientation to the membrane surface. Electrostatic interactions occur between anionic phospholipid groups and positively charged peptides. In contrast with other models, peptides are not internalized into the hydrophobic core and the lipid bilayer transforms into a micelle forming a transient hole. [83]
- **Inverted micelle model.** It is formed between two cell membrane bilayers, as a hexagonal structure in which the CPP is surrounded by the hydrophobic part of the membrane. Also, there is interaction between positively charged CPP and the negatively charged part of the

cell membrane. For instance, HIV-1 TAT peptide is effectively internalized through inverted micelle formation. [84]

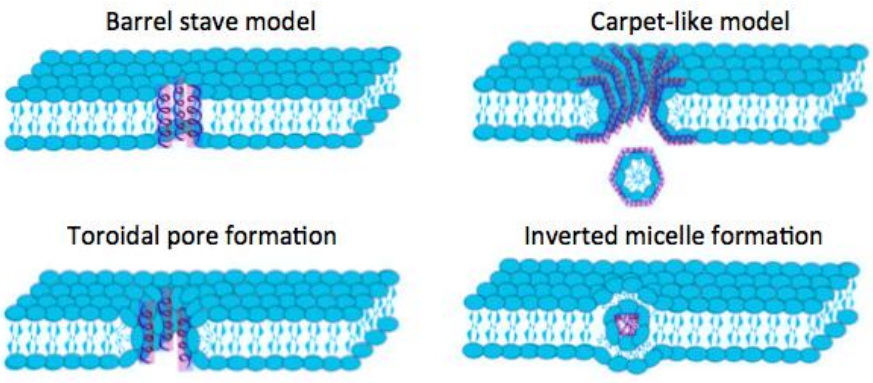

Figure 4. Models of direct penetration. Modified from [85]

Endocytosis

Endocytosis is an energy-dependent process that includes several pathways as phagocytosis when there is an uptake of large particles (diameter >500nm), and pinocytosis when there is internalization of the fluid that surrounds the cell. At the same time pinocytosis includes micropinocytosis, clathrin or caveolin-dependent/independent endocytosis and dynamin dependent/independent endocytosis. [86]

Caveolae, also known as caveolins or clathrin-coated vesicles, are cell membrane indentations formed by cholesterol binding proteins. Clathrin and caveolin are proteins present in the intracellular part of cell membrane that are involved in the invagination of the membrane and formation of vesicles coated by these proteins. Except for these endocytic pathways, clathrin and caveolae independent pathway also facilitate internalization of nanoscale systems, as shown in **Figure 5**.

Another endocytic mechanism is macropinocytosis that results in the formation of vesicles called macropinosomes, formed during the folding of the plasma membrane, which transform as lysosomes in the end. [87]

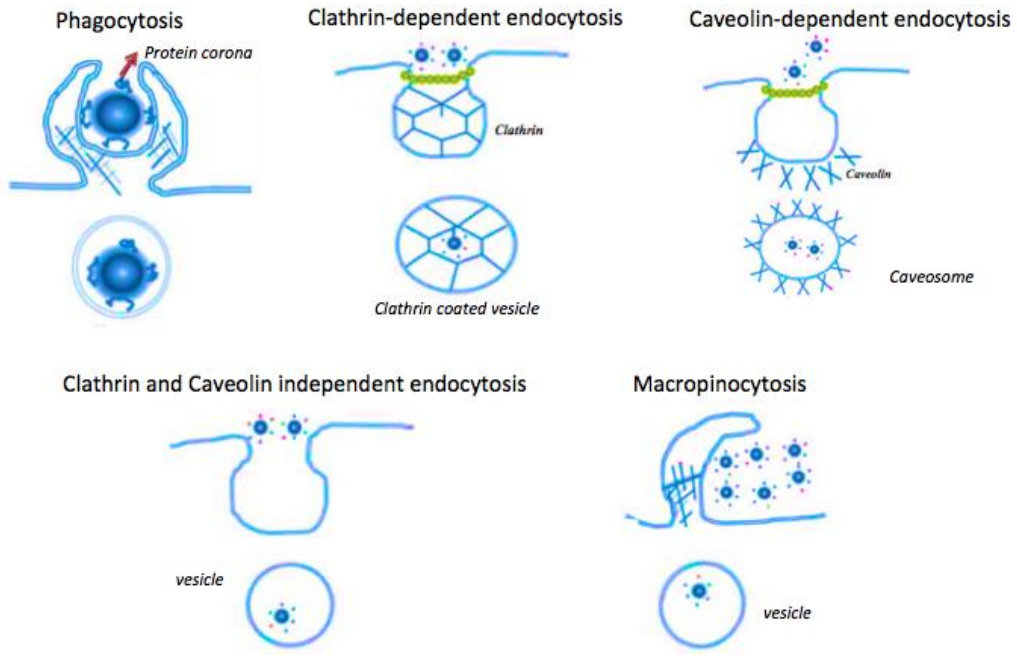

Figure 5. Cell uptake by endocytic pathways. Modified from [88]

Active Targeting

Unspecific drug exposure in the tumor area triggers systemic toxicities that are common when conventional cancer treatments are employed. For this reason, novel therapeutic options as engineered nanoparticles (NPs) that carry the drug selectively to the tumor tissue by containing an epitope recognition domain, are proposed[89]. Surface modified nanomedicines are thought to detain a circulating stem cells procedure known as metastasis. Furthermore, receptor-based active targeting nanoparticles represent an optimal-delivery strategy. Different targeting agents have been studied to

actively target receptors that are overexpressed in tumors, such as tumor specific antibodies, aptamers, peptides, proteins fragments or receptor ligands that can be successfully conjugated.

In this way, NPs can be *decorated* with different targeting groups that bind specifically to receptors located in the membrane of tumoral cells in order to enhance the particle uptake in the malignant cells, based in a ligand-receptor-mediated endocytosis. It is also possible to add targeting moieties that do not bind receptors located on the external membrane of the cells but recognize internal organelles, **Figure 6**. There are three levels of active targeting; tissular targeting, cellular targeting and intracellular or organelle targeting. Depending on the location of the receptor that the targeting ligand recognizes. [90]

Another option to overcome common physical barriers is double vectorization. The combination of tissular and cellular targeting in a unique nanocarrier must improve accumulation and the uptake in malignant cells[91]. Actually, applying a peptide that targets two or more receptors can be advantageous as well.

There are three main steps proposed as a strategy to improve the accumulation in tumor tissue[92];

1) Modification of physical conditions of tumor mass.

2) Selectively targeting of the payload towards tumoral stroma or vasculature tissue.

3) Firstly kill cancer cells that belong to the external shell of the tumor primary layers.

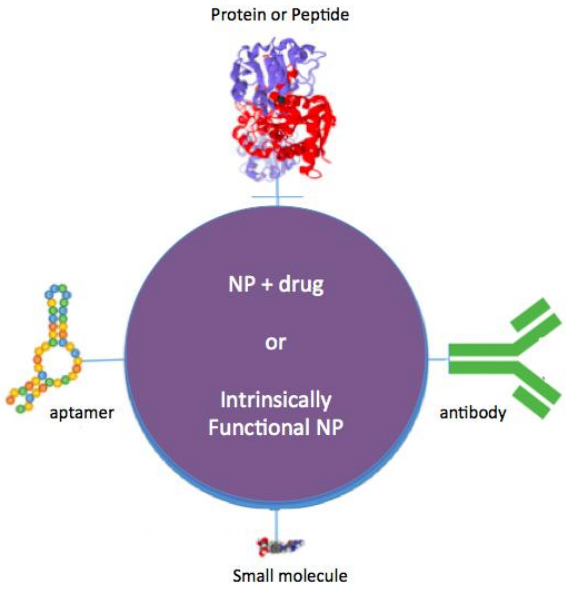

Figure 6. Nanotechnology applied in cancer treatment medicines. Adapted from [93]

Ligand based Targeting

A strong interaction between ligand-receptor may confer more specificity to the delivery system, thus minimizing non-specific interactions that could cause toxicity in peripheral tissues. The attached ligands actively take NPs to the tumor cells.

A screen study is firstly realized to find ideal ligands that can serve as targeting agents. Peptide based agents are a recent development with a wide range of essential functional groups. Bioactive peptides are short structured peptides, mostly derived from proteins and exhibiting essential biological properties[94]. Structure-function characteristics of bioactive peptides include anticancer characteristics. Structural features such as the presence of amino acids, net charge and chain length determine the potential peptide activity. A diversity of reports show that different peptides exhibit high target affinity. [95]

For example, the protein domains CXCL2, vCCL2, V1 (an amino terminal peptide of vCCL2) and the peptide T22 were studied to promote a receptor-mediated delivery to cancerous cells overexpressing CXCR4; finding T22 as the most efficient ligand[50]. Another example, chlorotoxin (CTX), with a compact and stable structure, binds preferentially to MMP-2 and annexin A2 presenting antiangiogenic activity for cancer treatment purposes; or even as an insecticide as another use.[96]

Antibody Targeting

Antibodies (Ab) were the firsts target-specific agents used to diagnose or treat cancer pathologies owing to their high selectivity and binding affinity[97]. The capability of the Ab employed to bind the target cells with a robust affinity in order to internalize the therapeutic component is determinant. Some targets include overexpressed proteins in specific cancer types such as the chemokine receptor 4 (CXCR4), Matrix metalloproteinase (MMP-2), annexin A2, human epidermal growth factor receptor 2 (HER2), or others. Targeting nanomedicines has attracted scientists' attention inclusively as immune-targeted strategy. In the same way theragnosis, simultaneous diagnosis and treatment is gaining relevance in nanotechnology.

Aptamers

DNA/RNA- based aptamers are small size synthetic oligonucleotides capable of binding a specific target. Small molecules, as folate, can be used to target different receptor overexpressed by tumors. Moreover, adverse effects like anemia or neuropathy were considerably reduced using these nanosytems. [93]

Types of Nanoparticles

When considering nanotechnology for therapeutic applications, it must be taken into count that the design must entail a biologically inert behavior, stability under physiological conditions, and a proper structure capable of allowing the nanosystem to move through the body and internalize in the target tissue. In addition, nanosystems must avoid undesired toxicity. A delicate balance between the properties and activities is required to construct a successful nanomedicine.

Therapeutic biomaterial platforms

Nanomedicine is traditionally defined as the technology that applies submicron sized molecular devices or nanoparticles ranging from 5 to 500nm in at least one dimension. Some examples are represented on **Figure 7**. Proteins, polymers, carbohydrates, quantum dots, carbon nanotubes, graphene, magnet, metal and mesoporous nanoparticles are examples of materials intended to be applied in the treatment of incurable current diseases, such as cancer[98]. Engineered functional systems are useful in biomedicine due to their molecular scale size and therapeutic behavior. Specifically, nanoparticles containing drugs have shown an important therapeutic action related to their saturation solubility, settling resistance and high surface interaction[99]. Nanoparticle size, shape and composition play an important role in the circulation time required to accomplish effectively the deliver, avoiding degradation and clearance. Different uptake efficiencies can be described depending on the nanoparticle complexion as large sizes are required to prevent leakages in blood capillaries, but in the same way, small sizes are suitable for escape from macrophages by the spleen or liver[100].

Biocompatible nanoparticles used as therapeutic carriers can improve, in theory, drug biodistribution, stability, targeting and reduce side effects. The truth is that only a fraction of the injected dose of a targeted nanoparticle reaches the tumor after intravenous administration.

By a design virtue, protein nanoparticles can combine a high level of functional sophistication leading to the formation of effective nanomedicines. Converting a drug into a nanosize material by crystallization, gives the opportunity for poor water solubility and dissolution rate compounds to have better effect biologically. A polymeric layer or other types of encapsulation can increase the particle stability, as well as, by introducing them in an aqueous dispersion[101]. The capability of functionalize any nanomaterial surface with different moieties, peptides or nucleic acids is very attractive. [102]

Liposomes are spherical vesicle nanoconstructs constituted by a bilayer structure of amphiphilic phospholipids compatible with both hydrophobic and hydrophilic drugs. Integrating a polymer layer in the surface has faced the disadvantages such as low stability, low encapsulation efficiency and a wide size distribution. [91]

Polymeric structures as nanoparticles, nanofibers, micelles, dendrimers or hydrogels offer good physical and chemical properties combined with good biological responses where drug encapsulation or covalent bindings are used to attach therapeutic agents to the polymeric construct. The principal polymers employed are polylactic acid, polyethylene glycol, polycaprolactone, collagen, gelatin, albumin, alginate and chitosan[103, 104]. Electrospun nanofibers have been used in tissue engineering and in periodontics as antimicrobial materials; nowadays its application as drug delivery systems

offers the advantages of a modifiable conformation, which could mimic biological entities. [105]

Finally, protein-based constructs have demonstrated to be highly active and effective as cancer therapeutic systems. Cationic peptides, when fused to His-tagged proteins, promote interactions that led to self-assemble protein-only nanoparticles. Full stability in vivo has been observed after systemic administration for drug delivery purposes. Chemical synthesis is an option to engineer these protein constructs. Moreover, obtaining recombinant proteins helps to overcome a low production scale rate and improves in a very significant way the biological compatibility and effective behavior. [106]

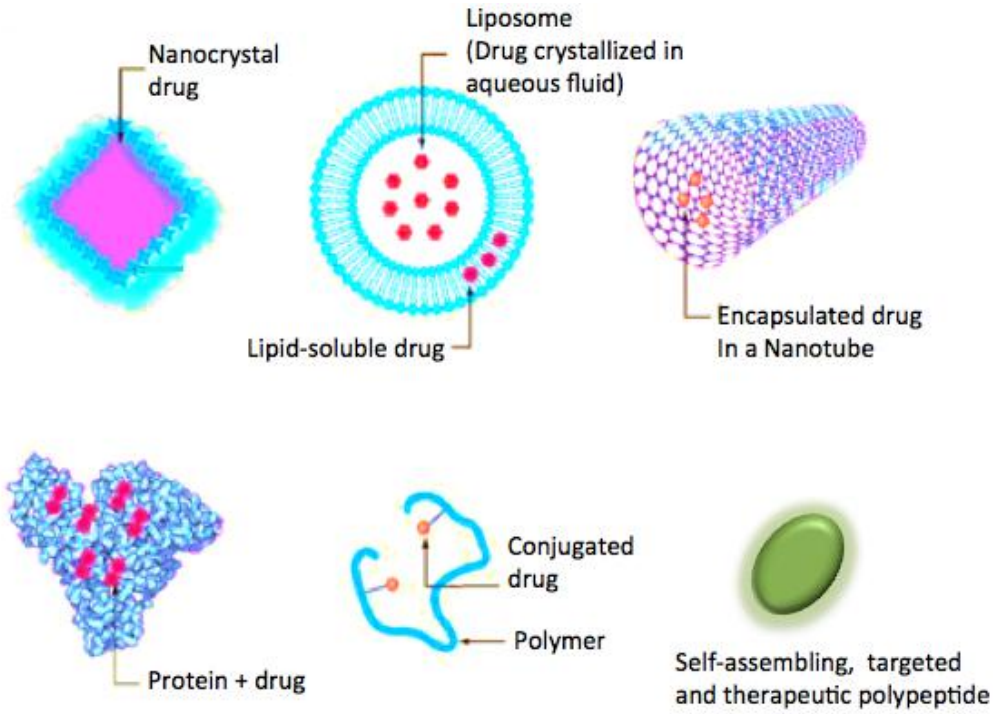

Figure 7. Nanotechnology based drug delivery platforms examples. Modified from[107, 108].

Protein-based nanoparticles offer one of the best morphometric and functional plasticity and versatility among all biomaterials. The good tumor

permeability and retention effect reduce systemic toxicity; due to the presence of targeting ligands evading in this way undesired side effects[109]. Moreover, drug biomaterials as vehicle-free nanomedicines with a good cell permeability and low renal clearance have been also designed to target specific cancer receptors and perform their cytotoxic activity after tumor internalization. [110]

Nanoparticle types

NPs can be broadly classified as organic or inorganic. Organic NPs can be subclassified in liposomes, polymeric micelles, polymeric NPs and dendrimers. Inorganic NPs include iron-oxide NPs, gold NPs, mesoporous silica NPs, carbon NPs and quantum dots. Moreover, many variables can be contemplated in a NP design, represented in general in **Figure 8**, such as the materials involved or composition, size, targeting and shape.

A new generation of NPs is achieved applying ***protein-based technologies*** for nanoscale drug design that offer functional and structural versatility allowing high selectivity in the delivery process through specific interaction with cell surface markers. Smart vehicles or selective drug carriers, in form of viral mimetics, done by the combination of functional peptides with cage-like supramolecular assemblies. [108]

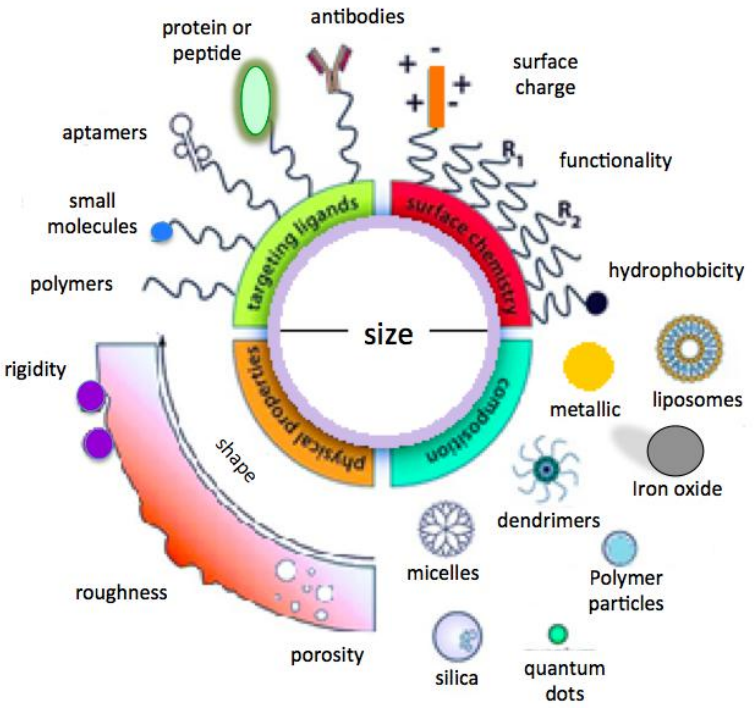

Figure 8. NP design options for intracellular and therapeutic applications. Adapted from [111]

Protein-based Nanoparticles

Protein nanoparticles are of paramount importance due to the advantages they show over other types of NPs. They are often biocompatible, biodegradable and non-toxic, they influence in the drug biodistribution decreasing side effects, and offer the possibility of diverse routs of administration (oral, nasal, intraocular, etc.). Moreover, the high drug loading capability when used as carriers and they metabolize easiness, close with a flourish.

The use of natural proteins depends on its bioavailability and its physicochemical stability, while synthetic or recombinant proteins are more versatile. The oligomerization of monomeric proteins led to the formation of self-assembled structures known as protein NPs. Protein building blocks are

mainly achieved by recombinant technologies, and this nanoparticle type is considered safe for biomedical applications [112]. Protein NPs diameters range between 20-100 nm, similar to virus sizes, and for this reason are known as virus-like particles (VLPs).

When recombinant proteins are produced, the C-terminus of the peptide chain (usually comprises six-histidines, His-tag) is useful in the isolation of the engineered peptide by affinity chromatography technique. Being functionally versatile, protein materials can perform complex activities when a selective cationic ligand is introduced at the N-terminus in a well-consolidated architectonic design. [113]

Protein-drug constructs

A robust strategy for delivering a potent cytotoxic compound to tumor cells improving the therapeutic efficacy of the agents is achieved applying protein-drug conjugates. They offer several strengths because they have a non-immunogenic nature, and low molecular weight agents confer good cell penetration in solid tumors. However, there are some shortcomings as the stability, protein-drug conjugation ratio and linker properties. Plasma stability has also been improved through linker modifications and protein engineering[114]. Typically, protein-drug conjugates are conformed by a target ligand, a spacer, a cleavable bridge and a therapeutic payload that is delivered after the tumor cell penetration.

The targeting ligand performs the functions of an antibody. When binding affinity and target selectivity are increased, the required dose to achieve high efficacy is reduced, thus toxicity is also minimized [115]. Size generally

influences drug delivery mechanisms as permeability, retention effects and excretion through the kidneys. When the cargo has a low molecular weight, it is easier to be released and diffused independently into tumors. However, when molecules are smaller than 40kDa they can be easily excreted from the body. [116]

Between the targeting ligand and the therapeutic payload there is a linker, which may contain a spacer and a cleavage bridge. The spacer maintains the connection between the construct but it can also increase the potency measured in terms of relative affinity [117]. Firsts-generation spacers were conformed by carbohydrate units, acidic residues or saccharoamino acid residues. Later, glutamic acid and glutamine were functionalized as epimerization-inert modules. More recently, valine-citrulline and valine-alanine spacers exhibited greater serum stability and superior therapeutic activity. Moreover, a good spacer design contributes in decreasing cytotoxicity, as the penetration of polar conjugates in cell membranes is more difficult without receptor-based endocytosis. [118]

Highly potent conjugates contain a cleavage bridge in their linker structure as it permits the release of the parent drug at the desired site after penetrating the target cell. The cleavage bridge must be stable enough to maintain its conformation during the transportation through the vasculature to the tumor. Effective triggering methods are mostly related to disulfide-based linkers as their reduction from oxidized counterparts by intracellular excesses of glutathione (GSH), thioredoxin, peroxiredoxins and nicotinamide adenine dinucleotides assure the cleavage in cancer cells. However, another triggering method is based on designing conjugates sensitive to pH changes making

use of acetals and hydrazones that can be hydrolyzed in the acidic environment of the endosomes. [119]

The active drug must collect some important criteria such as effective cytotoxicity, capacity of being released, minimum multidrug interactions and high binding affinity. After escaping from the encapsulating endosome successfully, drug molecules accumulate in cancer cells.

Successful examples in the market

Different cytotoxic therapeutic agents such as vinblastine, paclitaxel, mitomycin C, epothilone, tubulysin or campotethecin derivative SN-38 have been included in the design of protein-drug conjugates. As the active agent is determinant in the therapeutic construct performance, different protein-drug conjugations can be classified according the payload employed. [120]

Intrinsically Functional NPs

Previously, nanocarriers were applied to improve the specific drug delivery and the biodistribution. Other advantages include protection against degradation and aid in intracellular uptake. Nevertheless, the drug concentration at which cancer cells are treated is determined by the carrier-loading capacity. Last challenges led to the investigation of self-delivery systems like self-assembly proteins or small interfering RNA. Synergistic combination of therapies entails that the distinction between the active agent and the carrier is set aside [121]. The first models were conformed by the combination of different molecules resulting in a complex mixture of additional molecular systems to provide required functions; leading in very heterogenic constructs. Many protein-based compounds are approved by medicine agencies, as they are themselves therapeutically useful. [122]

Targeted self-assembling therapeutic protein NPs

When the nanoparticle is active because the material itself presents therapeutic properties, leakage is drastically diminished which entails fewer side effects related to toxicity. In addition, a nanoarchitectonic strategy based on a core protein with a cationic N-terminal domain plus a C-terminal tag results in a construct which charge distribution helps the self-assembling and oligomerization of proteins as toroid NPs stable in plasma. This kind of NPs is intrinsically functional therapeutic opportunities[110]. It was demonstrated that cytotoxic protein segments maintain their therapeutic potential when fused to carrier proteins even when they are oligomerized. [122]

Antimicrobials

Naturally occurring antimicrobial peptides (AMPs) are of special interest as the multi-resistant bacterial infections are a barrier for antibiotics efficacy. AMPs´ cell lytic activity is related to pore formation in the cell membrane induced by the selective peptide binding to negative charged cell surfaces and the subsequent membrane permeabilization [123]. Characteristics related to their low molecular mass such as low solubility, rapid metabolic excretion and limited stability increase the difficulty in applying AMPs in systemic therapies aside from topical formulations. GWH1 has very promising results when included in protein-only NPs and proved to perform high antimicrobial potential and antitumor activity both *in vitro* and in animal cancer models *in vivo*. For instance, GWH1 was incorporated to a recombinant only-protein self-assembling NP platform. [106]

Proapoptotic Proteins

The human origin of some proteins contributes to the suppression of an immunotoxic reaction. The apoptotic pathway of a cell, as an antitumor strategy, can be stimulated by extracellular proapoptotic proteins, and also by the BLC-2 intracellular protein family. Proapoptotic proteins are classified into BH3-only proteins (PUMA, BIM, BID; pore-forming proteins for instance) and into multidomain proteins (BAX, BAK). [124]

Toxins and Venoms

Engineered constructs with natural protein toxins and venoms have become promising anticancer nanomedicines. Toxins are very potent molecules that can proceed from plants, amphibians or microorganisms. Plant toxins can be ribosome-inactivating proteins (RIPs) capable of depurating a specific adenine residue in the 23S/25S/28S rRNA stem-loop irreversibly, as they are N-glycosidases, blocking protein translation and leading the cell to death. Some important RIPs are ricin, saporin, abrin, trichosanthin, bouganin, and gelonin. Animal Venoms are a mixture of complex and highly bioactive proteins and peptides with different pharmacological effects when used for anticancer therapeutics. Among its actions, venom proteins can alter normal cell-cycles, induce apoptosis and necrosis, inhibit cell growth, depolarizate the cell membrane or even disrupt the cell. [124]

Ricin, in **Figure 9**, is a very potent molecule as it can inactivate 1500–2000 ribosomes per minute that was originally extracted from the *Ricinus communis* plant. It is a RIP type II of ≈65 kDa protein with two chains linked by a disulfide bond; chain A (RTA) which has the N-glycosidase activity, and chain

B (RTB) with lectin properties targeting cell surfaces[125]. However, just 5% of the protein internalized by endocytosis achieves to be translocated by retrograde transport to the trans-Golgi network, backward through the Golgi apparatus to the endoplasmic reticulum and finally to the cytosol. Most of the protein cell-internalized is degraded or ejected out by lysosome transportation.

RTB has the ability to bind a wide diversity of cell types, making ricin dangerous, as it can target unspecific cell types. The function of both chains is represented on **Figure 10**. Despite some studies have been done with ricin for HIV therapy agents, most investigations are focused on RTA only, by means of two approaches; as a vaccine for an anti-ricin protection by the activation of antibodies, and as an anticancer therapeutic agent. [126]

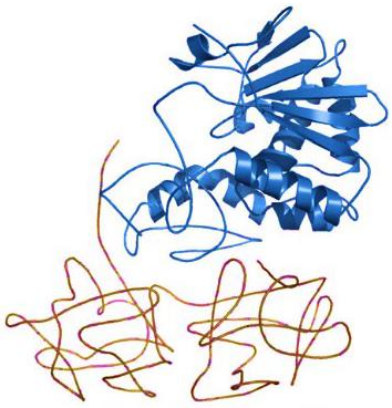

Figure 9. Crystal structure of ricin holotoxin. *RTA colored in blue and RTB colored in orange.* Adapted from [127]

The U.S. government has shown interest in the development of anti-ricin vaccines, as the toxin can be a possible bioterrorism tool[128]. Ricin is classified as a category B select agent by the Center for Disease Control and Prevention (CDC)[127]. Rivax™ is Soligenix's proprietary vaccine developed in *E. coli* as a recombinant RTA mutated firstly at two points: V76M and Y80A in order to

eliminate the vascular leak syndrome (VLS) [129]. RVEc with the C-terminal domain deleted is a derivative of the (RTA 1-33/44-198). It provided protection against poisoning in mice and showed to be relatively stable to thermal denaturation.[130]

When RTA is directed against carcinogenic cells, it is important to have a cell-selective and a specific-directed biological behavior. A frequent limitation observed in clinical trials is related to immunogenic responses that diminish the effect of the NPs in which ricin is involved after continuous dosages. Also the VLS presence has been overcame through specific mutations in RTA that have proved better results [131]. Some encouraging research in nanoconstructs employing ricin is explained below.

Combotox is a 1:1 mixture of two monoclonal antibodies (MAbs) linked to deglycosylated RTA (HD37-dgRTA and RFB4-dgRTA) directed against CD19 and CD22 [132]. Clinical trials phase II and I were evaluated where the maximum tolerated dose was 5 mg/m^2 due to the damage produced to the vascular endothelial cells by ricin (VLS). Despite this, three young patients achieved complete remission while six of them presented a decrease of more than 95% in their peripheral blood blast counts.

RTA has also being conjugated to the Pokeweed antiviral protein (PAP), other plant toxin, and expressed by recombinant methods in *E.coli* in two different orders: Ricin-A-Chain/PAP-S1 and PAP-S1/Ricin-A-Chain. The first arrangement was more active that the second one, but similar to PAP-S1 in a cell free prokaryotic environment. Moreover, both arrangements showed much stronger activity in a cell free eukaryotic environment than PAP-S1 by itself. [133]

Now, It is known that RTB is useful not only to attach RTA to cells surfaces but also helps it to get into the cytosol. When the holotoxin is together, the toxicity is greater than when the active chain is by itself. Nevertheless, RTB presence makes immunotoxins behavior very unspecific causing damage in healthy tissues. A construct of an adenovirus conjugate of Green fluorescence protein (GFP) and RTB was studied. The plasmid pAdCMV-RTB was transformed into the *E. Coli* strain BJ5183 that harbored a plasmid with an adenovirus genome, pAdEasy-1. This resulted in the production of pAdEasyCMV-RTB, containing a kanamycin resistant gene inserted within the adenovirus genome. This last plasmid was extracted and used to transfect HEK293 cells, whose efficiency could be monitored due to the GFP fluorescence. By the other hand RTA was expressed in *E. coli* as a 6xHis-tagged fusión protein. The cytotoxicity studies confirmed that RTA or AdGFP-RTB by themselves were inactive in HEK293, HeLa, SMMC7721, and HL7702 cells. Nonetheless, when RTA was put in touch with AdGFP-RTB-infected cells, a cell mortality of 60% was achieved. [134]

In the same way, a very interesting toxin combination was performed using the hepatitis C virus (HCV) to design two NS3 protease-activated zymogenized chimeric toxins. Diphtheria toxin A (DTA) or RTA, respectively, were combined to HCV NS3 protease-cleavable linker, and then fused to the binding and translocation domain of the exotoxin A. Zymoxins therapeutic behavior in HCV infected cells was inferior compared to the high cytotoxicity activity shown when applied to cells expressing NS3. Thus, acute infections with abundant presence of activating protease can be possibly treated with this conjugates. [136]

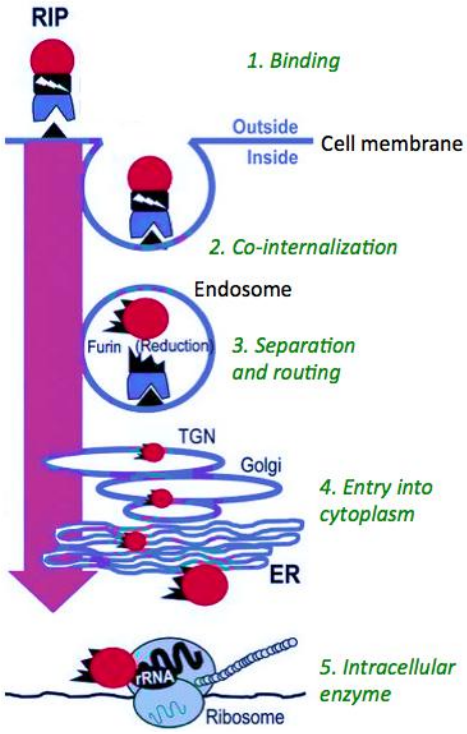

Figure 10. **RIPs pathway against carcinogenic cells.** *1) RIP binds to cell surface antigen and accumulate on target cells. 2) The internalization is generally mediated by the bound of target antigen. 3) Once inside the cell, toxins should be released from their targeting moieties to avoid degradation and perform its function. 4) The intracellular route is by reaching the endoplasmic reticulum (ER) through the trans-Golgi network (TGN). 5) The inhibition of protein synthesis is achieved by cleaving ribosomal rRNA.* Modified from [135]

Chlorotoxin (CTX) is a 4kDa neurotoxin derived from the Israeli yellow scorpion's venom, *Leiurus quinquestriatus,* conformed by 36 amino acids. It has shown advantageous properties such as preferential affinity to bind certain cancer cells, high cell penetration and good stability. CTX is a promising candidate for differentiating between molecular profiles and radiologic features of GBM, and serves as a platform for developing novel noninvasive techniques[137]. Molecular targets for CTX encompass voltage gated chloride channels, the calcium-dependent phospholipid-binding

protein Annexin-2 A2, and the inducible extracellular enzyme matrix metalloproteinase (MMP)-2. Because CTX blocks specifically and efficiently the chloride channel activity, cancer cells tend to stop growing by this inhibition[138]. Consequently, CTX is considered a therapeutic agent, but not toxic enough to eliminate carcinogenic cells by itself[139]. CTX offers some structural advantages including; four disulfide linkages, shown in **Figure 11**, that impart a stable tertiary structure and a tyrosine residue that can be conjugated for imaging purposes. But the most important characteristic is that it amazingly binds to brain tumor cells with zero interaction with normal brain.

Chloride channels are almost absent in healthy tissue or in non-glial tumors, which makes CTX ideal for glioma treatment.

And if that was not enough it selectively and specifically acts on MMP-2, protein associated with the enzymatic degradation of the ECM. As CTX can decrease the surface expression of MMP-2 and also inhibit its activity, thereby it reduces the overall invasion ability of glioma cells through compact extracellular spaces in normal brain tissue. MMP-2, a zinc-dependent endopeptidase, is member of an enzyme family that has a tissue allostasis maintenance role[140, 141].

Annexin-2 (A2), overexpressed at the cell surface in GBM, is one CTX´s molecular target[64]. The annexin family is a group of calcium-dependent phospholipid-binding proteins with a plasma membrane reparation function and they also carry out the vesicle fusion and trafficking[142]. A2 has many roles in cellular functions such as angiogenesis, apoptosis, cell migration, proliferation, invasion and cohesion.

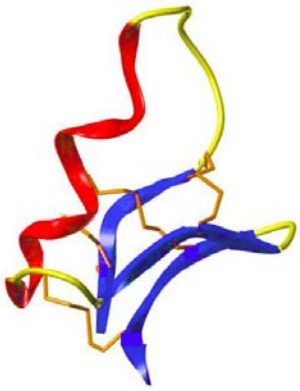

Figure 11. Structure representation of CTX. *The disulfide bridges are colored in orange.* Adapted from[143]

Different agents constructed employing CTX are successful nowadays. Some examples are mentioned next.

^{131}I-TM601 Synthetic chlorotoxin covalently attached to the radioisotope iodine-131 is in clinical trials phase I and II. It was found to bind the surfaces of Panc-1 cells depending on the A2 expression. [144]

Also, chlorotoxin Cy5.5 bioconjugate for intraoperative visualization of cancer foci (Tumor Paint) was approved by the FDA. It was studied in 9L cell line, rat glioblastoma, where its specificity was demonstrated by the previous addition of the MMP2 inhibitor, 1,10-phenanthroline (50μM) in order to block the receptor entry. [14]

Objectives

General Objectives

- Design, production and purification of effective recombinant self-assembling protein-only nanoparticles (NPs) based on the toxin ricin A chain (RTA) and the venom peptide chlorotoxin (CTX), capable of being architectonically stable and functional.
- Take advantage of the intrinsic properties of RTA to direct NPs specifically to acute myeloid leukemia cells through the effective peptide T22 and evaluate its activity in $CXCR4^+$ cells *in vitro* and *in vivo*.
- Evaluate the application of CTX as a targeting carrier system for glioblastoma therapy by its integration to the building blocks of the protein-engineering platform.

Results

Article 1

Selective CXCR4^{+} Cancer Cell Targeting and Potent Antineoplastic Effect by a Nanostructured Version of Recombinant Ricin

***Raquel Díaz**, Victor Pallarès, Olivia Cano-Garrido, Naroa Serna, Laura Sánchez-García, Aïda Falgàs, Mireia Pesarrodona, Ugutz Unzueta, Alejandro Sánchez-Chardi, Julieta M. Sánchez, Isolda Casanova, Esther Vázquez, Ramón Mangues, and Antonio Villaverde.*

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An acute myeloid leukemia (AML) successful treatment requires novel drugs capable of targeting leukemia stem cells that are those that regenerate the aberrant expression of undifferentiated myeloid blasts. [146]

Combining the outstanding protein-only self-assembling nanoparticle platform where the effective peptide T22 that binds CXCR4 receptor has been deeply studied, with the intrinsic cytotoxic characteristics of an improved ricin A chain, a potent 11 nm soluble nanomedicine named T22-mRTA-H6 was obtained by recombinant methods. When the cytotoxicity of this novel construct was evaluated in CXCR4+ cells, an IC50 of 13 ± 0.5 x 10^{-9} M was determined; and when applied in AML animal models, it showed a forceful antitumor activity without side toxic effects.

The last behavior was compared to the same compound in its insoluble version where the protein effect was limited by a moderate liberation of the nanostructured protein contained inside the inclusion bodies. Anyhow, self-assembled, self-targeted vehicle-free recombinant modified ricin was achieved to be engineered and effective in difficult-to-treat AML disease.

FULL PAPER
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Selective CXCR4+ Cancer Cell Targeting and Potent Antineoplastic Effect by a Nanostructured Version of Recombinant Ricin

Raquel Díaz, Victor Pallarès, Olivia Cano-Garrido, Naroa Serna, Laura Sánchez-García, Aïda Falgàs, Mireia Pesarrodona, Ugutz Unzueta, Alejandro Sánchez-Chardi, Julieta M. Sánchez, Isolda Casanova, Esther Vázquez, Ramón Mangues, and Antonio Villaverde**

Under the unmet need of efficient tumor-targeting drugs for oncology, a recombinant version of the plant toxin ricin (the modular protein T22-mRTA-H6) is engineered to self-assemble as protein-only, CXCR4-targeted nanoparticles. The soluble version of the construct self-organizes as regular 11 nm planar entities that are highly cytotoxic in cultured CXCR4+ cancer cells upon short time exposure, with a determined IC50 in the nanomolar order of magnitude. The chemical inhibition of CXCR4 binding sites in exposed cells results in a dramatic reduction of the cytotoxic potency, proving the receptor-dependent mechanism of cytotoxicity. The insoluble version of T22-mRTA-H6 is, contrarily, moderately active, indicating that free, nanostructured protein is the optimal drug form. In animal models of acute myeloid leukemia, T22-mRTA-H6 nanoparticles show an impressive and highly selective therapeutic effect, dramatically reducing the leukemia cells affectation of clinically relevant organs. Functionalized T22-mRTA-H6 nanoparticles are then promising prototypes of chemically homogeneous, highly potent antitumor nanostructured toxins for precise oncotherapies based on self-mediated intracellular drug delivery.

1. Introduction

Cancer is a major, growing, and unsolved health problem worldwide, with an incidence of 454.8 new cases per 100 000 (men and women) per year, and a mortality of 207.9 per 100 000 men and 145.4 per 100 000 women (U.S. data; https://www.cancer.gov/about-cancer/understanding/statistics). Only in 2018, 1 735 350 new cancer cases and 609 640 cancer deaths are projected to occur in the United States.[1] Conventional cancer treatments continue to be based on potent small molecular weight chemicals administered systemically. Since these drugs are not targeted to cancer cells they do not preferentially accumulate in tumor or metastasis. Biodistributed across healthy tissues, they promote severe hepatic and renal damage that often results in numerous life-threatening side effects.[2] In the line

R. Díaz, Dr. O. Cano-Garrido, N. Serna, L. Sánchez-García, Dr. M. Pesarrodona, Dr. J. M. Sánchez, Dr. E. Vázquez, Prof. A. Villaverde
Institut de Biotecnologia i de Biomedicina
Universitat Autònoma de Barcelona
Bellaterra, 08193 Barcelona, Spain
E-mail: Antoni.Villaverde@uab.cat

R. Díaz, Dr. O. Cano-Garrido, N. Serna, L. Sánchez-García, Dr. M. Pesarrodona, Dr. E. Vázquez, Prof. A. Villaverde
Departament de Genètica i de Microbiologia
Universitat Autònoma de Barcelona
Bellaterra, 08193 Barcelona, Spain

R. Díaz, Dr. O. Cano-Garrido, N. Serna, L. Sánchez-García, A. Falgàs, Dr. M. Pesarrodona, Dr. U. Unzueta, Dr. I. Casanova, Dr. E. Vázquez, Prof. R. Mangues, Prof. A. Villaverde
CIBER de Bioingeniería
Biomateriales y Nanomedicina (CIBER-BBN)
Bellaterra, 08193 Barcelona, Spain
E-mail: ICasanova@santpau.cat

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/smll.201800665.

Dr. V. Pallarès, A. Falgàs, Dr. U. Unzueta, Dr. I. Casanova, Prof. R. Mangues
Biomedical Research Institute Sant Pau (IIB-Sant Pau) and Josep Carreras Research Institute
Hospital de la Santa Creu i Sant Pau
08025 Barcelona, Spain

Dr. A. Sánchez-Chardi
Servei de Microscòpia
Universitat Autònoma de Barcelona
Bellaterra, 08193 Barcelona, Spain

Dr. J. M. Sánchez
Instituto de Investigaciones Biológicas y Tecnológicas (IIBYT) (CONICET-Universidad Nacional de Córdoba), ICTA and Cátedra de Química Biológica
Departamento de Química
FCEFyN, UNC, Av. Velez Sarsfield 1611, X 5016GCA Córdoba, Argentina

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with the development of new and improved drugs, drug nanoconjugates, therapeutic antibodies, antibody–drug conjugates, tumor-targeted nanoscale vehicles, and tumor-targeted toxins (such as immunotoxins) are being designed to gain specificity and potency, with still limited therapeutic improvement.[3] The nanoscale size of the drug, potentially reachable by coupling to a vehicle, minimizes renal clearance and favors the enhanced permeability and retention (EPR) effect.[4] Among the set of tested new drugs, protein toxins emerge as a very appealing alternative.[3] Proteins are biocompatible macromolecules, easily produced by recombinant DNA technologies, and more than 400 protein species have been already approved for use in humans.[5] As versatile molecules, they are suitable for fine tuning through protein fusion technologies, to incorporate relevant functions for use as targeted drugs (such as ligands to specific cell surface tumoral markers).[6] Engineered versions of natural protein toxins have become promising antitumor agents. The *Corynebacterium diphtheriae* toxin fused to interleukin-2 (Denileukin diftitox, ONTAK) is an FDA-approved drug that targets leukemia and lymphoma cell types that display IL-2 receptors.[7] The exotoxin A from *Pseudomonas aeruginosa* has also been produced through recombinant methodologies in different versions (SS1P, LMB-2, or BL22), which are under clinical trials for the treatment of mesothelioma and leukemia.[8,9]

Compared to microbial toxins, plant toxins are extremely potent molecules.[3,10,11] Many of them (such as ricin, saporin, abrin, trichosanthin, bouganin, and gelonin) are ribosome inactivating proteins (RIPs). Being N-glycosidases, they irreversibly depurinate a single adenine residue in the 23S/25S/28S rRNA stem-loop. This action blocks protein translation and leads to fast cell death. Ricin, a RIP originally extracted from the seeds of *Ricinus communis* of ≈65 kDa, consists of two chains linked by a disulfide bond; the chain A (RTA) with N-glycosidase enzymatic activity and the chain B (RTB) with lectin properties which binds carbohydrate ligands on target cell surface.[12] A single ricin molecule is estimated to inactivate 1500–2000 ribosomes per minute,[13] being very promising as highly active cytotoxic protein drug. We have previously identified the peptide T22, an efficient ligand of the cell surface marker CXCR4 (a cytokine receptor selectively overexpressed in metastatic cells of many cancer types[14–19]), as a targeting agent for the precise tumor delivery of protein-only self-assembling nanoparticles.[20,21] Some of these constructs have been built by the controlled oligomerization of proteins with cytotoxic activity, such as pro-apoptotic factors,[22] anticancer peptides,[22] and microbial toxins.[23] In this context, we intended to confer $CXCR4^+$ cell-targeted delivery of ricin assembled as protein nanoparticles to determine their selectivity in cell internalization and their performance as cytotoxic drugs. This has been done through in vivo administration of either soluble CXCR4-targeted protein nanoparticles formed by ricin as building blocks or to particular protein-releasing amyloidal aggregates formed by CXCR4-targeted ricin, named bacterial inclusion bodies (IBs),[24] that might represent a steady source of functional protein for advanced therapies.[25–27]

2. Results

The recombinant T22-mRTA-H6 (**Figure 1**A) was successfully produced in *Escherichia coli* Origami B, purified by His-based one-step affinity chromatography and detected as a single protein species with the expected molecular mass of 35.91 kDa (Figure 1B) that was fully confirmed by mass spectrometry (not shown). The pure protein was straightforward observed by both, dynamic light scattering (DLS) and field emission scanning electron microscopy (FESEM), as ≈11 nm entities occurring in the storage buffer without further treatment (Figure 1C,D), indicating the spontaneous formation of self-assembled nanoparticles. This was the expected outcome as the combination of cationic peptides at the amino terminus and polyhistidines at the carboxy terminus has been proved to be optimal to promote protein oligomerization as regular nanostructures,[28] irrespective of the core protein segment (ricin, in the case of T22-mRTA-H6, Figure 1A). Treating the material with SDS resulted in monomers of 5.5 nm (Figure 1C), which represented the probable building blocks of the nanoparticles. In the related self-assembling protein T22-GFP-H6, in which the sizes of the building block and the assembled version are both equivalent to those of T22-mRTA-H6, the use of small-angle X-ray scattering and other sophisticated analytical methods[29] as well as in silico modeling[30] have revealed that the nanoparticle was formed by ≈10 monomers. Being estimative, this figure also fits to T22-mRTA-H6. The analysis of T22-mRTA-H6 nanoparticles by circular dichroism (CD) revealed a structural composition in which α-helix predominates (29.2%, Figure 1E). However, a Thioflavin T (Th T) assay has also revealed the occurrence of intermolecular β-sheet interactions (Figure 1F) that might contribute to the stability of protein nanoparticles, and that is also compatible with the extent of important β-sheet structure found in the CD (Figure 1E). Since the nanostructured ricin was intended to be delivered in tumoral tissues, we wondered if the nanoparticles could still be stable in the abnormal pH values observed in the tumor environment that have been reported to range from ≈6.3 (intracellular) to 7.4 (extracellular).[31,32] As observed, T22-mRTA-H6 remained fully assembled under these conditions (Figure 1F), what supports the usability of construct from the stability point of view.

In order to test the functionality of the recombinant ricin in such assembled form, cultured $CXCR4^+$ HeLa cells were exposed to different concentrations of ricin-based nanoparticles. These materials showed a potent, dose-dependent cytotoxicity that essentially abolished cell viability at 100×10^{-9} M (**Figure 2**A). After 72 h of exposure, the IC50 was determined to be $13 \pm 0.5 \times 10^{-9}$ M. To confirm if, as expected, T22-mRTA-H6-mediated cell death was dependent on its cell binding and internalization of the protein via the cell surface receptor CXCR4 and its ligand T22, we tested if a potent CXCR4 antagonist, AMD3100,[33] could be able to recover cell viability when used as a competitor of the toxin, at a molar ratio of 10:1. As observed (Figure 2B), AMD3100 dramatically enhanced cell viability in T22-mRTA-H6-treated cells proving a specific, receptor-mediated penetration of the nanoparticles into target cells. To further confirm such precision cell entry mechanism, we decided to expose nontumoral ($CXCR4^-$) 3T3 cells and representative $CXCR4^-$ and $CXCR4^+$ tumoral cell lines to T22-mRTA-H6, and also to a conventional chemical drug used in the treatment of several cancer types but specially of acute myeloid leukemia (AML), namely, cytosine arabinoside

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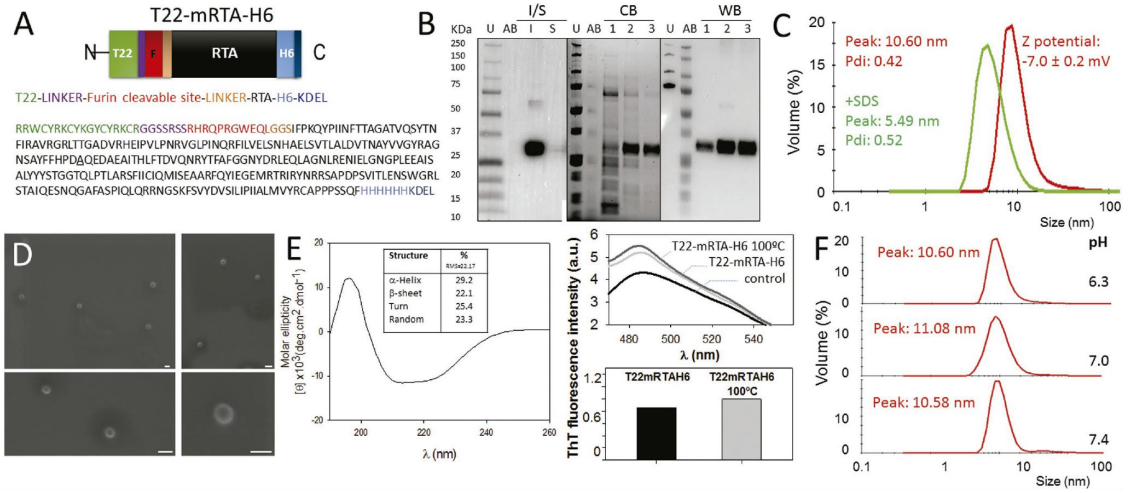

Figure 1. Physicochemical properties of T22-mRTA-H6. A) Modular scheme and amino acid sequence of T22-mRTA-H6. mRTA is the modified fragment A of ricin, described in the Experimental Section, in which the Asn residue 132 has been replaced by Ala (underlined). Sizes of the boxes are only indicative. B) Fractioning between insoluble (I) and soluble (S) cell fractions in total cell extracts, revealed by WB, upon protein production at 37 °C for 3 h. SDS-PAGE analysis of T22-mRTA-H6 upon one-step affinity purification, revealed by Coomassie blue (CB) staining and by Western blot (WB) using an anti-his antibody. U and AB stand for Unstained and All Blue markers, respectively (Bio-Rad, Refs. 161-0363 and 161-0373), and 1, 2, and 3 indicate, respectively, the unspecific elution peak and two peaks with increasing level of purity. Protein in peak 3 was used in further experiments. C) Hydrodynamic size (and Z potential) of T22-mRTA-H6 nanoparticles formed spontaneously upon purification (red line), determined by DLS. Pdi is polydispersion index, and all figures indicate nm. The size of the monomer, determined upon disassembling the material with 1% SDS for 40 min, is also indicated (green line). D) FESEM imaging, at different magnifications, of T22-mRTA-H6 nanoparticles. Bars represent 20 nm. E) Far UV CD of T22-mRTA-H6 in sodium carbonate-bicarbonate buffer at pH 8 measured at 25 °C. In the middle plot, ThT fluorescence emission spectra alone (black line) or in the presence of T22-mRTA-H6 (light grey line) and T22-mRTA-H6 previously heated at 100 °C (dark gray line). λ_{ex} = 450 nm. In the plot at the bottom, ThT fluorescence emission at 490 nm of T22-mRTA-H6 (black bar) and T22-mRTA-H6 previously heated at 100 °C (gray bars). F) Size of T22-mRTA-H6 nanoparticles dialyzed against 51×10^{-3} M sodium phosphate, 158.6×10^{-3} M trehalose dehydrate, 0.01% polysorbate-20 buffer at different pH values, determined by DLS.

(Ara-C).[34] These cell lines, with different levels of CXCR4 expression (Figure 2C), supported different levels of protein internalization mediated by the specific interaction between T22 and CXCR4 (Figure 2D). This was determined through the uptake of T22-GFP-H6, a self-assembling fluorescent protein closely related to T22-mRTA-H6 that contains the same ligand of CXCR4 also accommodated at the amino terminus of the polypeptide.[28] It must be noted that as predicted, CXCR4 expression and T22-mediated protein internalization showed a parallel behavior (compare Figure 2C,D). Then, when they were finally comparatively tested, the ricin-based protein nanoparticle promoted specific cell death only in $CXCR4^+$ cancer cells but not in normal cells, at a dose (100×10^{-9} M) at which Ara-C did not show any toxic effect on any of these cell lines (Figure 2E). This observation proved not only the effective targeting of the protein drug but also its superior cytotoxicity compared to an equimolar dose of the model chemical drug.

At this stage, we wanted to confirm that the cytotoxicity promoted by T22-mRTA-H6 was linked to the uptake of the nanoparticles inside $CXCR4^+$ cells, and triggered from within. This was reached by exposing HeLa cells to ATTO-labeled nanoparticles and monitoring internalization. As observed (**Figure 3**A), nanoparticles were internalized by cells at least up to 24 h. As expected for an active version of ricin, apoptosis was detected though both annexin affinity assay and by Hoechst staining (Figure 3B), and the number of apoptotic cells seemed to peak at around 15–24 h postexposure. In addition, mitochondrial damage was confirmed by the significant increase in the number of cells with lowered JC-1 red fluorescence at 15 and 24 h after treatment with T22-mRTA-H6 (Figure 3C), indicative of a depolarization in the mitochondrial $\Delta\Psi$ linked to apoptotic induction. Interestingly, cell damage occurred without a detectable increase in reactive oxygen species (ROS, Figure 3D), while the formation of apoptotic bodies in ricin-exposed HeLa cells was clearly caspase-dependent (Figure 3E). The combination of these data indicates that T22-mRTA-H6-mediated cell death occurs by a classical caspase-dependent apoptosis pathway.

The suitable cell-targeting of the nanostructured version of ricin conferred by the peptide T22 (Figure 2), and the fact that most of the T22-mRTA-H6 protein was obtained in insoluble form (Figure 1B), prompted us to evaluate if the insoluble version of ricin might also exhibit cell-targeted cytotoxicity. In this context, we have recently described how the presence of T22 and other cell ligands, in recombinant proteins that form bacterial IBs,[27] allow an efficient and specific cell penetration of the whole protein clusters. In the same conceptual line, bacterial IBs formed by self-assembling proteins might contain quasi-native forms of nanoparticles or assembling precursors.[36] IB proteins retain functionalities of the soluble protein version and can be gradually released from the

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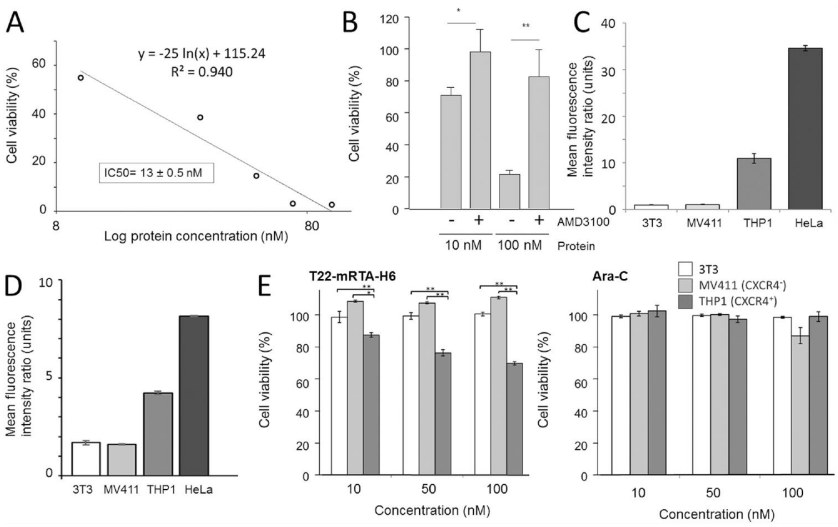

Figure 2. Cytotoxicity and CXCR4 specificity of T22-mRTA-H6 nanoparticles. A) Viability of cultured CXCR4$^+$ HeLa cells upon 72 h of exposure to T22-mRTA-H6 nanoparticles at different concentrations, presented as a dose–response curve. B) Inhibition of cell death in HeLa cells exposed to different concentrations of T22-mRTA-H6 nanoparticles for 72 h, mediated by the CXCR4 antagonist AMD3100 (always at an excess molar ratio of 10:1). C) Levels of CXCR4 membrane protein determined by flow cytometry of different cell lines (3T3, MV411, THP1 and HeLa), expressed as mean fluorescence intensity ratio ± SE. D) Extent of internalization of 100×10^{-9} M T22-GFP-H6 in the different cell lines at 1 h of exposure. Results are expressed as mean fluorescence intensity ratio ± SE. E) Viability of cultured CXCR4$^-$ 3T3 cells upon 48 h of exposure to T22-mRTA-H6 nanoparticles and the small molecular weight antitumoral drug Ara-C, at different concentrations. The commercial CXCR4$^-$ and CXCR4$^+$ human AML cell lines (MV411 and THP1, respectively) are included as controls. Ara-C showed cytotoxicity above 100×10^{-9} M (not shown). The standard error is represented in all bars. The level of significance is indicated by superscripts ($^*p < 0.05$, $^{**}p < 0.01$).

aggregates when exposed to cells[26] or when implanted in vivo by local injection.[25] The ultrastructural morphometry of insoluble version of T22-mRTA-H6 was observed in a nearly native state by FESEM as conventional IBs, namely, pseudo-spherical protein clusters with an average diameter size ranging from 400 to 600 nm (**Figure 4**A).

When exposing HeLa cells to increasing amounts of T22-mRTA-H6 IBs, a mild cytotoxic effect was indeed observed (Figure 4B), although the differences in cell viability, when comparing with untreated cell cultures, were in the limits of significance. In addition, the insoluble version of T22-GFP-H6 (forming similar IBs[27]), a self-assembling CXCR4-targeted protein devoid of any cytotoxic domain, also promoted a transient and mild reduction of cell viability. However, in this case, cells showed an immediate recovery at longer time exposures that, in contrast, was not found associated to T22-mRTA-H6. Despite previous data about the potential of functional protein release from IBs,[25] the biological effect of T22-mRTA-H6 IBs was, in our hands, only moderate.

The antitumor effect of both T22-mRTA-H6 soluble nanoparticles and T22-mRTA-H6 IBs was evaluated in a disseminated AML animal model. NSG mice were injected with THP1-Luci cells to generate leukemia dissemination in mice. 2 d after cell injection through the vein tail, we performed a single-dose injection in the mice hypodermis (SC) of 1 mg of T22-mRTA-H6 IBs in two mice (IB-T22mRTA group). In a different mouse group, we started daily intravenous administrations of 10 µg of soluble T22-mRTA-H6 (T22mRTA group) to one mouse or buffer alone (VEHICLE group) to three mice, for a total of ten doses. No effects on mice weight were observed during the treatments (data not shown). The progression and dissemination of leukemia was assessed by monitoring BLI using the IVIS Spectrum. From day 6 and until the end of the experiment, the mouse treated with soluble T22-mRTA-H6 (T22mRTA) showed lower luminescence emission than the VEHICLE group (**Figure 5**A). Thus, as measured by BLI, treatment with soluble T22-mRTA-H6 inhibited the dissemination of AML cells in mice, compared to the vehicle group, after the fourth, sixth, eighth, and tenth doses of T22-mRTA-H6 at 10 µg per dose (which corresponded to day 6, 8, 10, or 13 after injection of cells, respectively). In contrast, no differences in BLI were found between mice treated with T22-mRTA-H6 IBs (IB-T22mRTA) and the control VEHICLE mice (Figure 5A).

In a next step, the antitumor activity of nanoparticles was analyzed in affected organs ex vivo 14 d after the injection of cells when mice presented signs of advanced disease. The analyses with the IVIS Spectrum showed that the treatment with soluble T22-mRTA-H6 nanoparticles (T22mRTA) decreased BLI in the bone marrow (backbone and hindlimbs), liver, and spleen, in contrast to the findings in mice treated with buffer alone (VEHICLE) (Figure 5B). However, the treatment with T22-mRTA-H6 IBs (IB-T22mRTA) did not show changes in BLI in the same tissues in comparison to control mice (VEHICLE) (Figure 5B).

In addition, we evaluated the dissemination of leukemic cells in the affected organs of the animal by IHC of CD45, a human leukocyte marker that detects AML THP1 cells. Results correlated with BLI analyses showing that treatment with soluble T22-mRTA-H6, differently from those registered after

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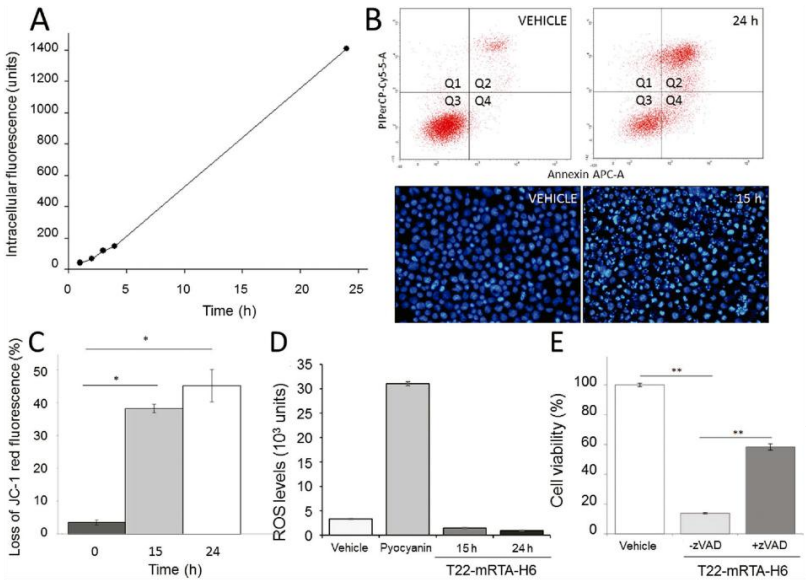

Figure 3. Cell penetrability and intracellular toxicity of T22-mRTA-H6 nanoparticles. A) Intracellular fluorescence in cultured HeLa cells exposed to 100×10^{-9} M of ATTO488 stained T22-mRTA-H6. Extracellular fluorescence was fully removed by a hash trypsin treatment as described.[35] B) Under the same conditions, the externalized phosphatidylserine was detected by Annexin V Detection Kit (APC, eBioscience) in cells exposed to nonstained T22-mRTA-H6. Dead cells were spotted with propidium iodide (PI). Quadrant Q1 shows HeLa cells marked with PI. Q2 shows cells marked with Annexin V and PI. Q3 shows cells without PI nor Annexin V. Q4 shows cells marked with Annexin V. Therefore, dead cells are shown in Q1 and Q2 while living cells in Q3 and Q4. Apoptotic cells are shown in Q4. At the bottom, Hoechst staining of HeLa cell under the above conditions. Images were obtained by fluorescence microscopy (×400). C) Loss of JC-1 Red fluorescence in T22-mRTA-H6-treated cells as described above, indicative of a change in the mitochondrial $\Delta\psi$. D) Levels of cellular ROS detected with a fluorescence microplate assay. HeLa cells were treated with either buffer, T22-mRTA-H6 (100×10^{-9} M, for 15 or 24 h) or 100×10^{-6} M Pyocyanin (1 h) as a positive control. Values are expressed as relative fluorescence units ± SE. E) Inhibition of caspases with zVAD-fmk reverses the antitumor activity of T22-mRTA-H6 in HeLa cells. Cells were pretreated for 1 h with 100×10^{-6} M zVAD-fmk and then exposed to 100×10^{-9} M T22-mRTA-H6 for 48 h. Cell viability is expressed as the percentage of cell survival compared with the control. Values are mean ± SE. *Vehicle* indicates treatment with buffer. The level of significance is indicated ($*p < 0.05$, $**p < 0.01$).

T22-mRTA-H6 IBs treatment, reduced the dissemination in the infiltrated tissues, by detecting lower number of CD45 positive cells in bone marrow, liver, and spleen in the mouse treated with soluble T22-mRTA-H6 (Figure 5C). Finally, we performed H&E staining of the infiltrated organs and additional organs not affected by leukemia cells. We did not observe any sign of toxicity in any of the affected or unaffected tissues, neither with the soluble T22-mRTA-H6 nor with the T22-mRTA-H6 IBs treatments (**Figure 6**). As it occurred in vitro, IBs caused, if any, just a mild biological effect.

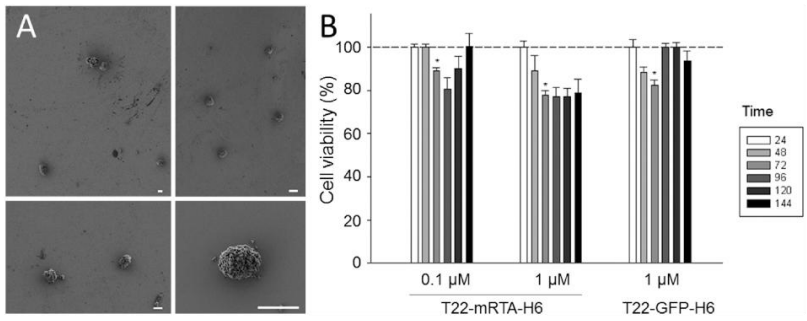

Figure 4. Properties of T22-mRTA-H6 IBs. A) FESEM images of isolated T22-mRTA-H6 IBs at different magnifications. Bars indicate 1 μm. B) Viability of cultured CXCR4⁺ HeLa cells upon different times of exposure to T22-mRTA-H6 IBs and to control, nonfunctional IBs formed by the related protein T22-GFP-H6. Exposure time is indicated in hours. The standard error is represented by a black line. The level of significance is indicated by superscripts ($*p < 0.05$).

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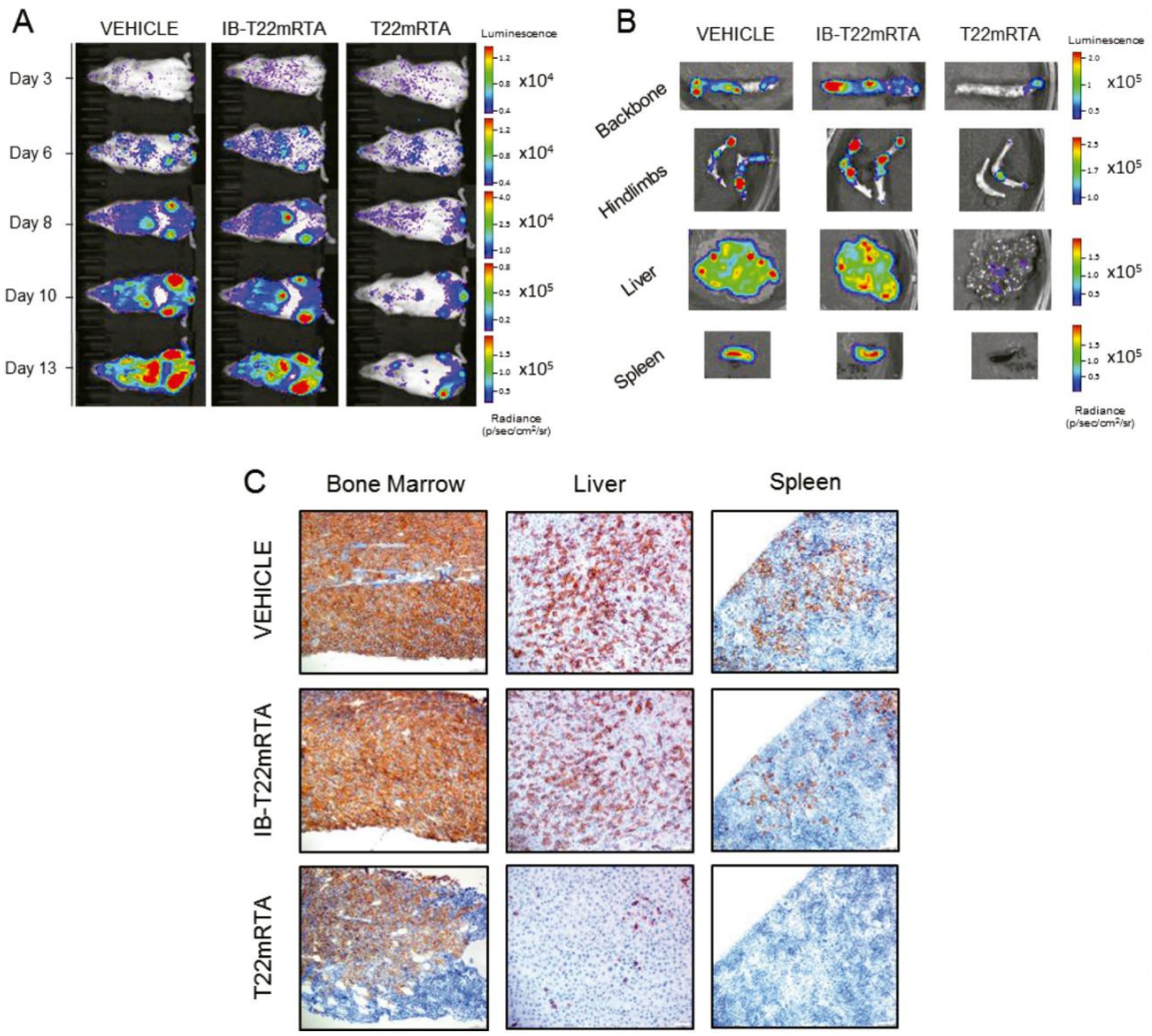

Figure 5. Antitumor activity of T22-mRTA-H6 in a disseminated AML mouse model. A) Follow-up of bioluminescence emitted by mice treated with soluble T22-mRTA-H6 nanoparticles (T22mRTA), T22-mRTA-H6 IBs (IB-T22mRTA), or buffer (VEHICLE) during the 14 d of the experiment, analyzed by IVIS Spectrum. B) Levels of luminescence detected ex vivo in IVIS Spectrum in the tissues infiltrated with leukemic cells such as backbone, hindlimbs, liver, and spleen of mice treated with buffer (VEHICLE), T22-mRTA-H6 IB (IB-T22mRTA), or soluble T22-mRTA-H6 (T22mRTA). C) Detection of CD45 positive cells by IHQ in spleen, liver, and bone marrow of mice treated with buffer (VEHICLE), T22-mRTA-H6 IBs (IB-T22mRTA), or soluble T22-mRTA-H6 nanoparticles (T22mRTA). T22mRTA, mouse treated with soluble T22-mRTA-H6; IB-T22mRTA, mouse group treated with T22-mRTA-H6 IBs; VEHICLE, group treated with vehicle. Bars indicate 50 µm.

3. Discussion

Functional recruitment in single-chain modular polypeptides is a promising strategy for the generation of self-targeted and self-delivered drugs that are chemically homogenous and produced in a single step in recombinant cell factories.[37] Protein drugs represent a big sector in the pharmacological market.[5] Their easy industrial biofabrication and scalability combined with the intrinsic biocompatibility and functional versatility, approachable by genetic engineering, make proteins a very convenient category of tunable pharmaceuticals.[38,39] In oncology, cytotoxic proteins selected from nature have been engineered and adapted to act as antitumor agents, by means of different approaches that must necessarily consider cell targeting.[3] Immunotoxins are relevant representatives of how protein toxins can be targeted by simple fusion technologies in monovalent complexes, with relevant potential for precise cell killing.[40–42] However, proper targeting is not regularly achieved in current nanomedicine[43] and the amount of cell targeted drugs that reach the intended tumor tissues, especially in oncology, is rather limited (usually <1%).[44] Specifically, immunotoxins have not so far fulfilled the requirements regarding a

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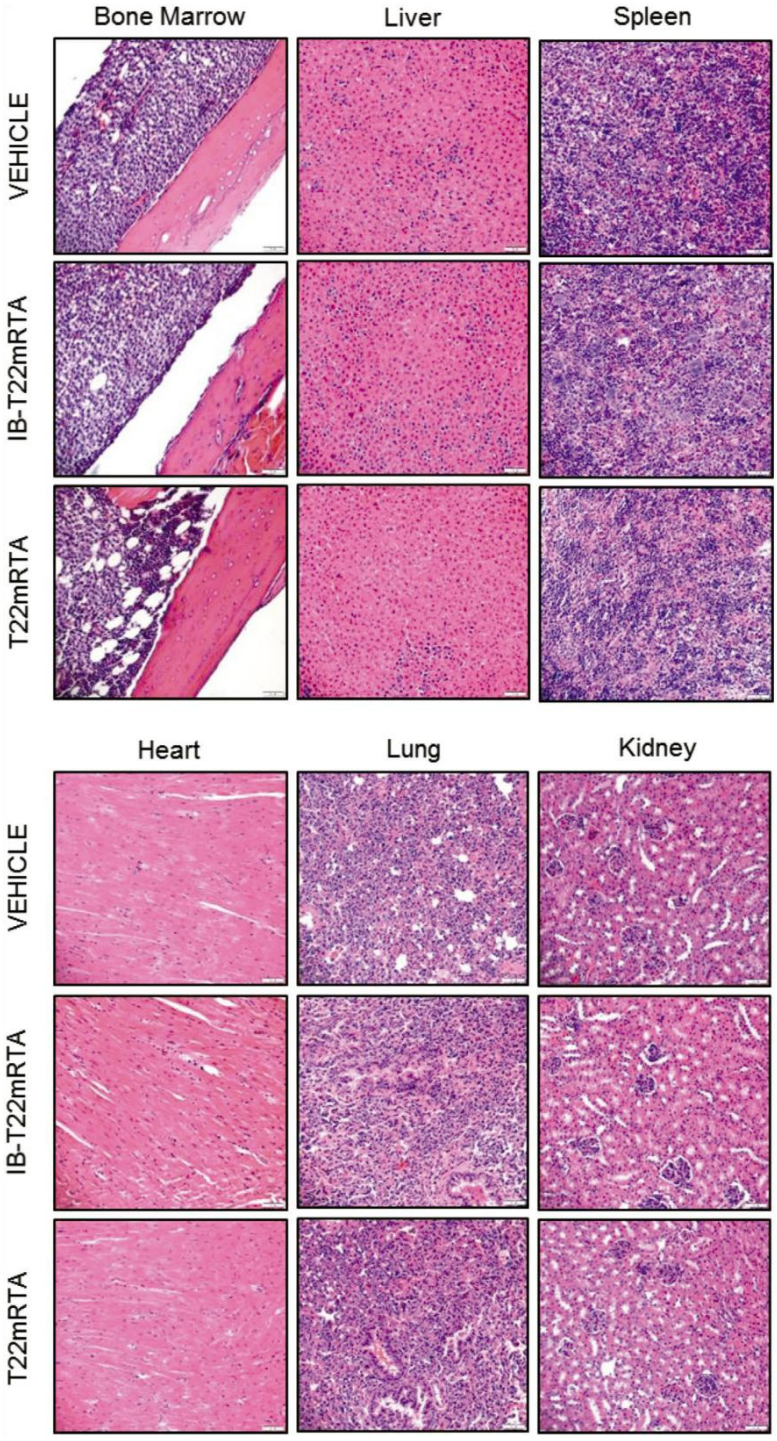

Figure 6. Histopathology in the disseminated AML mouse model after a treatment with T22-mRTA-H6. Hematoxylin and eosin staining of normal (heart, lung, kidney) and leukemia infiltrated organs (bone marrow, liver, spleen). Images were taken in the microscope with a 20× objective and an Olympus DP72 digital camera. H&E, hematoxylin and eosin; T22mRTA, mouse treated with soluble T22-mRTA-H6; IB-T22mRTA, mouse group treated with T22-mRTA-H6 IBs; VEHICLE, mouse group treated with buffer. Bars indicate 50 µm.

convenient therapeutic index, as side toxicity is still relevant.[3] The combination of highly potent toxins with effective targeting is then necessary for a highly precise and selective cell killing that might still be optimized by a regular and multivalent display of the targeting agent on the surface of the drug.[45] Also, formulating a protein drug within the nanoscale size should favor the enhanced permeability and retention effects,[4] minimizing the biological barriers in the drug delivery process.

Under these premises, we have engineered the highly potent plant toxin ricin as a CXCR4-targeted, protein-only nanoscale drug with a multivalent presentation of the ligand, the peptide T22, reached through the regular self-assembling of ricin as stable 11 nm nanoparticles (Figure 1D,F). A related modular protein, namely, T22-GFP-H6, that self-assembles as 12 nm nanoparticles, has been modeled as oligomerizing in ≈10 subunits accommodated in a toroid architecture, thus ensuring a sufficient multivalent display of the ligand.[29,30] According to the similarities in the molecular mass of the building block and in the final size of the resulting nanoparticles, T22-mRTA-H6 seems to self-arrange in a similar pattern (Figure 1). Ricin has been largely considered as a drug component in cancer therapies,[46] and previously explored in form of immunotoxins with moderate efficacy.[47–49] In the nanoconstruct generated here, ricin is highly active and fully potent on target cells, indicative of that oligomerization is not preventing functionality.

This particular approach highly increases the selectivity of the cytotoxic potential of ricin against $CXCR4^+$ cancer cells because of the combination of three main and critical effects.

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First, the specific uptake of the therapeutic protein was achieved because of the multivalent display of the CXCR4 ligand, T22, on the nanoparticle and exclusive CXCR4 receptor overexpression in the target cancer cell membranes. This fact prevents internalization and toxicity on normal cells with low or negligible levels of CXCR4. Second, the avoidance of the severe side effects that appeared on previous clinical trials testing ricin anticancer effect that led to their discontinuation.[13,46] Specifically, we incorporated the mutant (N132A) ricin A chain as functional building block of the nanoparticle, to suppress the potential vascular leak syndrome. We also excluded the use of ricin B-chain to block the severe toxicity associated with its nonspecific binding to glycoproteins or mannose receptors expressed on the membrane of nontumor cells (e.g., Kupffer cells of the liver sinusoids). Finally, the enhanced delivery of the biologically active ricin A chain to the cytosol of target cells (Figure 3A) was reached because of the addition of the furin cleavage site that releases the active domain from the nanoparticle in the endoplasmic reticulum, and a KDEL sequence which allows the translocation of the biologically active toxin to the cytosol, avoiding its lysosomal degradation. The endosomal delivery of the protein drug would also prevent the development of multidrug resistance that mainly relates to drug efflux by cancer cells through the ATP-binding cassette (ABC) transporters activity, overexpression of ABC transporters associating with poor response to therapy.[50] Low molecular weight drugs enter cells by diffusion across membranes, which renders them vulnerable to their efflux by ABC transporters. In contrast, the nanoscale size of oligomeric ricin is expected to avoid passive diffusion. Entering $CXCR4^+$ cells through endocytic vesicles, the protein achieves high intracellular concentration in absence of (or reversing) the multidrug resistance phenotype that might have been observed for a free small drug. This effect, associated with the entry route, has been reported for doxorubicin-loaded polymeric nanoparticles and doxorubicin-polymer conjugates, among others.[51,52]

The combination of these three crucial effects in basic cellular pathways makes for a dramatic increase of ricin A antineoplastic activity. Thus, the previously reported IC50 of untargeted ricin A in HeLa cells (IC50 36 μg mL (1×10^{-6} M))[53] is here reduced about 100-fold (IC50 = 13×10^{-9} M) because of selective CXCR4 cancer cell targeting, KDEL sequence, and furin site incorporation into the nanoparticle. The reached IC50 (Figure 2) is in the same nM range than that described by other highly lethal toxins (such as diphtheria toxin derivatives,[54–56] *Pesudomonas* exotoxin,[57] or neurotoxins[58]). However, this engineered version is highly promising for the further development of the present prototype as an efficient oncological nanostructured drug, since it keeps the full selectivity for the cell surface cytokine receptor CXCR4 (Figure 2) while keeping a nanostructured organization with a multivalent presentation of the surface receptor (Figure 1). In addition, in a molar basis, T22-mRTA-H6 is more cytotoxic on AML $CXCR4^+$ cells than Ara-C (Figure 2E), a basic chemical drug included in most AML treatment protocols.[34] Importantly, the precise cytotoxic activity of T22-mRTA-H6 nanoparticles is conserved in vivo after systemic administration, which leads to a dramatic blockade of leukemic cell affectation in the clinically relevant organs (bone marrow, liver, and spleen) in the $CXCR4^+$ AML model (Figure 5). These findings were associated with absence of any detectable systemic (not shown) or histological toxicity in off-target organs during the experiment time (Figure 6). It could not be fully excluded that in longer treatments ricin (as well as other recombinant toxins or therapeutic fusion proteins) may induce an immune response, that if involving antigens shared with endogenous protein might lead to adverse effects.[59,60] However, the modified version of ricin used here avoids the vascular leak syndrome (VLS), the major concern in the clinical trial of a ricin A-antibody (CD19/CD12) immunotoxin (https://clinicaltrials.gov/ct2/show/NCT01408160). In this context, further de-immunization might be feasible, if required, to improve the clinical performance of T22-mRTA-H6 or derived drugs, ensuring low immunogenicity and avoidance of autoimmune diseases. This could be done by an approach similar to that carried out for diphtheria and *P. aeruginosa* toxins. These microbial proteins, components of most of third generation immunotoxins under clinical evaluation, are successfully engineered by the removal of nonessential sequences and by the genetic elimination of antigenic T and B cell epitopes, without compromising their antitumor activity.[60]

Combining the impressive therapeutic effects observed in vivo and the fact that CXCR4 is a tumoral marker relevant in more than 20 human neoplasias,[61] its overexpression correlating with aggressiveness,[62–66] T22-mRTA-H6 nanoparticles combine selectivity, cytotoxicity, nanoscale size, and multivalent display in a chemically homogeneous entity devoid of any external carrier or vehicle that might impose limitations to the biocompatibility of the whole construct.[4]

4. Conclusion

One of the most potent toxins in nature, ricin, has been genetically instructed to self-assemble as stable 11 nm homomeric nanoparticles and to selectively kill CXCR4-overexpressing cells, by using a promising protein engineering toolkit. The resulting nanoscale material has been shown as highly cytotoxic and highly selective over $CXCR4^+$ cells, resulting in an unusually strong and efficient antitumor activity in a mouse model of the difficult-to-treat disseminated acute myeloid leukemia, in complete absence of side toxicity. This analysis opens a plethora of possibilities to combine highly toxic proteins with a highly selective tumor-targeting platform, that within the nanoscale, would fulfill the emerging concept of self-assembled, self-targeted vehicle-free recombinant drugs for precision medicines.

5. Experimental Section

Genetic Design and Protein Production: The recombinant protein T22-mRTA-H6 (Figure 1A) was designed to include the highly specific CXCR4 ligand T22[20] at the amino terminus followed by a mutated version of the ricin A chain, and a hexahistidine tail at the carboxy terminus. The mutation N132A was introduced to suppress the vascular leak syndrome in potential future in vivo administrations, keeping the cytotoxic activity. In addition, a furin cleave site was also incorporated to allow the release of the accessory N-terminal region in the endosome and the intracellular activity of ricin in a quasi-native sequence format. A KDEL motif was also incorporated to favor endosomal escape.[67] The plasmid construct

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pET22b-T22-mRTA-H6, encoding the protein under the control of the bacteriophage T7 promoter, was generated by GeneArt and transformed into *E. coli* Origami B cells.

Production and Purification of Soluble Protein: Recombinant bacteria were cultured in lysogeny broth (LB) medium with 100 μg mL^{-1} ampicillin, 15 μg mL^{-1} kanamycin, and 12.5 μg mL^{-1} of tetracycline, at 37 °C and 250 rpm. The recombinant gene expression was induced by adding 0.1×10^{-3} M isopropyl-β-thiogalactopyronaside (IPTG) when the OD of the culture reached a value between 0.5 and 0.7. Cultures were subsequently incubated overnight at 20 °C and 250 rpm. Cells were harvested and centrifuged (5000 *g*, 15 min, 4 °C). The cell pellet was resuspended in wash buffer (51×10^{-3} M sodium phosphate buffer, pH = 8, 158.6×10^{-3} M trehalose dihydrate, 0.01% Polysorbate-20, 15×10^{-3} M imidazole, 300×10^{-3} M NaCl) in presence of protease inhibitor cocktail Complete EDTA-Free (Roche). Bacterial cells were sonicated twice at 10% amplitude and once at 15% of amplitude for 10 min each round, centrifuged (15 000 *g*, 45 min, 4 °C) and soluble fraction purified by affinity chromatography with a HiTrap Chelating HP column in an AKTA purifier FPLC (GE Healthcare). After the samples were filtered (0.22 μm) and injected into the column, the fractions to be collected were eluted at ≈30% elution buffer (51×10^{-3} M sodium phosphate, pH = 8, 158.6×10^{-3} M trehalose dihydrate, 0.01% Polysorbate-20, 500×10^{-3} M imidazole, 300×10^{-3} M NaCl). The buffer exchange was done in Centricon Centrifugal Tubes Ultracel 10000 NMWL. T22-mRTA-H6 was found to be highly stable in 51×10^{-3} M sodium phosphate pH = 6.2, 60 mg mL^{-1}α-trehalose dehydrate, 0.01% polysorbate-20. Protein purity was analyzed by SDS electrophoresis on TGX Stain-Free gels (Bio-Rad), followed by Western blotting using an anti-His monoclonal antibody (Santa Cruz Biotechnology). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on TGX Stain-Free Gels (Bio-Rad) was conducted to analyze the protein. Samples were diluted in denaturing buffer (0.53 M Tris Base, 5.52 M glycerol, 0.27 M SDS, 2.84 M β-mercaptoethanol, 7.99 M urea) at a 3:1 molar ratio, boiled at 96 °C for 10 min and loaded into the gels lanes. For the Western blot, an anti-His monoclonal antibody was used (Santa Cruz Biotechnology) followed by a goat anti-mouse IgG (H+L)-HRP secondary antibody (Ref. 170-6516) conjugate (Bio-Rad, Ref. 170-6516). Images were observed using ChemiDoc Touch Imaging System. 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/)

Production and Purification of Insoluble Protein: Recombinant bacteria were cultured in LB at 37 °C and 250 rpm until the OD reached between 0.5 and 0.7, and gene expression was induced by 1×10^{-3} M IPTG. Then, cells were further incubated to allow gene expression for 3 h at 37 °C and 250 rpm. After sedimentation (5000 *g*, 15 min, 4 °C), the pellet was resuspended in 0.22 μm filtered lysis buffer (Tris 1 M pH = 8, NaCl 4 M, EDTA 50×10^{-3} M) in presence of protease inhibitor cocktail Complete EDTA-Free (Roche), the protease inhibitor phenylmethane sulfonyl fluoride (PMSF, 100×10^{-3} M), and 50 μg lysozyme mL^{-1}, followed by an incubation at 37 °C and 250 rpm for 2 h. Cells were disrupted in a French Press (5 rounds at 1200 psi) and kept at −80 °C overnight. Samples were thawed and treated 0.2 μL Triton X-100 mL^{-1} cell culture for 1 h at room temperature with agitation. Then, after sedimentation (15 000 *g*, 15 min, 4 °C), pellets were resuspended in the same volume of filtered lysis buffer. The following reagents were then added to the sample: 1 μL $MgSO_4$ (1 M) mL^{-1} cell culture, 1 μg DNAse mL^{-1} cell culture The culture was then incubated for 1 h at 37 °C and 250 rpm agitation. As a sterility assay, LB plates were seeded with 100 μL of culture at 37 °C, overnight, and the suspension of insoluble protein was frozen at −80 °C overnight. The suspension was frozen and thawed daily until no bacterial colonies appeared in the plates. Then, after sedimentation (15 000 *g*, 15 min, 4 °C), the supernatant was discarded, and each pellet was resuspended in filtered ultrapure water and aliquots were made. Finally, after sedimentation of insoluble material (15 000 *g*, 15 min, 4 °C), supernatants were discarded and pellets were stored at −80 °C.

Quantitative Protein Analysis: Protein purity was analyzed by SDS-PAGE on a Chemi Doc Touch Imaging System (Bio-Rad). Briefly, both soluble and insoluble samples were mixed with in denaturing buffer (0.53 M Tris Base, 5.52 M glycerol, 0.27 M SDS, 2.84 M β-mercaptoethanol, 7.99 M urea) at a ratio 3:1, boiled for 5 or 45 min, respectively, and loaded onto the gels. For the Western blot, an anti-His monoclonal antibody was used (Santa Cruz Biotechnology) followed by a goat anti-mouse IgG (H+L)-HRP secondary antibody conjugate (Bio-Rad). Gels were scanned at high resolution and bands were quantified with Quantity One Software (Bio-Rad) using a known protein standard of soluble recombinant T22-mRTA-H6.

Quantitative and Qualitative Analyses of Soluble Protein: Protein molecular weight was verified by mass spectrometry (MALDI-TOF), and concentration determined by Bradford assay (Dye Reagent Concentrate Bio-Rad kit). Volume size distribution of protein nanoparticles was determined by DLS. For that, a 50 μL aliquot (stored at −80 °C) was thawed and the volume size distribution of nanoparticles was immediately determined at 633 nm (Zetasizer Nano ZS, Malvern Instruments Ltd.). Far-UV CD was determined at 25 °C in a Jasco J-715 spectropolarimeter to assess the secondary structure of T22-mRTA-H6, which was dissolved at 0.35 mg mL^{-1} in 166×10^{-3} M sodium bicarbonate buffer, pH 8. The CD spectra were obtained in a 1 mm path-length cuvette over a wavelength range of 190–260 nm, at a scan rate of 50 nm min^{-1}, a response of 1 s, and a bandwidth of 1 nm. Six scans were accumulated. The magnitude of secondary structure was analyzed using the JASCO spectra-manager analysis software. To investigate potential intermolecular β-sheet structure in the protein nanoparticles, conventional methods for Thioflavin T (ThT) staining were adapted. Briefly, protein aliquots (10 μL) were added to 90 μL of 50×10^{-6} M (Sigma Aldrich) in phosphate buffered saline (PBS), pH 7.4 and stirred for 1 min. The final protein concentration was 0.17 mg mL^{-1}. ThT was excited at 450 nm and the fluorescence emission spectra were recorded in the range of 460–565 nm with a Varian Cary Eclipse spectrofluorimeter. The cross-β-sheet structure was monitored by the enhancement of the free dye fluorescence emission.

Cell Culture and Determination of Cell Viability and Apoptosis: HeLa cells (ATCC-CCL-2) were cultured at 37 °C in a 5% CO_2 humidified atmosphere in MEM-α media supplemented with 10% fetal calf serum (Gibco Thermo Fisher Scientific (TFS)). They were seeded in an opaque 96-well plate (3×10^4 cells/well) for 24 h. When insoluble T22-mRTA-H6 was assayed, the media was supplemented with 2% penicillin, 10 000 U mL^{-1} streptomycin (Gibco, TFS). The next day soluble T22-mRTA-H6 was added and cells were exposed for 24, 48, and 72 h). Cells were also exposed to insoluble protein version during 24, 48, 72, 96, 120, and 144 h. Cell viability was determined by CellTiterGlo Luminescent Cell Viability Assay (Promega) in a Multilabel Plater Reader Victor3 (Perkin Elmer). For the CXCR4 specificity assay, the CXCR4 antagonist AMD3100[33] was added at 10:1 molar ratio 1 h before the incorporation of the protein. Antagonist and protein were incubated in a final volume of 10 μL that were mixed with 90 μL of culture media. All soluble protein experiments were done in triplicate and insoluble protein with six replicates.

On the other hand, the AML cell lines THP1 (ACC-16) and MV411 (ACC-102), as well as 3T3 mouse fibroblasts (ACC-173), were purchased from DSMZ (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). THP1 was cultured in RPMI-1640 medium supplemented with 10% FBS, 10 mmol L^{-1} L-glutamine 100 U mL^{-1} penicillin, 10 mg mL^{-1} streptomycin, and 0.45 μg mL^{-1} fungizone. (Gibco, TFS). 3T3 cells were cultured with DMEM medium adding the same supplements. Cells were kept at 37 °C in a humidified atmosphere of 5% CO_2. Cell viability assays with these cell lines were performed using the XTT Cell Viability Kit II (Roche Diagnostics) and absorbance was read in a spectrophotometer at 490 nm (BMG Labtech). The effect of the caspase inhibitor zVAD-fmk was evaluated pretreating for 1 h cells seeded on 96-well plates (100×10^{-6} M zVAD-fmk) and then exposing them to 100×10^{-9} M T22-mRTA-H6 for 48 h. The antitumor drug Ara-C (cytosine β-D-arabinofuranoside hydrochloride) was purchased from Sigma Aldrich. To allow the follow-up of AML in mice, THP1 AML cell line was transfected with a plasmid encoding the luciferase gene that confers

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bioluminescence that can be noninvasively imaged (BLI) to the cells. Briefly, THP1 cells were harvested in 24-well plates, treated with 0.5 μg of DNA plasmid and mixed with Lipofectamine LTX and PLUS reagents (A12621, Invitrogen, TFS) in Opti-MEM Reduced Serum Medium (Gibco, TFS) according to the manufacturer's instructions. 48 h later BLI levels were tested incubating cells with luciferin in an IVIS Spectrum In Vivo Imaging System (PerkinElmer, Waltham, MA). Finally, transfected cells were selected with 1.5 mg mL^{-1} geneticin (G418 Sulfate, Gibco, TFS) and BLI was analyzed periodically to check the preservation of the plasmid in cells, called THP1-Luci cells. Internalization of T22-GFP-H6[30] in 3T3, MV411, THP1, and HeLa was determined by fluorescence-activated cell sorting (FACS Calibur, BD). Cells were exposed for 1 h to T22-GFP-H6 at 100×10^{-9} M. Then, cells were washed with PBS and trypsinized (1 mg mL^{-1} trypsin, Life Technologies) in order to remove nonspecific binding of nanoparticles to the cell membrane. Finally, levels of intracellular GFP fluorescence were quantified by flow cytometry. Mean fluorescence intensity ratios are given as mean fluorescence intensity of the treated samples divided by the mean fluorescence intensity of the vehicles.

To evaluate cell apoptosis, nuclear staining was performed with the Hoescht 3342 dye (Sigma-Aldrich) in HeLa cells exposed to 100×10^{-9} M T22-mRTA-H6 or buffer for different times. Once the incubation was finished, the media was collected and centrifuged to obtain the suspended cells. They were rinsed with PBS and centrifuged again. The adhered cells were trypsinized and pulled together with those previously obtained. These cells were fixed (3.7% *p*-formaldehyde in PBS, pH 7.4) for 10 min at −20 °C, washed with PBS and resuspended in 10 μL of PBS. Finally, cells were mounted on a slide with ProLong Gold Antifade Mountant with DAPI and observed for the appearance of the nuclei under a fluorescence microscope. In addition, externalized phosphatidylserine protein-exposed cells were detected by Annexin V Detection Kit (APC, eBioscience) while dead cells were spotted with propidium iodide (PI), according to supplier instructions. Cell internalization was monitored using ATTO-labeled protein as described elsewhere.[23]

Determination of ROS Levels and Mitochondrial Damage: On the other hand, levels of cellular ROS were measured with the Cellular ROS Detection Assay Kit (Abcam). In brief, HeLa cells were exposed to 100×10^{-9} M T22-mRTA-H6 (15 or 24 h) or buffer. Then, cells were washed and incubated with ROS Detection Solution for 1 h at 37 °C, in the dark, adding 100×10^{-6} M Pyocyanin (1 h) to the positive controls. Afterward, levels of fluorescence were read with a microplate reader (BMG Labtech) at Ex = 488 nm and Em = 520 nm. Values were expressed as relative fluorescence units after subtracting the background fluorescence of blanks. Finally, to measure mitochondrial membrane potential ($\Delta\psi m$), a mitochondrial potential detection kit (BD MitoScreen, BD Biosciences) according to manufacturer's instructions was used. Labeled cells were analyzed by flow cytometry and the data were expressed as percentage of cells containing depolarized mitochondria (loss of JC-1 red fluorescence).

Flow Cytometry: CXCR4 membrane expression was determined by fluorescence-activated cell sorting (FACS Calibur, BD). Cells were washed with PBS 0.5% BSA and incubated either with PE-Cy5 mouse anti-CXCR4 monoclonal antibody (BD Biosciences) or PE-Cy5 Mouse IdG2a isotype (BD Biosciences) as control. Results of fluorescence emission were analyzed with software Cell Quest Pro and expressed as the ratio between the mean fluorescence intensity of each sample and the isotype values.

Electron Microscopy: The ultrastructure of soluble (in form of nanoparticles) and insoluble (in form of IBs) T22-mRTA-H6 was observed by FESEM). Insoluble protein was resuspended in PBS and sonicated at 10% amplitude 0.5 s ON/OFF for 1 min. Drops of 10 μL of either soluble protein in storage buffer or insoluble protein in PBS were deposited during 1 min on silicon wafers (Ted Pella), excess of liquid eliminated, and air dried. Samples without coating were observed with an in-lens detector in a FESEM Zeiss Merlin (Zeiss) operating at 1 kV. Representative images were obtained at a wide range of magnifications (from 100 000× to 450 000×).

Antineoplastic Effect in a Disseminated AML Mouse Model: NSG (NOD-scid IL2Rgammanull) female mice (five weeks old) were obtained from Charles River Laboratories (Wilmington, MA) and housed in microisolator units with sterile food and water *ad libitum*. After one week in quarantine, NSG mice were intravenously (IV) injected with luciferase-transfected THP1 cells (THP1-Luci; 1×10^6 cells/200 μL) and divided randomly into three different experimental groups. One group (VEHICLE; $n = 3$) was IV injected with $NaCO_3H$ pH = 8 buffer, a second group (T22mRTA; $n = 1$) was administered with 10 μg of T22-mRTA-H6. Both groups were injected with a daily dose for a total of ten doses. A third group (IB-T22mRTA; $n = 2$) was subcutaneously (SC) injected once with 1 mg of T22-mRTA-H6 IBs. These treatments started 2 d after the IV injection of THP1-Luci cells in mice, which generated the disseminated AML model. Evolution of AML dissemination was monitored in IVIS Spectrum three times per week until the day of the euthanasia. Weight of the animals was measured the same day of BLI analysis. All mice were euthanized the day that the first of them presented relevant signs of disease such as 10% weight loss or lack of mobility. Animals were intraperitoneally injected with luciferin, and after 5 min mice were killed by cervical dislocation. Tissues were excised and the BLI levels of the organs ex vivo analyzed. After that, they were preserved in formaldehyde 3.7% and paraffin embedded for further immunohistochemistry analyses. The analysis and detection of BLI was performed using radiance photons in Living Image 4.4 Software both in in vivo and ex vivo studies. All procedures were conducted in accordance with the guidelines approved by the institutional animal Ethics Committee of Hospital Sant Pau.

Histopathology and Immunohistochemical Staining: Sections of paraffin-embedded samples of infiltrated (liver, spleen, hindlimbs, and backbone) and normal (lung, heart, and kidney) organs were hematoxylin and eosin (H&E) stained and the presence of toxicity was analyzed. Moreover, in order to detect AML cells in infiltrated tissues, immunohistochemical analysis with anti-human CD45 antibody (DAKO) was done in paraffin-embedded tissue samples. Staining was performed in a Dako Autostainer Link 48, following the manufacturer's instructions. Two independent observers evaluated all samples, using an Olympus BX51 microscope (Olympus). Images were acquired using an Olympus DP72 digital camera and processed with CellD Imaging 3.3 software (Olympus).

Statistical Analysis: Quantitative data are expressed as mean ± standard error (SE). Previously to perform statistical analyses, all variables were tested for normality and homogeneity of variances employing the Shapiro–Wilk and the Levene test, respectively. Comparisons of soluble protein cytotoxicity effects and competition assays were made with Tukey's test. Meanwhile, protein cytotoxicity assays were assessed by Mann–Whitney U tests. Significance was accepted at $p < 0.05$.

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Conflict of Interest

Some of the authors hold a patent on the use of the peptide T22 as cell-targeted delivery agent.

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Article 2

Engineering a recombinant chlorotoxin as cell targeted cytotoxic nanoparticles

***Raquel Díaz**, Laura Sánchez-García, Naroa Serna, Alejandro Sánchez-Chardi, Olivia Cano-Garrido, Julieta M. Sánchez, Ugutz Unzueta, Esther Vázquez and Antonio Villaverde*

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Cell-targeting nanostructured proteins increase the drug specificity and reduce undesired side effects. Cytotoxic proteins are promising drug components against cancer cells. Among them, chlorotoxin (CTX) is a venom compound that has gained interest also as a targeting agent. [147]

As a T22-GFP-H6 analog that has been studied in depth[50], two recombinant versions of CTX-GFP-H6 have been proposed. The incorporation of CTX to the protein-engineering platform, promotes the self-assembling of core proteins and therapeutic proteins via functional recruitment. Moreover, CTX binds effectively to the cell surface matrix metalloproteinase-2 (MMP-2) and the annexin-2, abundantly present in different types of cancer, specifically in glioma.

Two CTX-GFP-H6 proteins with and without additional positive charges demonstrated to self-assemble as 12 nm NPs, to be stable and fully fluorescent. The CTX targeting abilities were preserved in these NPs and a receptor-dependent uptake by HeLa and U87MG cells was reported with slight toxicity. The CTX-GFP-H6 biocompatible and biodegradable NPs are proposed as a possible therapeutic carrier nano-system.

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Engineering a recombinant chlorotoxin as cell-targeted cytotoxic nanoparticles

Raquel Díaz[1,2,3], Laura Sánchez-García[1,2,3], Naroa Serna[1,2,3], Alejandro Sánchez-Chardi[4], Olivia Cano-Garrido[1,2,3§], Julieta M. Sánchez[1,2,5], Ugutz Unzueta[2,6*], Esther Vazquez[1,2,3] and Antonio Villaverde[1,2,3*]

Cytotoxic proteins have a wide applicability in human therapies, especially in those conditions that require efficient and selective cell killing, such as cancer [1]. Chlorotoxin (CTX) is a small (4 kDa) basic peptide from the venom of the yellow scorpion *Leiurus quinquestriatus* [2], which blocks small-conductance chloride channels [3] thus paralyzing the scorpion prey. Being not extremely potent as a cytotoxin (for instance when compared with ribosome-inactivating proteins), it has gained interest as a targeting agent, as the peptide shows a preferential binding to glioma cells mediated by the cell surface matrix metalloproteinase-2 (MMP-2) and the annexin-2. The expression of these proteins is increased in gliomas and other cancer cell types [4]. Upon exposure, CTX blocks the chloride channel activity but it also inhibits and downregulates MMP-2 [5], hampering the glioma tissue migration and invasion potency and inhibiting the metastasis [6]. Despite the efforts to develop chlorotoxin-derivatives and analogues that may enhance the cytotoxic effect of the natural peptide, the most promising strategy to improve patient mean survival time appears to be the use of chlorotoxin as a targeting agent for the delivery of anti-tumor agents. In this context, CTX has been explored in drug delivery as a component of drug formulations that have entered in clinical trials or are already FDA-approved [7]. Indeed, CTX has been largely explored as a partner in drug conjugates [8] or in form of fusion proteins [9] for the treatment and diagnosis of gliomas and other malignant tumors.

Recently, we have developed a protein engineering platform based on functional recruitment [10] to promote the self-assembly of reporter proteins such as the green fluorescent protein (GFP) [11], and therapeutic proteins such as pro-apoptotic factors [12] or microbial [13] and plant toxins [14] in form of therapeutic or theranostic nanoparticles [15]. These category of constructs, based on the fusion of N-terminal cationic stretches [16], form fully functional non-amyloid nanoparticles (ranging from ~10 to 60 nm) [17], that are highly stable upon *in vivo* administration and show a proper biodistribution and accumulation in tumoral tissues [15,18]. Lacking natural cell-targeting properties, these constructs have been genetically empowered to bind $CXCR4^+$ cells by the addition of the CXCR4-binding peptide T22 [19]. We were interested in knowing how a protein-only nanostructured version of CTX would keep the cell binding and internalization abilities of this peptide.

In this context, we designed the modular protein CTX-GFP-H6 (Fig. 1; see all used methods in the Supplementary information). Being cationic, CTX was expected to act as an architectonic tag in combination with the carboxy terminal histidine tail. In addition, we were in-

[1] Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain
[2] Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain
[3] CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), Bellaterra, 08193 Barcelona, Spain
[4] Servei de Microscòpia, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain
[5] Universidad Nacional de Córdoba, Facultad de Ciencias Exactas, Físicas y Naturales. ICTA and Departamento de Química, Cátedra de Química Biológica. Córdoba. Argentina. CONICET, Instituto de Investigaciones Biológicas y Tecnológicas (IIBYT), Córdoba, Argentina. Av. Velez Sarsfield 1611, X5016GCA Córdoba, Argentina
[6] Biomedical Research Institute Sant Pau (IIB-Sant Pau) and Josep Carreras Research Institute, Hospital de la Santa Creu i Sant Pau, 08025 Barcelona, Spain
[§] Present address: Biomedical Research Institute Sant Pau (IIB-Sant Pau) and Josep Carreras Research Institute, Hospital de la Santa Creu i Sant Pau, 08025 Barcelona, Spain
[*] Corresponding authors (emails: uunzueta@santpau.cat (Unzueta U); antoni.villaverde@uab.cat (Villaverde A))

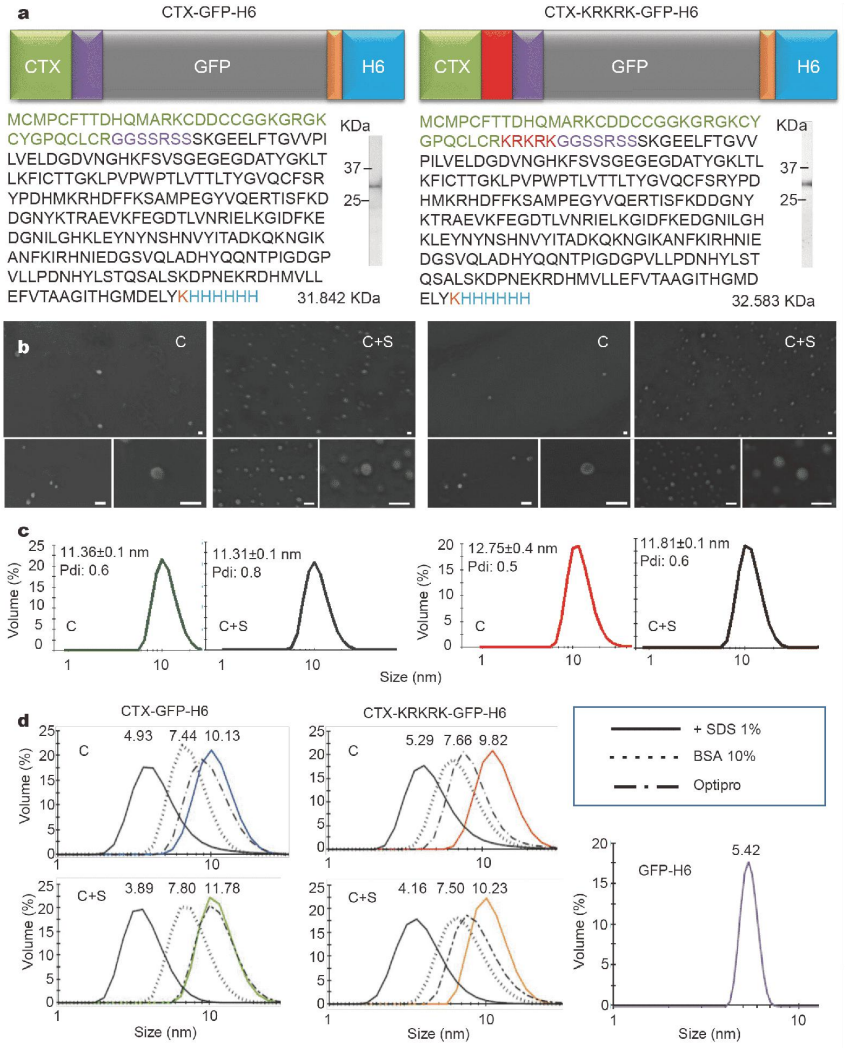

Figure 1 Modular organization of CTX-based building blocks and nanoparticle characterization. (a) Schematic representation of the fusion proteins showing the amino acid sequences, where CTX (green) is placed at the amino termini and a hexahistidine tail (H6, blue) at the carboxy termini. Linker regions (purple) were placed in both cases between CTX and GFP (grey), to ensure fluorescence emission of the fusion protein. A cationic (red) region was inserted in CTX-KRKRK-GFP-H6 downstream the CTX. Siding amino acid sequences, we show the Comassie blue staining of proteins upon elution from affinity chromatography and polyacrylamide gel electrophoresis. Relevant molecular weight markers are indicated. At the bottom, the molecular weights of the whole constructs as determined by matrix-assisted laser desorption/ ionization time of flight mass spectrometry. (b) Field emission scanning electron microscopy images of purified protein, showing their nanoarchitecture. Particles were diluted in two buffers, in which nanoparticles were tested for stability, namely carbonate buffer (C) and carbonate buffer plus 333 mmol L^{-1} NaCl (C+S). Bar size is 20 nm in all panels. (c) Dynamic light scattering plots showing the hydrodynamic size of nanoparticles. The peak value and the polydispersion index (Pdi) are indicated. Determinations were done on the material dissolved in buffer C and C+S. (d) The hydrodynamic size of the particles in these buffers was also determined in presence of 10% BSA and in Optipro cell culture medium. SDS (at 1%), that promotes the disassembling of protein-only nanoparticles was alternatively added to the buffer to identify the size of the building blocks. The size of the parental GFP-H6 is also indicated in nm. Untreated nanoparticles are shown by coloured plots. All the experiments were performed at pH 8. The peak value of the samples in SDS, BSA and Optipro are specified over the respective plots.

terested in investigating whether CTX can retain its natural biological activities as a targeting agent in such a macromolecular organization. Since the cationic characteristic of CTX is only moderate, we generated the alternative fusion CTX-KRKRK-GFP-H6, in which additional cationic residues were inserted between CTX and GFP (Fig. 1a), to favour nanoparticle formation. Such strategy was previously observed as useful to promote oligomerization of blood-brain-barrier (BBB) crossing peptides as brain-targeted, protein-only nanoparticles [20]. These two CTX-containing proteins were produced and stored in carbonate buffer, which had been previously shown to be optimal for the stability of self-assembling protein nanoparticles in cell cultures [13]. We also tested two salt concentrations, as the ionic strength might have a significant role in nanoparticle formation [18]. As observed in the inset (Fig. 1a), both proteins were produced in bacteria as a single molecular species of the expected molecular mass, and spontaneously assembling as regular nanoparticles of ~12 nm (Fig. 1b, c). The addition of sodium dodecyl sulphate (SDS), that promotes the disassembly of protein-only nanoparticles, revealed the actual size of the building blocks (around 3.8–5 nm, probably protein monomers and/or dimers), very similar to that of the parental GFP-H6 (5.4 nm, probably dimers, Fig. 1d). The salt content did not have any detectable impact on the particle size and stability but the buffer with salt seemed to promote or increase nanoparticle density or amount (Fig. 1b, c). A rich culture media such as Optipro did not show any significant impact on the size of the materials, while 10% bovine serum albumin (BSA) decreased the particle size, probably by slightly destabilizing protein-protein contacts without inducing their full disassembling (Fig. 1d). The resulting nanoparticles were fully fluorescent, with specific emission values of 2,550.6 ± 2.8 units/μg and 2,027.8 ± 8.1 units/μg for CTX-GFP-H6 and CTX-KRKRK-GFP-H6 respectively (not shown). Such intrinsic fluorescence emission allowed the monitoring of the materials in subsequent assays in cell cultures.

The spontaneous self-assembling of the engineered CTX, in both versions, prompted us to further investigate whether the toxin, in such oligomeric form, could mediate cell binding and internalization. Two cell lines previously identified as targets for CTX, namely HeLa (overexpressing annexin-2) and U87MG (overexpressing MMP2) [21–24], were selected to examine cell penetrability of the nanoconstructs, using the intrinsic green fluorescence as a monitoring tool. As observed (Fig. 2a), CTX-KRKRK-GFP-H6 nanoparticles were much more efficient than CTX-GFP-H6 in cell internalization, in both cell lines. Moreover, regarding to the CTX-KRKRK-GFP-H6 protein version, a high salt content significantly improved cell penetration of the material, in particular when observing the uptake in U87MG cultures. In this cell line, penetrability of the protein nanoparticles was globally much higher than in HeLa cell line.

To ensure that CTX, in form of nanoparticles, had not lost its cell targeting activities, we explored the selectivity of cell penetrability by inhibiting annexin-2 binding during cell interaction. As observed (Fig. 2b), cell uptake in HeLa cells was significantly reduced by both a monoclonal antibody and a polyclonal serum against the cell surface protein acting as a CTX receptor. Since the antibodies acted over the penetration of both proteins in both buffers, we deduced that both the cationic stretch added to CTX-KRKRK-GFP-H6 and the high salt content enhanced the penetrability of the protein (Fig. 2a) by a receptor-dependent mechanism (Fig. 2b). Both cell penetrability and receptor specificity were observed at levels comparable to those shown by T22-GFP-H6 (Fig. 2c). This protein contains the peptide T22 that selectively and uniquely binds the cell surface cytokine receptor CXCR4, expressed in HeLa cells [19]. In that case, inhibition of cell uptake by the CXCR4 antagonist AMD3100 [25] was more effective than the mediated by the anti-annexin-2 antibodies over CTX-carrying constructs probably because the unique target of T22 compared to the dual binding sites of CTX. On the other hand, GFP-H6 was unable to penetrate cultured cells (Fig. 2c), supporting again the role of CTX in the penetrability of the nanoparticles.

At this stage, we determined the viability of cells exposed to CTX nanoparticles. Although classified as a toxin, chlorotoxin has displayed no obvious cytoxicity when administered to humans, which is important for drug development. Indeed, biological activities of chlorotoxin are mainly related with targeting ability, inhibition of migration and invasion of glioma cells and also, with antiangiogenic properties [27].

Unexpectedly (Fig. 3), the nanostructured CTX-GFP-H6 had a significant cytotoxic impact on both lines, being the CTX-KRKRK-GFP-H6 version more cytotoxic than its counterpart CTX-GFP-H6, and U87MG cells more sensitive than HeLa. CTX-GFP-H6, when added to 0.1 μmol L^{-1}, had a surprising but robust positive impact on cell viability, the number of viable cells reaching 120% of the control samples. This fact was not observed in U87MG cells (Fig. 3b), in which cell death was only moderate, dose-independent, protein-independent, al-

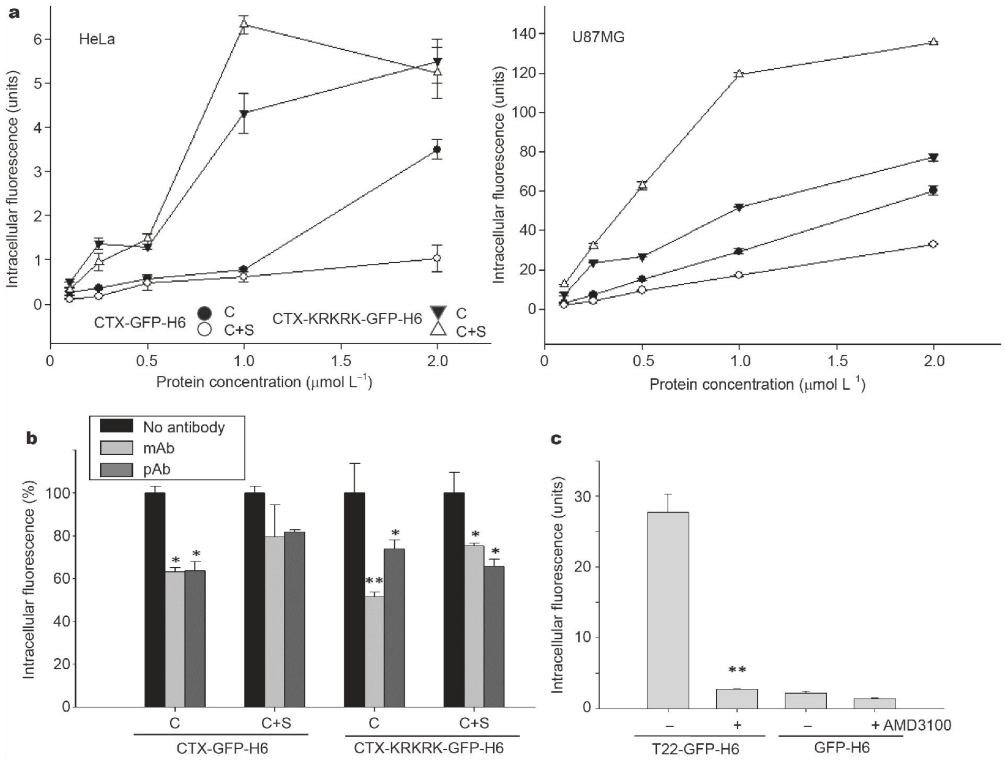

Figure 2 Cell penetrability of CTX-based nanoparticles. (a) Internalized nanoparticles in two alternative cell lines, namely HeLa and U87MG cells, 24 h after exposure to different protein amounts. Intracellular fluorescence was corrected by the specific emission to result in data representative of protein amounts. Cells were submitted to a harsh trypsin treatment before measurements to remove externally attached protein as described [26]. Nanoparticles were administered as dissolved in either C or C+S buffer. *Y* axis scales might be not precisely comparable. (b) Selective antibody-mediated inhibition of nanoparticle uptake in HeLa cells, by an anti-annexin-2 monoclonal antibody (mAb) and polyclonal antibody (pAb). The statistical analysis was performed using an ANOVA Tukey's multiple comparisons test (*p< 0.05; **p< 0.01). Normality was confirmed by Shapiro-Wilk W where $p > 0.05$. Comparisons were done always with samples without antibody. (c) Internalization of the related CXCR4-binding T22-GFP-H6 nanoparticles in ($CXCR4^+$) HeLa cells, and inhibition by the CXCR4 antagonist AMD3100 at an excess molar ratio 10:1. The parental GFP-H6 protein is unable to enter cultured cells. All the experiments using HeLa cells were performed at pH 7.0–7.4, and those using U87MG cells at pH 6.8–7.2.

though significantly modulated by the salt content of the protein storage buffer. The toxicity of CTX was milder than that of the potent microbial toxin PE24 from *Pseudomonas aeruginosa* [13] (Fig. 3c), while the CTX-less GFP-H6 showed no effect on cultured cells (Fig. 3c).

In summary, we have constructed two recombinant versions of CTX, that fused to a His-tagged GFP assemble as stable, fully fluorescent protein nanoparticles of regular size (12 nm, Fig. 1). In this oligomeric form, the protein retains its ability to penetrate target cells, as determined here in two cell lines that display suitable receptors for CTX, namely HeLa and U87MG [21,23,24,28]. The cell uptake is receptor-dependent, as it is inhibited when annexin-2 is sterically blocked by both a monoclonal antibody and an anti-annexin-2 sera (Fig. 2b). The CTX version that contains some additional cationic residues (KRKRK, Fig. 1) inserted between the targeting peptide and GFP, shows an enhanced cell penetrability when compared with the plain CTX fusion (Fig. 2a). Since the uptake of the cationic construct is still receptor-mediated (Fig. 2b), it cannot be merely attributed to a higher electrostatic affinity of the nanoparticles to the cell membrane. In fact, it has been reported that single amino acid substitutions (Lys to Arg) that enhance the cationic nature of CTX result in a more stable version of the peptide and in an enhanced cell penetrability, probably

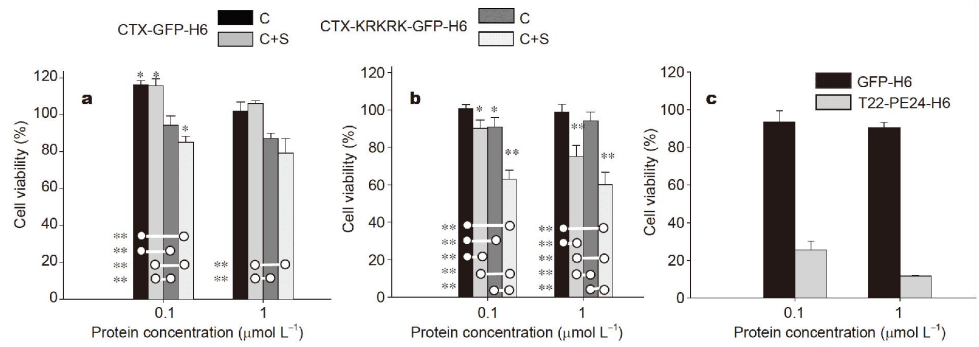

Figure 3 Cell viability upon exposure to CTX-based nanoparticles. HeLa cells (a) and U87MG cells (b) were exposed to protein nanoparticles for 72 h. Nanoparticles were administered as dissolved in either C or C+S buffer. The statistical analysis was performed using an ANOVA Tukey's multiple comparisons test (*p< 0.05; **p< 0.01). Normality was confirmed by Shapiro-Wilk W where p > 0.05. Symbols at the top of the bars indicate the comparison with the control (100%). Symbols at the left of the bars indicate comparisons between protein pairs, indicated by white linkers. (c) HeLa cell viability upon exposure to control, non-toxic GFP-H6 protein and cytotoxic T22-PE24-H6 nanoparticles. All the experiments using HeLa cells were performed at pH 7.0–7.4, and those using U87MG cells at pH 6.8–7.2.

associated to such higher structural stability [7]. Interestingly, the presence of salt dramatically enhances up to three fold the already improved cell penetrability of the cationic CTX version (Fig. 2a) that is accompanied by a slight tendency to an increased cytotoxicity *in vitro,* at least in HeLa cells (Fig. 3).

Importantly, the nanostructured version of CTX retains the tumor cell-targeting properties of this protein, with high cell level of specificity, and excellent cell penetrability. In addition, a mild but significant cytotoxicity is associated to the constructs. The cell killing properties of the CTX nanoparticles appear to slightly be cell line dependent, and also influenced by the engineered cationic segments and the salt content of the media (Fig. 3). In this regard, both CTX nanoparticle versions developed here appear as promising biocompatible and biodegradable carrier systems to load anticancer drugs or therapeutic proteins for targeted therapy of glioma. However, in addition, the unexpected dual role of CTX as driver and cell killing agent is highly promising for a true functional recruitment in the generation of nanostructured, multifunctional and smart therapeutics [10]. This is also in the line of designing chemically homogeneous vehicle-free drugs, at the nanoscale, that is now an emerging and appealing concept in the context of innovative tumor targeted drugs [29].

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Author contributions Díaz R performed most of the protein production and characterization experiments assisted by Sánchez-Garcia L, Serna N, Cano-Garrido O and Sánchez JM. Serna N, Díaz R and Sánchez-garcía L designed the fusion proteins and Sánchez-Chardi A performed the electron microscopy studies. All authors have discussed the data and prepared the figures and methods. Unzueta U, Vazquez E and Villaverde A conceived the study, supervised the experiments and organized the figures. The manuscript was mainly written by Villaverde A.

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Raquel Díaz studied chemical engineering at the University of Sonora, (Mexico, 2008) and achieved a one-year academic exchange at the University of British Columbia (Canada, 2007-2008). Later she fulfilled her Master's study in materials science at the University of Sonora (Mexico, 2012) and is currently studying her PhD in biotechnology at the Autonomous University of Barcelona (Spain, 2019), particularly in the cancer research investigation line.

Ugutz Unzueta developed his PhD in Biotechnology at the Nanobiotechnology group led by Prof. Villaverde at the Autonomous University of Barcelona and he is currently a post-doctoral researcher at Oncogenesis and Antitumoral drugs group at Sant Pau Biomedical Research Institute in Barcelona. His research line is mainly focused on the design, production and characterization of self-assembling protein nanoparticles and nanoconjugates for targeted cancer nanomedicines.

Antonio Villaverde graduated in biological sciences in 1982 and got his PhD in 1985. Since 1987, he is Professor of Microbiology at the Universitat Autònoma de Barcelona in Spain, where he got a Full Professorship in 2002. He leads the Nanobiotechnology group in this university and in the CIBER-BBN, and he is devoted to the design of protein-based materials for biomedical applications. He founded the journal *Microbial Cell Factories* in 2002 being its Editor-in-Chief for 15 years.

重组氯毒素构建细胞靶向的活性纳米颗粒

Raquel Díaz[1,2,3], Laura Sánchez-García[1,2,3], Naroa Serna [1,2,3], Alejandro Sánchez-Chardi[4], Olivia Cano-Garrido[1,2,3§], Julieta M. Sánchez[1,2,5], Ugutz Unzueta[2,6*], Esther Vazquez[1,2,3], Antonio Villaverde[1,2,3*]

摘要 功能性蛋白质在纳米尺度的可控寡聚化提供了通过重组DNA技术来设计和生产改良材料和药物的可能性. 氯毒素(CTX), 作为一种重组的蝎毒素, 由于其优先结合癌细胞的能力而引起人们的兴趣. 本研究将氯毒素设计并自组装为12 nm的常规纳米颗粒, 这些纳米颗粒可穿透具有和天然毒素相同受体特异性的培养细胞. 这些生物相容且可生物降解的材料, 表现出与同时作为载体和治疗剂的重组毒素相应的温和但仍然显著的细胞毒活性, 有希望成为用于细胞靶向治疗胶质瘤的药物载体. 此外, 对CTX侧区域的修改可有效影响纳米颗粒的性能, 说明基于CTX的构建体可通过常规基因工程来调节其多重功能性.

Discussion

Discussion

Cancer research requires therapeutic medicines that specifically inhibit the abnormal cellular proliferation and that stop their potential to spread to other parts of the body. Uncontrolled clonal proliferation in hematologic malignancies, such as AML, affecting blood, bone marrow and lymphatic system have shown important recovery when received treatment with cytotoxic drugs. In the other hand, solid cancer treatment calls for inhibition of the malignant local invasion through the extracellular matrix (ECM) and metastasis, which promotes the establishment of secondary tumors. Solid cancer therapy´s progress is determined not only by the tumor size increase or shrinkage, but also by its anti-metastatic reaction, as in glioblastoma (GBM). In each cancer type, clonal proliferation, cell migration and invasion mechanisms are distinct; either way, complete therapies should be dichotomized into anti proliferative effects and metastasis inhibition.

The invasive phenotype is the most important cancer feature. This novel anticancer proposal as a drug system based on protein-only NPs with the ability to selectively act over malignant cells, avoids healthy tissue due to its specific interaction with an overexpressed receptor particularly in cancerous cells. As the recombinant NPs designed according to the self-assembling targeting protein-engineering platform, they interfere with all modes of cancer cells invasion; therefore they impede their ability to metastasize. Nowadays there are no drugs in the market capable of completely eliminate in a selective way the metastatic cancer cells but with these only-protein NPs (T22-mRTA-H6, CTX-GFP-H6, CTX-KRKRK-GFP-H6) it has been observed a

positive effect in the metastatic process which involves cancer cells motility, migration and invasion reduction. [124, 125]

Particularly, when cytotoxic proteins are involved in the composition of conventional drug chemotherapy, the cancer cell population has been reduced[108, 125]. Nevertheless, damaged healthy tissues related to adverse toxic effects during the treatment have limited its application. The protein platform promotes the propensity of building block proteins to undergo self-assembly and form stable spherical nanostructures. By means of incorporating key segments of toxins and venoms to the recombinant protein platform it is possible to enhance the natural characteristics of the proteins upgrading its functionality by submitting them to a favorable architectonical morphology with a targeting peptide assuring specificity.

The ductility of last technique offers the possibility of obtaining soluble or insoluble NPs depending on the process conditions implemented. Both types of NPs with its own particular characteristics have proved to be effective. In case of producing soluble protein NPs as nanomedicine, it can be administered intravenously more advantageously in comparison to current chemotherapeutic compounds as they offer fully biodegradability and biocompatibility directed to the target cells[148]. In the other hand, when insoluble inclusion bodies (IBs) are produced, functional protein contained inside the NP is released gradually over time in the surrounding area either *in vitro* incubation or *in vivo* local injection or systemically administrations. Inclusion bodies can maintain robust physicochemical conditions along larger periods of time producing favorable therapeutic effects.

When evaluating ricin A chain in T22-mRTA-H6 in comparison with other important nanoconstructs as immunotoxins (ITs), it is important to mention

the murine IgG monoclonal antibodies anti-CD19 (HD37) and anti-CD22 (RFB4) [149], both coupled to deglycosylated ricin A chain (dgRTA), with IC_{50}= 3 x 10^{-9} M and no IC50 for the second immunotoxin as it is was able to kill half of the malignant cells population. Despite when ITs were applied together in a 1:1 mixture known as Combotox their cytotoxicity was improved in pre-B-ALL cells. And when tried *In vivo* in mice and inclusively in humans (phase I), vascular leak syndrome (VLS) was the dose-limitating toxicity (DLT). Conversely, T22-mRTA-H6 by itself achieved excellent cytotoxic effects on HeLa cells, and no significant systemic or histological toxic effects adrift when studied in mice models (Article 1, Fig. 6).

Ricin is a potent toxin originally isolated from the seeds of the *Ricinus communis* plant and it is composed of the catalytic chain A (RTA) and the lectin chain B (RTB). The use of the protein as a drug is segmented in two applications; as a potent anticancer compound or directed for the development of anti-poison remedy to avoid intoxication. As anti-proliferative effector, the limitations observed are mainly adverse toxic effects such as Vascular Leak Syndrome (VLS) related to epithelial cells damage but a non-relevant reduction in its cell killing capacity after several doses[150,151,152]. The implementation of a mutated ricin A chain (mRTA) to the protein platform enriched its intrinsic properties through its coupling to the astounding peptide T22 which unparalleled binds to the receptor CXCR4 overexpressed in AML. T22-mRTA-H6 (Article 1, Fig. 1 A) was evaluated in both possibilities; soluble NPs and IBs by means of the transformation of *E. coli* Origami B cells, as most part of the protein was obtained in insoluble version (Article1, Fig. 1 B). Recombinant IBs were satisfactorily produced and purified, and their spheroid average diameter size ranged between 400-600

nm according to the field emission scanning electron microscopy (FESEM) (Article1, Fig. 4 A). IBs showed a partial cytotoxic effect *in vitro* and *in vivo* related to the unenhanced functional protein release, which can be possibly improved by a chemical or conditional modification (Article1, Fig. 4 B). Otherwise, purified soluble CXCR4-targeted ricin A chain NPs with an average diameter of 11 nm, confirmed by DLS and FESEM (Article 1, Fig. 1 C-D), were functional when evaluated in CXCR4+ HeLa cells with an IC50 of 13 ± 0.5 x 10^{-9} M (Article1, Fig. 2). The receptor-dependent cell uptake was verified through the previous interaction of HeLa cells with AMD3100 in a molar ratio 10:1, a strong CXCR4 antagonist (Article 1, Fig. 2 B). The administration of T22-mRTA-H6 was way more effective than the effect produced by the equimolar dose of Ara-C, the common drug used to treat different types of leukemia including AML, which showed null cytotoxicity (Article 1, Fig. 2 E). The internalization was monitored by an ATTO-labeled version of the protein, and apoptosis was confirmed in the Annexin V affinity assay and Hoechst staining, as expected for an active ricin version (Article 1, Fig. 3 A-B). T22-mRTA-H6 was also evaluated by a vein tail injection systemic administration in an AML mouse model where no side systemic or histological toxicity was observed in healthy organs but an important blockage of the leukemic cell affectation in bone marrow, liver and spleen (Article 1, Fig. 5-6). Immunogenic responses after further administrations of the modified targeted ricin cannot be excluded; nevertheless engineering compounds in the absence of antigenic T and B epitopes sequences that do not compromise the functionality, such as the mutant (N132A) ricin A chain (mRTA) functional building block has been used[131, 153, 154]. Homogeneous T22-mRTA-H6 NPs combine selectivity, nanoscale, cytotoxicity and

multivalent display in a fully biocompatible structure without any external carrier or material added fulfilling the newfangled concept for precision medicines that involve self-assembled, self-targeted and efficient vehicle-free recombinant drug. [108]

Apart from the above, another severe cancer is GBM, not only because it is located in the brain but also because of the local complications present such as the difficult to trespass blood brain barrier (BBB) [55]. Taking advantage of the *Leiurus quinquestriatus* yellow scorpion venom component named chlorotoxin (CTX), renown as a specific cancer targeting, it was also incorporated to the recombinant protein platform. It is reported that CTX as a venom peptide presents some cytotoxicity, nevertheless not potent enough to inhibit cancer cells; moreover it is actually approved as part of grand glioma marker. [155]

As an example, a synthetic CTX compound (TM-601) designed for gliomas treatment, covalently linked to iodine 131 for targeting radiation to malignant cells, has been evaluated in clinical trials phase I, where it selectively binds to the tumor [156, 137]. CTX targets are voltage gated chloride channels, calcium-dependent phospholipid-binding protein Annexin-2 and the inducible extracellular enzyme matrix metalloproteinase MMP-2. Because it is not potent enough to kill malignant cells, CTX is then evaluated as imaging agent or as tumor paint[157]. Nonetheless, it cannot be dismissed that it has a mild cytotoxic effect (Article 2, Fig 3).[158, 147]

Two different versions were engineered; CTX-GFP-H6 and CTX-KRKRK-GFP-H6, the last one with extra positive residues added to ensure the NP self-assembling formation and evaluate changes in its performance (Article2, Fig. 1 A). In this case T22 peptide was substituted by CTX, with the difference

that the target receptors for this case were cell surface matrix metalloproteinase-2 (MMP-2) and the annexin-2 instead of CXCR4[139]. Recombinant soluble NPs were incubated in HeLa (overexpressing annexin-2) and U87MG (overexpressing MMP2) cell lines, after confirming a spherical stable structure with an average diameter size of 12 nm by DLS and FESEM (Article2, Fig. 1 B-C). Both versions were fully fluorescent; which allowed the NPs monitoring. CTX-GFP-H6 presented mild cytotoxic effects in both cell lines, while CTX-KRKRK-GFP-H6 showed to be more cytotoxic in U87MG cells than in HeLa cells (Article 2, Fig. 3). It was not possible to find a behavior pattern related to both types of cell lines subjected to the CTX versions. Nevertheless when U87MG cells were exposed to CTX-KRKRK-GFP-H6 it was visually possible to observe an incredible amount of protein penetration, thus the internalization was studied. CTX in these oligomer forms preserved its natural targeting characteristics; this was confirmed by evaluating the selectivity of the cells uptake through monoclonal antibody and a polyclonal serum against the cell surface protein (acting as a CTX receptor), where a receptor-dependent internalization was confirmed (Article 2, Fig. 2 B).

Nanostructured CTX was successfully engineered in the targeted, self-assembled, vehicle-free, chemically homogeneous recombinant drugs concept. Unexpectedly, the dual role as a cytotoxic compound and as a targeting agent complicates the ideal application bound to this drug. Possibly more experiments with modified conditions could be evaluated to enhance the line of action.

Metastasis is a global challenge in cancer treatment[159]. Therefore, it can be said that cancer is still a non-curable disease specifically when dealing with difficult-to-treat types of cancer such as AML and GBM[160, 161]. Fortunately,

state-of-the-art nanomedicine approaches have offered to many patients the opportunity to recover without relapses. There is still a real need to obtain therapeutic strategies and nanomedicines capable of selectively direct cytotoxicity or attach specifically to the malignant cells for imaging applications. The possibility of obtaining cell stabilization, growth and spread inhibition and an anti-angiogenic effect is despairingly required. The invasion-metastasis cascade causes the majority of the patients' death[162]. Nanotechnology represents a real opportunity of designing highly sensitive nanostructured biomolecules with an important affinity for unique surface receptor proteins located in the cellular wall, provoking the desired accumulation only in tumors. Particularly, the oligomer organization of protein building blocks into multi modal nanostructured recombinant drugs has proved to be outstanding homogeneous, self-assembled, vehicle-free, self-directed medicines against cancer metastasis. There are still some potholes to pass such as some unwanted side effects or natural immunogenic responses that can be overcome by alternating the administration of differently composed drugs along time, or cancer resistance that can be attacked by drug combinations. Nevertheless, the unique advantages that the protein platform engineered drugs offer, surpass any current chemotherapeutic result. There are still doubts related to the effects of a long-term administration *in vivo* but the rational possibility of blocking the metastasis is a real hope that needs to be evaluated. [163]

1. Engineering recombinant protein-only nanoparticles anchoring adequate spherical structure and size distribution.

Oligomerization of building blocks protein parts or peptides requires an adequate morphology with a high structural stability in order to provide a good ability to perform over malignant cells. Modern protein engineering techniques were applied in the design of our nanoparticles and the results were meticulously evaluated by several experiments.

The equilibrated blending of a cationic peptide in the N-terminal and a six his-tag in the carboxi-terminal was also successful in T22-mRTA-H6 where robust spherical nanoparticles were obtained, and structurally corroborated for AML treatment (Article 1, Fig 1C and 1D). It is important to stand out that a modified version of ricin A chain (mRTA, N132A mutation) was used in order to diminish the VLS. In the same way, a furin cleavable site fragment was added in order to release the protein intracellularly and a KDEL sequence in C-terminal for an endoplasmic reticulum secretion prevention (Article 1, Fig 1A). The result of these combinations threw out a 100-fold cytotoxic improvement with a IC50 = $1x10^{-6}$ M in HeLa cells and high selective recombinant self-assembling NP with specificity action. The NP stability was verified at three pH values, 6.3, 7 and 7.4, where the spherical size showed to be consolidated (Article 1, Figure 1F).

When studying a new short protein CTX for GBM, as it is not as cationic as T22, some positively charged amino acids were also taken in count during the nanoconstruct design to ensure the NP self-assembling (Article 2, Fig 1a). Nevertheless, both CTX-GFP-H6 and CTX-KRKRK-GFP-H6 satisfactorily produced high quality NPs. Pertinent evaluations were not only focused on morphological features which are determinant in NPs´ behavior, but also

functional characteristics were also important to be monitored (Article2, Fig 1).

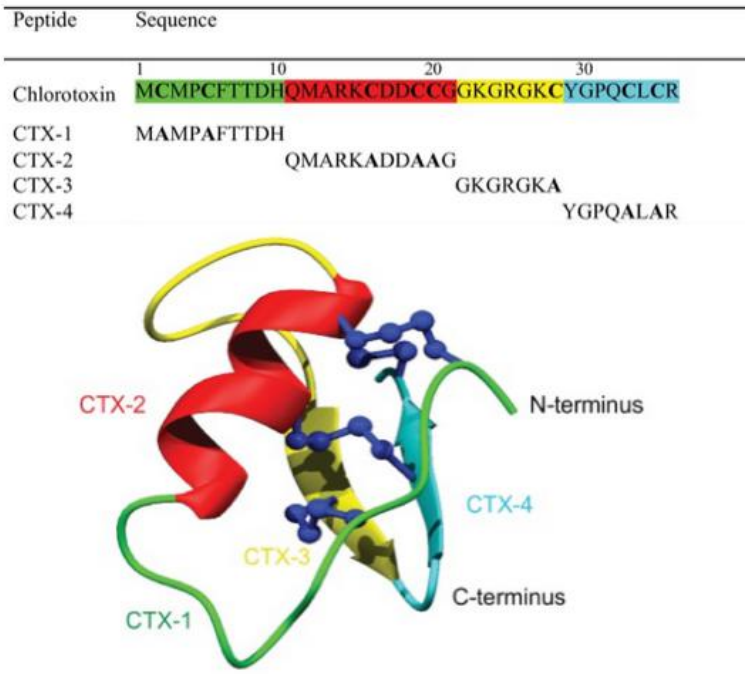

Figure 12. Schematic structural representation of CTX showing four fragments in different colors. Modified from [147]

Other CTX structural studies disclosed that different fragments[147] of the protein carried different cytotoxic behaviors concerning U87-MG cell invasion, shown below in **Figure 12**. Last results help us to explain the reason why in our studies chlorotoxin showed a range of cytotoxic performances in HeLa and U87MG as well, without setting aside the different buffers in where the NPs where located (Article 2, Fig 3a and 3b).

CTX peptide, at a concentration of 20 μM, has shown to be stable in 100% human male serum[145]. The CTX-GFP-H6 and CTX-KRKRK-GFP-H6 of our NPs structural stability was also evaluated in sodium dodecyl sulphate (SDS1%) because it promotes the disassembly of protein-only nanoconstructs, finding

the building-blocks size of 3.8-5 nm corresponding to monomers or dimers. In the same way, NPs´ structure was studied in bovine serum (BSA 10%) and in the cell culture medium OptiPro (Article 2, Fig 1d). It is important to point out that the nanoparticles were submerged in carbonate buffer, and in carbonate plus salt buffer (333 mmol/L of NaCl).

2. **Assert the targeting and specific behavior of the construct joined to its cytotoxic effects.**

The cationic peptide T22 targeting performance and the nanoparticle recombinant platform production success was already confirmed in several evaluations[50, 164, 165, 122] in conjunction with a His-tag in the C-terminal. For T22mRTAH6 NPs, different expression levels of CXCR4 receptor were found on 3T3, MV411, THP1 and HeLa cell lines. (Article 1, Fig. 2C and 2D) and the internalization pattern of NPs was achieved in concordance with the level of receptor expression. With these experiments the directed and specific receptor-mediated behavior was confirmed with better results than the conventional chemotherapeutic agent cytosine arabinoside Ara-C. Also, the CXCR4 antagonist AMD3100 was used to verify the specificity in a 10:1 molar ratio.

When the ricin NPs were tried *in vivo* using the disseminated AML mouse model, the bioluminescence emitted by mice (Article 1, Fig. 5A) was localized in the desired areas, while the *ex vivo* luminescence (Article 1, Fig. 5B) of affected organs further studied (backbone, hindlimbs, liver or spleen) showed a positive result when soluble NPs were injected in systemic administrations by the vein tail. Amazingly, histopathology experiments

confirm that no toxicity is observed in off-target organs, such as heart, lung, kidney (Article 1, Figure 6).

When CTX was conceived as the targeting and therapeutic part, potentially both functions in one, the protein design objective was to evaluate its ability to interact only with carcinogenic cells. Two different antibodies, anti-Annexin A2 mAb and pAb were used to verify the receptor-dependent internalization behavior (Article 2, Fig 2b). Other investigators employed the MMP2 inhibitor, 1,10-phenanthroline (50μM) in order to block the receptor entry. [145]

The multifunctional toxin and venom compounds played successfully their actions in our research.

3. Overcoming the targeting recombinant self-assembling protein-only therapeutic NPs limitations.

Protein and peptides are prominent therapeutic agents for cancer. An important pharmacological restriction observed, even in clinical trials, is related to biological immunogenic responses after several doses, reproducibility issues, and no information about long-term toxicity and protein degradation. Polymeric encapsulation is a recurrent way to overcome immunogenicity[57]. However, dynamic delivery systems could show different performances than the studied ones without encapsulation, especially in targeting and specificity. PEGylation has shown satisfactory results in protein NPs[166] as the half-life of the drug could be amplified and the immunogenicity reduced.

Nevertheless, there is a better method called lipidation, which offers the effect of intracellular improvement by enabling intracellular uptake. Adding a lipid group to the protein design[167] consolidates lipidation. It is helpful for the

endosomal escape mediated by detached lipids and half-life improvement because it enables albumin binding to a drug molecule. Also, longer circulation times have been observed[168]. Other advantage is the possible administration of the drug via topical, pulmonary or orally, besides systemically[169]. For the lasts reasons the FDA has already approved more than 400 protein drugs[170]. Cytotoxic proteins lead a new generation of efficient and precise antitumor drugs.

Our NPs are promising as real therapeutic anticancer agents. Some chemotherapeutics in the market such as paclitaxel, cisplatin, doxorubicin and etoposide present disadvantages as low specificity and undesired side effects that have been mostly overtaken by the new generation of NPs of our research group. Many immunotherapeutic latest generation monoclonal antibodies (mAbs) were approved recently. Some examples include Ipilimumab (Yervoy®), trastuzumab (Herceptin®), alemtuzumab (Campath®), bevacizumab (Avastin®) and cetuximab (Erbitux®). Similarly, some Immune checkpoint inhibitors block the interactions between PD-1 receptors with cancer cells and on T-cells, such as Pembrolizumab (Keytruda®) and Atezolizumab (Tecentriq®). [171]

Modern protein-based or peptide-based NPs are conjugated or encapsulate protein derivations as active agents. Our group choice is betting for protein-only NPs, which assures full biocompatibility and biodegradability of the drug. The usage of serum proteins as a carrier is aimed at humanizing proteins which, as the next step of our NPs, GFP could be substituted by albumin for deeper *in vivo* experiments. Human serum albumin is a 67kDa protein abundant in the blood, which has a half-life of 19 days and is reabsorbed from systemic clearance with a low rate of metabolism by the

liver. An albumin-drug example that is already approved is Nab-paclitaxel (Abraxane®). A biological method known as one-bead one-compound (OBOC) is helpful for finding suitable peptides for a specific target[172]. The presence of functional peptides in NPs offers unique advantages, as high cell penetrability, Sandostatin LAR® for instance.

With the examples cited above it is remarkable that the research pathway by which our research group is focused, is convincing for achieving a useful nanomedicine in the market soon.

Conclusions

Conclusions

- The incorporation of the cytotoxic proteins ricin and chlorotoxin to the recombinant building block platform was successfully achieved.
- T22-mRTA-H6 evaluation confirmed the effectiveness of the targeting peptide and NP design, and showed that functional soluble protein is more effective for this particular construct than the insoluble counterpart. The targeting effectiveness was evaluated using a 10:1 molar ratio of AMD3100 (CXCR4 antagonist) and different cell lines with expression or no-expression of CXCR4, showing different levels of protein internalization in concordance with CXCR4 level.
- The most important *in vivo* ricin limitation against cancer, VLS and toxicity that leads to organ failure, has been completely overcome in soluble T22-mRTA-H6 NPs with consistent reduction of malignant cells in complete absence of systemic and histological toxicity when an intravenous dose of 10μg was applied daily for 10 days in AML mice model.
- IBs conditions could be regulated in order to upgrade the functional protein release from amyloidal aggregates to the maximum improving the cell viability experiments in order to eliminate more carcinogenic cells.
- CTX was subjected to an oligomer engineered nanostructured technology with a solid nanoparticle structural conformation corroborated by DLS and FESEM.
- CTX-KRKRK-GFP-H6 could be proposed as marker for brain tumor resection fluorescent-guided surgeries as, particularly, in U87MG cells

presented high internalization patterns in a receptor-dependent mechanism, analyzed by intracellular fluorescence quantification after 24h of exposure to the protein.

Annexes

Article

Protein-Based Therapeutic Killing for Cancer Therapies

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

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Cell killing therapy is important when the objective is to eradicate malignant cells. Conventional chemotherapeutics administered systemically at specific doses have achieved some advance. For this reason, cytotoxic proteins are useful for being incorporated as novel drug compounds.

The importance of cell-targeting is crucial in cancer therapy in order to direct cell-killing agent only to malignant tissue and avoid damaging healthy cells[173]. There is a wide plethora of cytotoxic proteins that may be engineered in nanotechnology, such as venoms[174], plant toxins[175], microbial and animal toxins[176], antimicrobial peptides[177], mAbs and proapoptotic proteins[178]. They are cost-effective as they can be produced by recombinant methods, and offer a versatile structural and functional performance because they can be self-assembled and targeted nanoscale prototypes.

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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

[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]Centro de Investigación Biomédica en Red (CIBER) de Bioingeniería, Biomateriales y Nanomedicina, 08193 Cerdanyola del Vallès, Spain
[4]Institut d'Investigacions Biomèdiques Sant Pau and Josep Carreras

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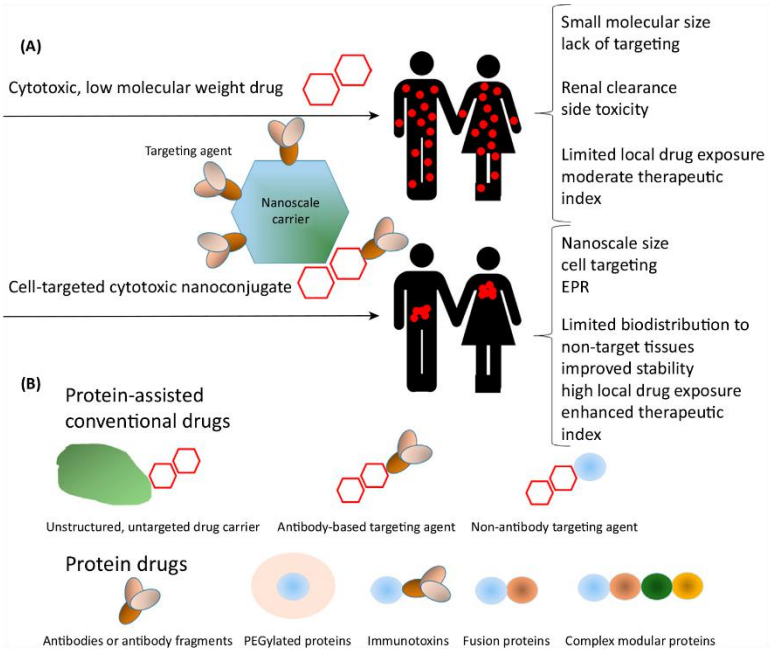

Figure 1. Spectrum of Current Antitumoral Drugs and their Relevant Features. (A) The chemotherapy of cancer is commonly approached by the use of low molecular weight chemicals (red symbols and dots) that display generic cytotoxicity to both tumor and healthy cells. Their low molecular size (usually <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

Research Institute, Hospital de la Santa Creu i Sant Pau, 08025 Barcelona, Spain

*Correspondence: rmangues@santpau.cat (R. Mangues) and Antoni.Villaverde@uab.cat (A. Villaverde).

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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

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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

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].

activated by its ligand VEGF for cell signaling.

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)-PE38]	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 NCT02271756
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 antiapoptotic 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	*Acanthoscumia 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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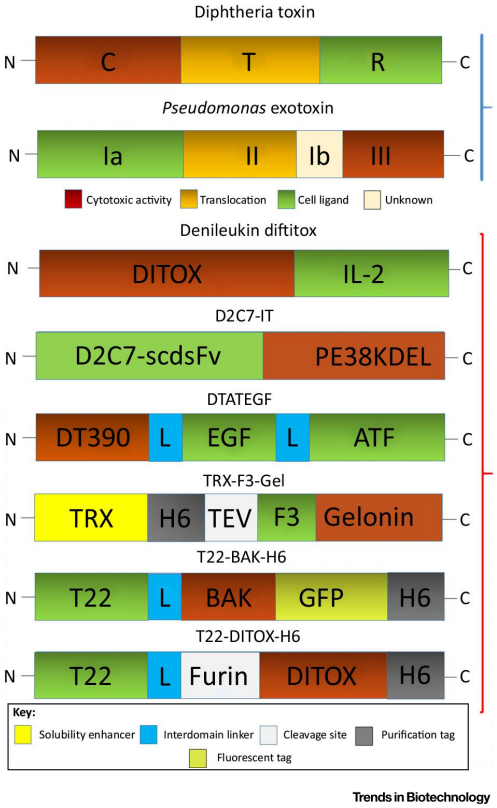

Figure 2. Modular Organization of Natural and Representative Engineered Toxins. Natural toxins (blue set) usually show a modular architecture, illustrated here by diphtheria toxin (DT) and *P. aeruginosa* exotoxin A (PE). Engineered versions (red set) have been adapted by modular protein engineering 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.

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

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

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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Glossary

- ACT (adoptive cell transfer) is the transfer of cells into a patient. The goal of the transfer is improve the immune system functionality. When T cells are extracted from the patient, genetically modified in vitro and returned to the same patient is called autologous cancer immunotherapy. Oppositely when cells are isolated and expanded from a donor and transplanted to the patient is known as allogenic therapy.
- AML (acute myeloid leukemia) is a cancer of the myeloid line of blood cells, characterized by the rapid growth of abnormal cells that interfere eith normal blood cells and gather in the bone marrow and blood.
- AML in remission. It starts with the first phase of AML treatment, called remission-induction therapy, where a high-dose chemotherapy is applied to kill leukemia cells in the blood and bone marrow. Complete remission is achieved when there are no signs of leukemia cells (blasts) in the bone marrow, there is absence of symptoms of AML and the blood count (number of blood cells) is considered normal.
- AML1-ETO is the product of the chromosomal translocation t(8;21)(q22;q22) that usually occurs in acute myeloid leukemia (AML) with granulocytic differentiation, FAB M2 subtype.
- Annexin A2 is a protein encoded by the ANXA2 gene and it is involved in diverse cellular processes (cell motility, linkage of membrane-associated protein complexes to the actin cytoskeleton, endocytosis, fibrinolysis, ion channel formation, and cell matrix interactions). It is a calcium-dependent phospholipid-binding protein whose function is to help organize exocytosis of intracellular proteins

to the extracellular domain. A2 is a pleiotropic protein because its function is dependent on place and time in the body.

- Ara-C (cytosine arabinoside) is a chemotherapy drug used to treat acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelogenous leukemia (CML), and non-Hodgkin's lymphoma.
- ATRX and Tert mutations. Telomerase reverse transcriptase (TERT) and the α thalassemia/mental retardation syndrome X-linked (ATRX) genes are involved in the classification and prognosis of glioma.
- BM (bone marrow) is a semi-solid tissue located within the spongy or cancellous portions of bones. BM comprises 5% of total body mass and it is the primary site of new blood cell production or hematopoiesis. It is composed of hematopoietic cells, marrow adipose tissue, and supportive stromal cells.
- bsAbs (bispecific antibodies) are protein that can simultaneously bind to two different types of antigens.
- CBF-AML (core binding factor). The CBF is a group of heterodimeric transcription factors that are composed of 1) a non-DNA-binding CBFβ chain (CBFB). 2) a DNA-binding CBFα chain (RUNX1, RUNX2, RUNX3). A transcription factor (TF) or sequence-specific DNA-binding factor is a protein that controls the rate of transcription of genetic information from DNA to messenger RNA, by binding to a specific DNA sequence.
- CD (common differentiation). The cluster of differentiation is a protocol used for the identification of cell surface molecules providing targets for immunophenotyping of cells. CD molecules can act as receptors or ligands. The CD system is commonly used as cell markers in

immunophenotyping, allowing cells to be defined based on what molecules are present on their surface. The markers are used to associate cells with certain immune functions.

- CDK4 (cyclin-dependent kinase 4) is a member of the cyclin-dependent kinase family. Enzymes help accelerating chemical reactions.
- CDK4/MDM2 amplification or non-amplified patients. Gene amplification is an increase in the number of copies of a gene without a proportional increase in other genes. A duplication of a region of DNA that contains a gene through errors in DNA replication and repair machinery can produce amplifications.
- Chromosome 8q22. A chromosome is a deoxyribonucleic acid (DNA) molecule with part or all of the genetic material (genome) of an organism.
- CN-AML (normal cytogenetics in AML). Cytogenetics studies how the chromosomes relate to cell behavior during mitosis and meiosis.
- CNS (central nervous system) consists of the brain and spinal cord. The CNS integrates the received information and coordinates the activity of all parts of the bodies.
- Codeletion of chromosomal arms 1p and 19q. Complete deletion of both the short arm of chromosome 1 (1p) and the long arm of chromosome 19 (19q) (1p/19q co-deletion) is the molecular genetic signature of oligodendrogliomas. This molecular alteration is the result of an unbalanced whole-arm translocation between chromosomes 1 and 19 with the loss of the derivative t(1p;19q), which occurs early in the pathogenesis of oligodendrogliomas. 1p/19q co-deletion is a

valuable diagnostic, prognostic and predictive biomarker for the management of oligodendroglial tumors.

- Codon. In DNA or RNA, a sequence of 3 consecutive nucleotides that codes for a specific amino acid or signals the termination of gene translation.
- CR (complete response) occurs when all target lesions have disappeared during the course of a treatment. SD (stable disease) occurs when there is no significant decrease or increase in the size of target malignant cells.
- CXCL12-CXCR4-ACKR3 axis can be a therapeutic target because it interferes with cell migration and modulates immune responses. Chemokine receptors are cytokine receptors located on the surface of certain cells that interact with a type of cytokine known as chemokine. There are 20 chemokine receptors discovered in humans. Chemokine receptors are divided into different families, CXC chemokine receptors, CC chemokine receptors, CX3C chemokine receptors and XC chemokine receptors that correspond to the 4 distinct subfamilies of chemokines they bind. Four families of chemokine receptors differ in spacing of cysteine residues near N-terminal of the receptor
- Danorubicin is a chemotherapy drug used to treating acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), chronic myelogenous leukemia (CML), and Kaposi's sarcoma.
- DFS (disease free survival) is related to the measure of time after treatment during which no sign of cancer is found.
- dgRTA (deglycosylated RTA). The removal of the sugar entity from a glycogen or glycoprotein, which has covalently-bonded carbohydrates.

- Difference between gliomas. A glioma is a type of brain tumor that grows from glial cells. Glial cells support nerve cells with energy and nutrients and help maintain the blood-brain barrier. There are various types of glial cells, each with a different function; astrocytes transport nutrients and holds neurons in place,

  oligodendrocytes provide insulation (myelin) to neurons, microglias digest dead neurons and pathogens and ependymal cells line the ventricles and secrete cerebrospinal fluid. Glioma is a general term that encompasses glial tumors, such as astrocytoma, oligodendroglioma, and glioblastoma, which are different in their aggressiveness, some are slow growing, and others are very invasive.
- DN MT3A mutations. DNMT3A gene mutation is associated with poor prognosis in AML.
- DNA loop. A displacement loop or D-loop is a DNA structure where the two strands of a double-stranded DNA molecule are separated for a stretch and held apart by a third strand of DNA. An R-loop is similar but the third strand is RNA rather than DNA.
- DNA/RNA-based aptamers. Aptamers are oligonucleotide or peptides that bind to a specific target molecule. Aptamers are classified as 1) DNA or RNA aptamers that consist of short strands of oligonucleotides. 2) Peptide aptamers that consist of short variable peptide domains, attached at both ends to a protein scaffold.
- Dysregulation of antigen presentation. Antigens (Ag) are structural molecules that bind specifically to an antibody only in the form of native antigen. It also refers to any molecule or fragment after

processing the native antigen that can be recognized by a T-cell receptor (TCR).

- Exon 4 codon 132 (R132H). Exon and introns are features of DNA, whereas codons are features of RNA.
- FLT3 gene (Fms-like tyrosine kinase 3 mutation) is a class III receptor tyrosine kinase expressed in early hematopoietic progenitors that are $CD34^{+}$/c-Kit^{+}. When bound by its ligand (FLT3 ligand or FL) the receptor dimerizes, leading to activation of the receptor's intrinsic tyrosine kinase activity. The activated kinase signals through a variety of pathways including the phosphatidylinositol 3-kinase (PI3K) and RAS signal-transduction cascades through phosphorylation of cytoplasmic substrates. Two major classes of FLT3-activating mutations have been identified in AML, internal tandem duplication (ITD) in the juxtamembrane domain and point mutations in the tyrosine kinase domain (TKD). Both classes of mutations result in ligand-independent constitutive activation of the receptor's kinase activity and induce factor-independent growth of the murine pro-B–cell line Ba/F3. FLT3 mutations are frequent cooperating lesions, being found not only in cytogenetically normal AML, but in APL, CBF AML, and MLL-rearranged leukemia as well. The tyrosine kinase pathway constitutes a very important cellular signal transduction pathway. Tyrosine kinases can be grouped into two classes: receptor tyrosine kinases and non-receptor tyrosine kinases (without extracellular binding domains). When cellular tyrosine phosphorylation is enhanced, for instance, by a growth factor to the receptor tyrosine kinase, this triggers a cascade of downstream signals, thereby affecting many different cellular

functions. Importantly, many of the cellular tyrosine kinases are frequently products of proto-oncogenes and their aberrant expression has been associated with many different human cancer types. One of the best-studied families of tyrosine kinases is the epidermal growth factor receptor (EGFR) family. The erbB family consists of four different types of receptor tyrosine kinase, including erbB-1 (also known as EGFR), erbB-2 (HER-2/neu), erbB-3 (HER-3), and erbB-4 (HER-4).

- Folate is one of the B vitamins.
- G-proteins are a family of proteins are involved in transmitting signals from a variety of stimuli outside a cell to its interior. Their activity is regulated by factors that control their ability to bind to and hydrolyze guanosine triphosphate (GTP) to guanosine diphosphate (GDP).
- GAGs (glycosaminoglycans) are long linear polysaccharides consisting of double disaccharide units.
- GBM (glioblastoma) is the most aggressive brain cancer.
- Gene HOX, a subset of homeobox genes, are a group of related genes that specify regions of the body plan of an embryo along the head-tail axis of animals. Hox proteins encode and specify the characteristics of position, ensuring that the correct structures form in the correct places of the body.
- GSH (glutathione) is an antioxidant capable of preventing damage to important cellular components caused by reactive oxygen species such as free radicals, peroxides, lipid peroxides, and heavy metals.
- HCV (hepatitis C virus) is an infectious disease caused by the hepatitis C virus (HCV) that primarily affects the liver.

- HIV-TAT peptide. Tat (trans-activator of transcription) is a protein that is encoded by the tat gene in HIV-1. Tat is a regulatory protein that drastically enhances the efficiency of viral transcription.
- IDH (isociatrate degydrogenase) is an enzyme that catalyzes the oxidative decarboxylation of isocitrate, producing alpha-ketoglutarate (α-ketoglutarate) and CO_2. IDH1 or IDH2 mutation occur in the vast majority of low grade gliomas and secondary high grade gliomas and they drive increased methylation. Gliomas with mutated IDH1 and IDH2 have improved prognosis compared to gliomas with wild-type IDH.
- IgG (Immunoglobulin G) is a type of antibody. Representing approximately 75% of serum antibodies in humans, IgG is the most common type of antibody found in blood circulation. IgG molecules are created and released by plasma B cells.
- Indoleamine 2, 3 dioxygenose Indoleamine-pyrrole 2,3-dioxygenase (IDO or INDO) is a heme-containing enzyme that in humans is encoded by the IDO1 gene.
- ITD (internal tandem duplications) mutations of the FLT3 gene (FLT3/ITD mutations) are the most frequent molecular abnormality in AML and are associated with a poor overall survival.
- LSCs (leukemia stem cells) in AML represent a low-frequency subpopulation of leukemia cells that possess stem cell properties distinct from the bulk leukemia cells, including self-renewal capacity and drug resistance. Due to these properties, LSCs are supposed to facilitate the development of relapse.

- mAbs (monoclonal antibodies) are antibodies that have monovalent affinity, in that they bind to the same epitope (the part of an antigen that is recognized by the antibody). In contrast, polyclonal antibodies bind to multiple epitopes and are usually made by several different plasma cell lineages. Bispecific monoclonal antibodies increase the therapeutic targets to two epitopes.
- MAPK (mitogen-activated protein kinase) A mitogen-activated protein kinase (MAPK or MAP kinase) is a type of protein kinase that is specific to the amino acids serine and threonine. MAPKs are involved in directing cellular responses to a diverse array of stimuli, such as mitogens, osmotic stress, heat shock and proinflammatory cytokines. They regulate cell functions including proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis.
- MDSCs (myeloid-derived suppressor cells) are a heterogenous group of immune cells from the myeloid lineage. MDSCs strongly expand in pathological situations such as chronic infections and cancer, as a result of an altered haematopoiesis. MDSCs interact with other immune cell types including T cells, dendritic cells, macrophages and natural killer cells to regulate their functions.
- Methylation is a process by which methyl groups are added to the DNA molecule. Methylation can change the activity of a DNA segment without changing the sequence. When located in a gene promoter, DNA methylation typically acts to repress gene transcription.

- MGMT (O6-methylguanine- DNA methyltransferase) is a protein that in humans is encoded by the O^6-methylguanine DNA methyltransferase (MGMT) gene. It is crucial for genome stability. It repairs the naturally occurring mutagenic DNA lesion O^6-methylguanine back to guanine and prevents mismatch and errors during DNA replication and transcription.
- MMP2, MMP9 (matrix metalloproteinases) are a family of proteolytic enzymes implicated in the invasion and metastasis of many kinds of cancer because of their ability to degrade components of extracellular matrix. Specific tissue inhibitors of matrix metalloproteinases (TIMPs) regulate their activity.
- NK-cells (Natural killer cells) are a type of lymphocyte (white blood cells) and a component of innate immune system. NK cells play a major role in the host-rejection of both tumours and virally infected cells.
- NPM1 mutation in AML is characterized by accumulation of myeloid cells in the bone marrow because of impaired differentiation and proliferation, resulting in hematopoietic insufficiency.
- Oligomerization refers to the process of converting a monomer or a mixture of monomers into an oligomer. Oligonucleotides are short DNA or RNA molecules, oligomers, that can be applied in genetic testing, research, and forensics.
- OS (overall survival) The length of time from either the date of diagnosis or the start of treatment, which patients diagnosed with the disease are still alive.
- Phosphatidylinositol 3-kinase Phosphoinositide 3-kinases (PI3Ks), are a family of enzymes involved in cellular functions such as cell growth,

proliferation, differentiation, motility, survival and intracellular trafficking, which in turn are involved in cancer.

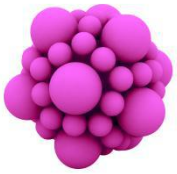
- Phospholipase C (PLC) is a class of membrane-associated enzymes that cleave phospholipids just before the phosphate group.
- rrAML (relapsed/refractory AML). In refractory leukemia most patients achieve a remission, an absence of signs and symptoms, after the initial treatment. However, some patients have residual leukemic cells in their marrow even after intensive treatment. This is referred to as refractory leukemia. For refractory AML, treatment options may include drugs that were not used during the first course of treatment. Stem cell transplantation may be used when remission is achieved, which may result in a more durable remission. In relapsed leukemia some patients reach remission and then have a return of leukemia cells in the marrow and a decrease in normal blood cells.
- RUNX1 Runt-related transcription factor 1 is a transcription factor that regulates the differentiation of hematopoietic stem cells into mature blood cells. In addition it plays a major role in the development of the neurons that transmit pain.
- STAT3 (transcription factor 3) is a member of the STAT protein family. A transcription factor (TF) is a protein that controls the rate of transcription of genetic information from DNA to messenger RNA, by binding to a specific DNA sequence.
- T-cells are a type of lymphocyte, which develops in the thymus gland and plays a central role in the immune response. T cells can be distinguished from other lymphocytes by the presence of a T-cell receptor on the cell surface. These immune cells originate as precursor

cells, derived from bone marrow, and develop into several distinct types of T cells once they have migrated to the thymus gland. A lymphocyte is one of the subtypes of a white blood cell that includes natural killer cells, T cells, and B cells.

- T22 peptide [Tyr5,12,Lys7]-polyphemusin II peptide, is a short, positively charged peptide, analog of polyphemusin peptide which exerts a strong anti- HIV activity. Polyphemusins are known to inhibit the growth of gram-negative bacteria and some fungi such as Candida Albicans. T22 peptide is a potent antagonist of CXCR4 (IC_{50} = 5.05 nM), a co-receptor used by HIV to penetrate cells. It has been suggested that T22 peptide exerts its effect against HIV-1 especially by blocking virus-cell fusion and that T22 might interact with HIV envelope protein (such as gp120) and T-cell surface protein.
- TET-2 (ten- eleven translocation 2) is a human gene that resides at chromosome 4q24, in a region showing recurrent microdeletions and copy-neutral loss of heterozygosity (CN-LOH) in patients with diverse myeloid malignancies. A gene is a sequence of nucleotides in DNA or RNA that encodes the synthesis of a gene product, either RNA or protein.
- TKI (Tyrosine kinase inhibitors) are a type of targeted therapy and they come as pills, taken orally. Four TKI drugs are approved as initial therapy; Imatinib mesylate (Gleevec®), Dasatinib (Sprycel®), Nilotinib (Tasigna®), Bosutinib (Bosulif®).
- VLS (vascular leak syndrome) is a dose-limiting toxicity effect of immunotoxin (IT) therapies. VLS is characterized by an increase in

vascular permeability accompanied by extravasation of fluids and proteins resulting in interstitial edema and organ failure.

- α-ketoglutarate----2-hydroxyglutarate α-Hydroxyglutaric acid (2-hydroxyglutaric acid) is an alpha hydroxy acid form of glutaric acid. α-Hydroxy acids (AHAs), are a class of chemical compounds that consist of a carboxylic acid substituted with a hydroxyl group on the adjacent carbon.



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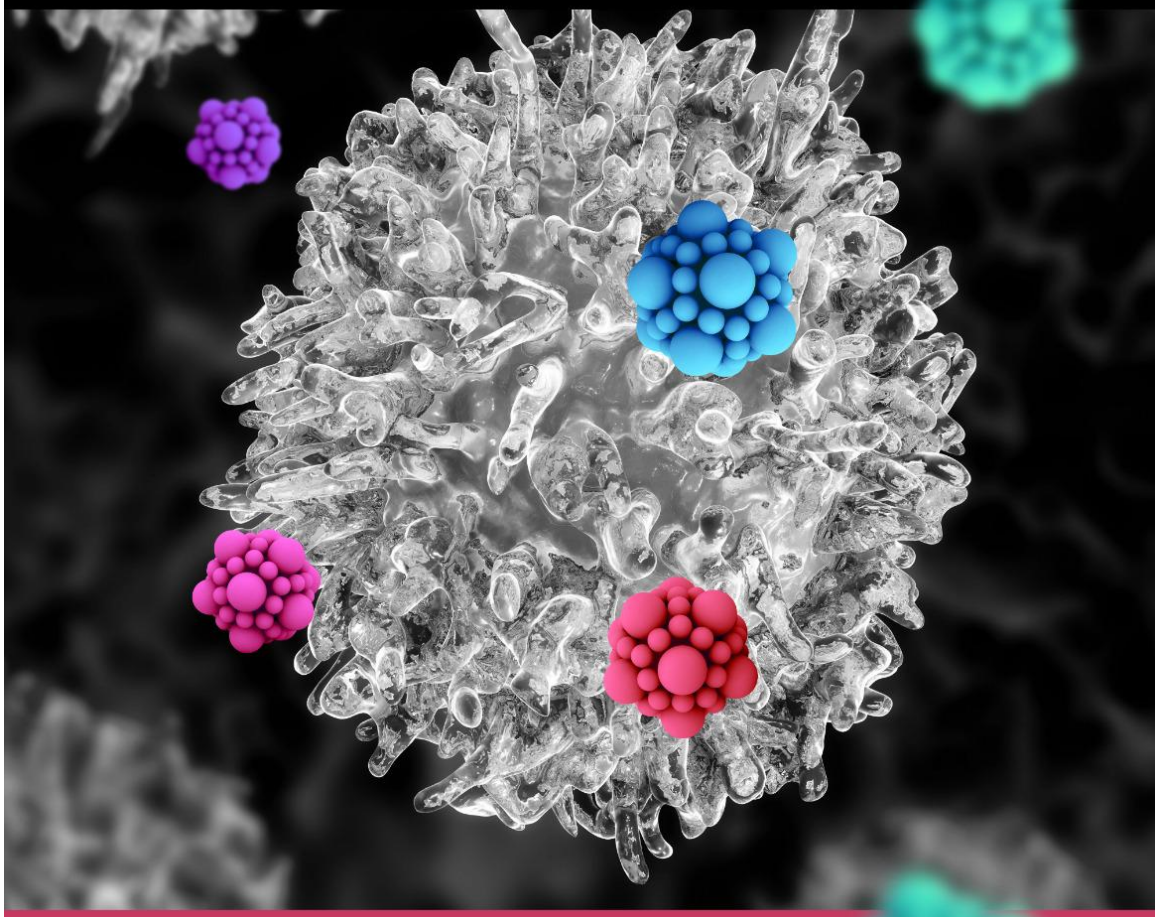

Raquel Díaz Ocaña

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