Design and engineering of tumor-targeted, dual-acting cytotoxic nanoparticles

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Abstract

The possibility to conjugate tumor-targeted cytotoxic nanoparticles and conventional antitumoral drugs in single pharmacological entities would open a wide spectrum of opportunities in nanomedical oncology. This principle has been explored here by using CXCR4-targeted self-assembling protein nanoparticles based on two potent microbial toxins, the exotoxin A from *Pseudomonas aeruginosa* and the diphtheria toxin from *Corynebacterium diphtheriae*, to which oligo-floxuridine and monomethyl auristatin E respectively have been chemically coupled. The resulting multifunctional hybrid nanoconjugates, with a hydrodynamic size of around 50 nm, are stable and internalize target cells with a biological impact. Although the chemical conjugation minimizes the cytotoxic activity of the protein partner in the complexes, the concept of drug combination proposed here is fully feasible and highly promising when considering multiple drug treatments aimed to higher effectiveness or when facing the therapy of cancers with acquired resistance to classical drugs.

Keywords: Recombinant proteins; nanoparticles; drug delivery; drug combination; hybrid materials

1. Introduction

Side toxicities linked to current chemotherapies for cancer limit the usable drug doses and prevent the drug from reaching effective local concentrations [1-4]. In this regard, tumor-targeted drug delivery is an unmet therapeutic need, although largely pursued through different developmental approaches [5-11]. Most of them are based on nanomedical concepts, in which a nanoscale drug carrier is functionalized with a ligand of a cell surface marker that is selectively overexpressed in target cancer cells [5, 12-14]. Such functional combination not only benefits from the targeting event endorsed by the ligand, but also from the whole nanoscale drug size that prevents renal filtration and exploits the enhanced permeability and retention (EPR) effect [9, 15-18]. However, targeting attempts have been in general unsuccessful *in vivo* (with only 1-2 % of the administered drug reaching the target) [19, 20]. Even more importantly, the non-therapeutic drug carrier, that represents around 90 % of the whole nanoconjugate (carrier plus drug) mass, imposes a limitation in the drug doses and generates severe concerns regarding the potential intrinsic toxicity of the vehicle and its prevalence and accumulation in both body and environment [21]. Therefore, the emerging concept of self-assembling drugs in the nanoscale, delivered in absence of any heterologous

carrier and acting simultaneously as drug and vehicle, is highly appealing but difficult to reach by conventional chemistry [21].

In such a challenging scenario, proteins represent multifunctional and biocompatible materials, versatile enough for the development of new generation, cell-targeted protein drugs at the nanoscale [22-24]. Protein engineering is not only resulting in the fabrication of a diversity of nano- and micro-scale protein materials [25], but it also enables the generation of vehicle-free nanoscale drugs for tumor-targeted delivery. Since many proteins show cell-killing activities [24], the incorporation of oligomerization peptides and tumor-homing peptides in form of modular proteins results in self-assembling, self-targeted and self-delivered drug nanoparticles that promote efficient and selective destruction of tumor tissues [26]. Recently, microbial [27] and plant [28] toxins, venoms [29] and also pro-apoptotic factors [30, 31] have been engineered under this concept as powerful and highly selective nanoscale drugs in oncology, that self-organize as robust protein-only nanoparticles upon their production in recombinant microorganisms, with sizes and superficial charges compatible with those favoring stability and cell delivery [32]. The assembling as nanostructured materials ensures not only high stability in blood [33], but also a proper organ biodistribution provided homing peptides are incorporated in the constructs [15]. Although the use of recombinant proteins as therapeutic agents might also pose some concerns regarding immunogenicity, the tendency to use human or humanized proteins as drugs [34-38] and the improved genetic procedures to de-immunize heterologous proteins [39-41], exemplified by the *Pseudomonas aeruginosa* exotoxin A [39], should solve this potential issue. In addition, the high number of biopharmaceuticals approved for use by the FDA and EMA [36, 42-44] demonstrate that other potential concerns linked to the presence of bacterial endotoxins and other contaminants from the cell factories can be smoothly solved even at large scale.

On the other hand, using the same protein engineering principle based on a cationic N-terminal peptide and a C-terminal polyhistidine, we have also generated self-assembling, biologically inert protein carriers for the targeted delivery of the conventional chemical drugs oligo-floxuridine (FdU) and monomethyl auristatin E (MMAE) [45, 46], to which they are chemically coupled [47]. In that case, the protein core of the vehicle consists of biologically irrelevant polypeptides such as GFP, which act as scaffold carriers. The high antitumoral and antimetastatic effect of the resulting nanoconjugates was excellent [45, 48], resulting in clinically promising products for further development in oncology. Combining concepts from both approaches, we wondered if it would be possible to use intrinsically cytotoxic protein nanoparticles (those based on toxins) as carriers of conventional small molecular weight

antitumoral drugs. This would allow integrating, in single tumor-targeted nanoparticles, the therapeutic value of the protein and the chemical drug. The generation of such hybrid nanoscale materials has been explored here by conjugating the above small molecular weight agents with tumor-targeted protein-only cytotoxic nanoparticles. The obtained data validate the proposed concept but also reveal methodological bottlenecks, offering clues for the further development of this category of innovative materials.

2. Material and methods

2. 1 Protein architecture, production and purification

Protein nomenclature was established according to their modular organization (Figure 1A). T22-DITOX-H6 is a CXCR4-targeted toxin comprising domains A and B from *Corynebacterium diphtheriae* toxin, responsible for cytotoxic and translocation functionalities, respectively. T22-PE24-H6 is a CXCR4-targeted toxin based in the de-immunized catalytic domain of *Pseudomonas aeruginosa* exotoxin A, including a C-terminal KDEL motif to control its intracellular location. Both constructs were produced and purified as previously described [26]. Protein integrity and purity were assessed by Matrix Assisted Laser Desorption Ionization – Time of Flight (MALDI-TOF) mass spectrometry, SDS-PAGE and Western blot using anti-His monoclonal antibodies (SC-57598 #B2613, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein concentration was determined by the Bradford assay.

2.2 In silico analyses

Three-dimensional models of monomeric entities were obtained by Robetta comparative modelling approach [49] through the Robetta web server (http://robetta.bakerlab.org). In both cases, the full amino acid sequences were introduced as queries. For T22-DITOX-H6, the template was the catalytic and translocation domains from *Corynebacterium diphtheriae* toxin (PDB ID 1MDT, residues 1-386, named here as DITOX) [50]. Likewise, for T22-PE24-H6, the C-terminal domain of *Pseudomonas aeruginosa* exotoxin A (PDB ID 1IKQ, residues 395-606) was used [51]. Parameters were set to 100 sampling models, 3 register shifts and a probability of 0.3 of sampling fragments within template regions. Secondary structure of residues 8-10 and 13-15 from both proteins was constrained to a beta sheet, according to its characterized structure [52]. After the modelling process, candidates were selected according to their estimated error. UCSF Chimera [53] was used to represent three-dimensional structures showing their secondary structure and atomic surface.

2.3 Chemical conjugation

FdU refers to a penta-oligonucleotide made of 5 units of 5-fluoro-2'-deoxyuridine (floxuridine) [32, 34]. T22-PE24-H6 FdU nanoconjugates were generated by the covalent binding of FdU molecules through protein lysine-amines in a two-step reaction, using a bifunctional cross-linker as described [47]. In short, thiolated FdU molecules were first reacted with the maleimide group of a 4-maleimido hexanoic acid N-hydroxy-succinimide ester (EMCS) bifunctional cross-linker in a 1:1 molar ratio for 10 min at RT. Then FdU-linker molecules were subsequently reacted with the protein in a 1:5 molar ratio, overnight at room temperature, generating an amide bond between the ester group of the bifunctional linker and the solvent-exposed Lysine-amines. Finally, T22-PE24-H6 FdU were dialyzed against their storage buffer (sodium carbonate buffer, 166 mM $NaHCO_3$, pH 8) using 12-14 MWCO membranes to remove remaining free FdU molecules and centrifuged at 15,000 g during 15 min to remove insoluble aggregates. The average conjugation efficiency was calculated by dividing the number of FdU molecules attached in the main population by the ratio of FdU molecules in reaction (1:5). The concentration of each protein in formulation was set to 2 mg/mL.

T22-DITOX-H6 was conjugated with maleimide functionalized Monomethyl Auristatin E (MC-MMAE, 911 g/mol, Levena Biopharma) through solvent solvent-exposed lysine-amines at four different protein : MC-MMAE molar ratios (1:5, 1:10, 1:20, 1:50) upon incubation for 4 h at room temperature in one-pot reaction [45]. As in FdU conjugates, T22-DITOX-H6 MMAE conjugates were then dialyzed against sodium carbonate buffer, using 12-14 MWCO membranes to remove non-reacted free MC-MMAE molecules, and finally centrifuged at 15,000 g during 15 min to remove insoluble aggregates. The average conjugation efficiency was calculated by dividing the number of MMAE molecules attached in the main population by the ratio of MMAE molecules in reaction at each case. The concentration of each protein in formulation was set to 2 mg/mL.

2.4 Physicochemical characterization

The molecular weight of protein conjugates was measured by MALDI-TOF, SDS-PAGE and anti-His Western blot, as done for purified proteins before conjugation. The Bradford assay was used to measure protein concentration. The ratio of FdU or MMAE units per protein was estimated through the analysis of MALDI-TOF spectra, taking into account their respective molecular weights. Volume size distribution and Z potential of proteins before and after drug

conjugation were measured in triplicate by Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS) respectively, at 633 nm, in a Zetasizer Nano ZS (Malvern Instruments Limited, Malvern, Worcestershire, UK). Experiments were performed at pH 8.0. Circular dichroism measurements were made with a JASCO J-715 spectropolarimeter (JASCO, Oklahoma City, OK) using a 0.2 mm path length quartz cell. Each spectrum was an average of ten scans. The protein concentration was adjusted to 0.2 mg/mL in sodium carbonate buffer, at pH 5.6 or pH 8 respectively. Scan speed was set at 50 nm/min with a 1 s response time. Measurements were obtained as ellipticity in millidegrees (mdeg) in the 200-260 nm region. The spectra were processed through a negative exponential with a sampling proportion of 0.1 and 1 polynomial degree.

2.5 Cell culture

$CXCR4^+$ HeLa (ATCC® CCL-2™), $CXCR4^+$ THP-1 (acquired from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), $CXCR4^-$ SW1417 (ATCC® CCL-238™) and $CXCR4^-$ PANC-1 cells (ATCC® CRL-1469™) were used for the functional characterization of protein and protein conjugates *in vitro*. HeLa and THP-1 cells were maintained in MEM-α and RPMI-1640 culture media respectively supplemented with 10 % fetal calf serum (Gibco, Thermo Fisher, Waltham, MA, USA). SW1417 and PANC-1 cells were maintained in DMEM supplemented with 10 % fetal calf serum. HeLa and THP-1 cells were cultured at 37 ºC in a 5 % humidified atmosphere, while SW1417 and PANC-1 were cultured at 37 ºC in a 10 % humidified atmosphere.

2.6 Characterization of cellular expression of CXCR4

To detect cell surface expression of CXCR4 in each cell line, fluorescence-activated cell sorting (FACS) analysis was performed as described [54]. Cell Quest Pro software (BD Biosciences, San Jose, CA, USA) was used to analyze data and results were expressed as mean fluorescence intensity (MFI) $\pm$ standard error of the mean (SEM). Two technical and two biological replicates were performed for each cell line. CXCR4 levels are shown in Supplementary Figure 1A.

2.7 Cell viability assay

Cytotoxicity of the recombinant protein toxins and their respective nanoconjugates with FdU or MMAE was determined by a CellTiter-Glo® Luminiscent Cell Viability Assay (Promega, Madison, WI, USA), carried out as previously described [46]. For that, HeLa or THP-1 cells were

cultured in opaque-walled 96-well plates at 3,500 cells/well or 30,000 cells/well respectively for 24 h at 37 ºC until reaching 70 % confluence. Cells were then incubated in presence of different concentrations (0.1, 0.5, 1, 5, 10 or 20 nM) of protein samples (T22-PE24-H6 and T22-PE24-H6 FdU for HeLa cells and T22-DITOX-H6 and T22-DITOX-H6 MMAE for THP-1 cells) in a final volume of 0.1 mL for 48 h. As a control, cells were co-incubated in presence of equimolar concentrations of targeted nanotoxins and the corresponding free drug. CellTiter-Glo ® Luminiscent Cell Viability Assay reactive was added at each well following supplier instructions. Experiments were performed in triplicate for each condition.

2.8 Cell death assays

To explore apoptosis, THP-1 cells and HeLa cells were seeded in 24-well plates at 75,000 cells/well and 30,000 cells/well respectively and incubated for 24 h at 37ºC. THP-1 cells were exposed to T22-DITOX-H6 and T22-DITOX-H6 MMAE nanoconjugates at 20 nM in a final volume of 0.25 mL, for 24 h. HeLa cells were exposed to T22-PE24-H6 and T22-PE24-H6 FdU at 20 nM in a final volume of 0.25 mL, for 48 h. Externalized phosphatidylserine in protein-exposed cells were detected by Annexin V Detection Kit (eBioscience™, ThermoFisher), and propidium iodide (PI) was used to track dead cells, following supplier instructions. Experiments were performed in duplicate for each condition. Cells were analyzed by fluorescence-assisted cell cytometry (FACS)-Canto system (Beckton Dickinson) and results were processed using Flowing Software. Quadrants were generated according to control results (Supplementary Figure 2). Left bottom refers to viable cells (Annexin V negative, PI negative). Right bottom refers to cells undergoing early apoptosis (Annexin V positive, PI negative). Right top refers to cells undergoing late apoptosis (Annexin V positive, PI positive). Left top refers to cells undergoing non-apoptotic cell death (Annexin V negative, PI positive).

2.9 Statistical analysis

An initial analysis of normality and lognormality was performed to establish data normal distribution by using Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov tests. From these premiere results, one-way ANOVA (multiple comparisons) or two-way ANOVA test was used to determine significances among all parametric data. In those, data sets were expressed as mean $\pm$ standard error of the mean (SEM), measurements performed at least in triplicate (n=3) and significance achieved when (**$p < 0.001$) or (*$p < 0.05$).

3. Results and discussion

T22-PE24-H6 and T22-DITOX-H6 are lysine-containing fusion proteins (Figure 1A, B, C) that when produced in *Escherichia coli,* self-assemble as regular multimeric nanoparticles with hydrodynamic sizes around 47 nm (Figure 1C, D). Such nanoparticle dimensions are within the range described as optimal for interaction with target cells and for subsequent internalization [55, 56]. Both events are expected to be favored not only because of the size of the material but also by the multiple display of any cell ligand contained in the oligomeric complex, that mimics the architecture of viral capsids [57]. Importantly, the Z potential of both particles was negative and significantly far from zero (Figure 1C). This fact ensured solubility and stability of the material and it is also expected to prevent the massive aggregation of the nanoparticles [58, 59], a potential issue regarding further development towards *in vivo* applications [60, 61].

The assembling process in this platform occurs through the formation of intermediate oligomers [62], that are detectable as a minor stable population in the case of T22-DITOX-H6 but not in samples of T22-PE24-H6 (Figure 1D). In the modular proteins that act as building blocks, T22 is a potent ligand of the tumoral marker CXCR4 that is overexpressed in cancer stem cells in more than 20 human neoplasias, and whose abundance is related to bad prognosis [63-68]. T22 empowers the nanoparticles with precise binding and $CXCR4^+$ cell internalization selectivity *in vitro* and excellent tumor targeting and biodistribution in animal models of human $CXCR4^+$ cancers [33, 69]. T22-PE24-H6, that is particularly effective over human cervix carcinoma epithelial cells (HeLa), is observed here as representative of solid tumors (Figure 1C). In contrast, T22-DITOX-H6 showed more potent antitumoral effects over the human monocytic cell line THP-1 derived from the non-solid human acute myeloid leukemia (AML) (Figure 1C).

The presence of solvent-exposed lysine residues in both proteins prompted to explore the possibility to use them as anchorage sites for the chemical conjugation of small molecular weight drugs, to generate dual acting nanoparticles and to reinforce their therapeutic potential. Pentaoligomers of 5-fluoro-2'-deoxyuridine monophosphate (named here as FdU), a drug used in the chemotherapy of solid tumors such as colorectal cancer [70-72], were then selected to be combined with T22-PE24-H6, since this drug is also highly effective over cultured HeLa cells [45]. Under this concept, a conjugation method previously developed to generate protein FdU nanoconjugates [45, 47] was adapted to T22-PE24-H6 (Figure 2A). The application of this two-step procedure resulted in a spectrum of complexes, in which those carrying 1 and 2 bound FdU molecules abounded (Figure 2B), but that also contained

increasing molar amounts of the drug at decreasing proportions (Figure 2B). The average conjugation efficiency of this reaction was about the 40 %. The conjugation pattern obtained here was similar to that described by other protein-drug pairs, such antibody-drug conjugates, which followed a Poisson distribution [73, 74]. The chemical conjugation did not modify the nanoparticle size (that remained stable at around 47 nm) and only moderately, the surface charge of the proteins that became slightly more negative (Figure 2C). These data ensured the stability of the materials even in the resulting hybrid versions and kept the particle size observed as optimal for interaction with target cells and for further penetrability via endosomal routes [56]. This size value, slightly higher than 20 nm but still within the nanoscale, would be also optimal for prolonged circulation upon systemic administration [75]. When the resulting nanoconjugates were tested in HeLa cell cultures, the obtained IC_{50} values were surprisingly lower using the protein alone than when using the nanoconjugate, under an experimentally robust test (Figure 2D). T22-PE24-H6 FdU nanoconjugates activity remained dependent on the presence of CXCR4 receptor, as $CXCR4^-$ cells viability was not affected when they were exposed to the highest nanoconjugate concentration used in this study (Supplementary Figure 1B). When performing a separate analysis of the cytotoxicity associated to the protein nanoparticle, the drug alone, the conjugate construct and the unconjugated mixture of protein and chemical drug, we were able to discriminate between the potency of each component. Under the experimental set up and at 5 nM, T22-PE24-H6 killed around 90 % of the cultured cells and the free FdU around 80 % (Figure 2E). A mixture of both uncoupled drugs tended to be more aggressive (even not showing significant differences) than T22-PE24-H6 alone, indicating a cumulative effect. However, the resulting nanoconjugates were hardly killing around 50 % of the cells when applied at the same molar concentration, being clearly less efficient than any of the separated components (Figure 2E). These results, initially unexpected, might suggest that the chemically coupling of FdU to the nanoparticles reduced the cytotoxic potential of the oligomerized protein although not that of the attached chemical partner. Despite being less toxic than protein alone, HeLa apoptosis pattern induced by T22-PE24-H6 FdU was similar to that of the toxin alone (Figure 2 F and Supplementary Figure 2A). Although only as a tendency, the FdU nanoconjugates seem to promote lower non-apoptotic cell death and a higher percentage of cells were found in early apoptosis. This may be related to the activity of FdU as effector in the conjugates.

In a second attempt, MMAE was tested as bound to T22-DITOX-H6. Conjugated Auristatin E is effective against several types of hematologic tumors such as leukemia and lymphoma [48, 76-80], and we wondered if the antitumoral activities of T22-DITOX-H6 could be enhanced by the

attached drug. THP-1 monocytes, convenient *in vitro* models of AML [81] were exposed to T22-DITOX-H6 MMAE conjugates (Figure 3A). Again, the conjugation resulted in a spectrum of differently loaded nanoparticles with a conjugation efficiency of 10 % (Figure 3B) whose Z potential (Figure 3C) and size (Figure 3D) were, in this case, more apparently affected by the cross-linking process than in the previous nanoconjugate. It must be noted that increasing amounts of the coupled MMAE, while progressively enlarging the material in a few nm, reduced the mean standard error of the material size, indicative of a more robust geometry of the oligomers in form of nanoconjugates. Also, increasing amounts of the coupled drug progressively reduced the Z potential in a more evident trend than in the PE24-based construct. However, these modifications were moderate in absolute values and still in the desirable ranges expected in medically oriented materials [16]. As in the case of T22-PE24-H6 FdU, the presence of the chemical drug minimized the cytotoxic effect of the protein nanoparticles in a dose-dependent effect (Figure 3E). However, T22-DITOX-H6, free and in a mixture, showed clear cell killing effects (Figure 3F). At that point, we wondered if the attached chemical might reduce the conformational flexibility of the protein oligomers [82]. Such a flexibility might be necessary for the membrane translocation of DITOX that occurs in the endosomal route of cell penetration, during the acidification of endocytic vesicles [83]. Still, both free and conjugated DITOX-based nanoparticles structurally responded to a pH downshift (Figure 3G), indicating that MMAE did not restrict the conformational versatility of T22-DITOX-H6. In that case, and contrarily to what had been observed in the previous construct, the cell death profile promoted by the nanostructured toxins and the nanoconjugates was dissimilar (Figure 3H, left), indicative of protein inactivation. The negative impact of the nanoconjugates on cell viability, that majorly occurred via apoptosis, progressively decreased when increasing the ratio of drug:protein (Figure 3H, right and Supplementary Figure 2B), what suggested a progressive inactivation of the toxin by conjugation and a limited effect of the chemical drug in this platform. Again, nanoconjugates remained CXCR4-dependent (Supplementary Figure 1).

The similar global behavior of PE24- and DITOX-based nanoconjugates regarding their capability to promote death of target cells, and specially, the progressive inactivation of DITOX by increasing amounts of coupled MMAE might be indicative of a critical role of lysine residues in the performance of the toxins, that might be sterically affected by the chemical conjugation with the drug. The progressive surface charge modification towards negative values observed in both conjugates, but especially in the DITOX-based construct (Figure 3C) that is richer in lysine residues (Figure 1), fully support a quenching of the electrostatic charges offered by this

amino acid due to the attached drug. Therefore, functional data presented here suggest that the formation of the nanoconjugates, with the applied conjugation strategy, probably results in a minimized intrinsic activity of the toxins, that are instead fully active in the protein-alone forms (Figure 1C, 2E and 3F). On the base of these findings, alternative models for the mechanics of conjugation-mediated reduction of protein cytotoxicity are presented in Figure 4, for both types of conjugates. The PE24-based construct contains 7 lysine residues (Figure 1A), distributed in T22, PE24 and KDEL domains (Figure 4A). T22 is responsible for the interaction with the cell surface receptor CXCR4 and further internalization of the complex [84-86], while KDEL, a segment from the Shiga toxin, promotes retrograde transport from the endosome to endoplasmic reticle via Golgi apparatus [87, 88] (Figure 4A). While a blocking of these functions by the linked FdU might be a potential hypothesis, the even mild effect on cell viability (Figure 2E) indicates to some extent internalization, since both PE24 and FdU act from within the cell. On the other hand, many lysine residues of DITOX are located in the vicinity of the toxin active site (Figure 4B), in a loop critical for cytotoxicity [89](Figure 4C), what would offer, in this particular case, a more evident support for such possibility. Interestingly, the conjugation of FdU to T22-GFP-H6 had no important effects of the florescence of the material [45], being lysine residues not involved in the GFP fluorophore [90] (Figure 4D). Again, this fact indirectly supports the fact that the chemical binding of a drug through lysines might alter protein functionality when these residues are critical for protein functionality but not when this amino acid is far from the active sites of the protein.

In summary, we have proposed a strategy to simultaneously deliver anticancer drug pairs, composed by a tumor-targeted protein nanoparticle and an antiproliferative drug, with specific activity for the same type of cancer. By taking this approach, we have constructed robust protein-based hybrid nanoparticles whose sizes are kept essentially invariable in comparison with the parental protein-only versions (Figure 2, 3), and in the optimal ranges regarding interaction with target cells, further penetration [56], and optimal circulation in blood envisaging future clinical applications [16, 75]. A regular pseudo-spherical geometry, the absence of important aggregation and a generic diameter between 10 and 100 nm of particles as those generated in this study favour their permanence in a region of blood vessels known as cell-free layer, responsable for the extended circulation time and absence of aggregation in organs such as liver and spleen [91]. Finally, all these properties are found specifically convenient in the nanomedicines of cancer, since they allow exploiting the enhanced retention and permeability (EPR) effect [17], preventing, in addition, the renal clearance of free chemical drugs with sizes below 6-8 nm [92]. On the other hand, DITOX-based nanoparticles are

stabilized by the conjugation of MMAE, as the mean standard error of the material hydrodynamic size, mainly expanded by a minority population of small nanoparticles (Figure 1D) is largely reduced in presence of increasing drug amounts (Figure 3D). Although the concept on which the approach is based has been fully validated, we have identified important bottlenecks in the fabrication process, which were not apparent when linking FdU or MMAE to a carrier nanoparticle based on GFP. In these cases, fluorescence was conserved and the functionality of the whole nanoconjugate was excellent both *in vitro* and *in vivo* [45, 48]. However, the sensitivity of lysine residues to chemical modification made the modified toxins suitable as drug carriers but not as active drug effectors. This hypothesis is particularly fitting in the case of DITOX, in which the active site is particularly enriched in this amino acid (Figure 4B, C). Such limitation might however be overcame by a more precise conjugational chemistry, taking insights and strategies from antibody-drug conjugates [93-96]. The use of cysteine residues as anchorage sites or the incorporation of cleavable peptide linkers are among the most promising approaches, although it might be not applicable to the current platform because of the disulfide bridges occurring in T22 are relevant for the CXCR4 specificity of the construct. Other more advanced site-directed conjugation strategies that are currently under development regarding ADCs [97] would allow to control the exact location of conjugated drug molecules within the nanostructured protein toxin and avoid its interference over the therapeutic effect associated to the protein partner of the nanoconjugate. While at the present stage, the used chemical conjugation might alter the refined cytotoxic activities of the protein constructs, the simultaneous administration of protein and chemical drugs, results, at least in cell culture, in a cumulative cytotoxic effect that should be explored *in vivo* (Figure 2, 3). The combination of protein and non-protein drugs might minimize the acquisition of pharmacological resistance to antitumoral drugs, an event that is largely affecting the recovery of oncological patients treated exclusively with conventional chemotherapy [98].

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