

Figure 1. CXCR4-targeted protein toxins. A. Modular organization and amino acid sequence of T22-PE24-H6 (top) and T22-DITOX-H6 (down), the intrinsically cytotoxic proteins used in this study. More details can be found elsewhere [26]. In both proteins, the CXCR4-targeting peptide T22 is represented in orange, the flexible linker GGSSRSS in grey and the hexa-histidine tag in turquoise. Toxic regions are colored in mild blue (PE24) and mild yellow (DITOX). KDEL sequence crucial for intracellular trafficking in T22-PE24-H6 is shown in green. Lysines, which are key residues for conjugation, are stressed in pink. Color legend is used hereafter. B. Three-dimensional model of each fusion protein, highlighting each functional region and the Lys residues. C. Key properties of T22-PE24-H6 and T22-DITOX-H6. Left panel displays data obtained from ProtParam webserver as well as the resulting nanoparticle peak size, polydispersion index (PDI) and Z potential, meanwhile right panel displays preliminary toxicity values of both proteins in HeLa and THP-1 cell lines after 48 h exposure to 10 nM protein. D. Hydrodynamic size distribution (in nm) of T22-PE24-H6 and T22-DITOX-H6 nanoparticles determined by DLS. Mean size (in bold) and \pm standard errors (SEM) are indicated in panel C. In the insets, field scanning electron micrographs of the nanoparticles. Reproduced from [26] with permission of Elsevier. Bars indicate 50 nm.

Figure 2. Physicochemical and functional characterization of T22-PE24-H6 and T22-PE24-H6 FdU conjugates. A. Scheme of T22-PE24-H6 FdU conjugation, a two-step process with an initial thioether bond formation between thiolated FdU and a bifunctional cross-linker followed by the reaction of an ester group with amino groups from protein solvent-exposed Lys. B. MALDI-TOF analysis of T22-PE24-H6 FdU. Vertical discontinuous bar in mild blue indicates the molecular weight of T22-PE24-H6. Horizontal bar on top indicates the number of FdU molecules conjugated in each peak. Each conjugation adds 2283 Da. Mild blue and deep blue refer to T22-PE24-H6 and T22-PE24-H6 FdU, respectively. Colour legend is conserved hereafter. C. Comparative physicochemical properties of T22-PE24-H6 and T22-PE24-H6 FdU. Top table indicates the Z potential (mV) and bottom bar chart indicates mean volume size (nm) obtained by Light Scattering. Data indicate mean \pm SEM. D. HeLa cells viability (%) upon incubation at a range of T22-PE24-H6 and T22-PE24-H6 FdU concentrations (0.1-20 nM). IC_{50} values and fitting R^2 are shown. E. HeLa cells viability (%) upon incubation at 5 nM of T22-PE24-H6 and T22-PE24-H6-FdU for 48 h. Equimolar incubation of free FdU and co-incubation of T22-PE24-H6 and free FdU are used as a control. Data sets are expressed as mean \pm SEM, at least $n=3$ and statistical significance achieved when (** $p<0.001$). F. HeLa cell apoptosis promoted by T22-PE24-H6 FdU (left) and T22-PE24-H6 FdU (middle) nanoconjugates, added to 20 nM and recorded 48 h after exposure. X-axes indicate annexin levels and Y axis propidium iodide levels. Quadrants were generated according to control results (Supplementary Figure 2A). At right, graphical comparison of both conditions to the control. Left bottom in the dot plot corresponds to viable cells (mild green), right bottom to early apoptotic cells (mild grey), right top to late apoptosis (dark grey) and left top to non-apoptotic death (black).

Figure 3. Physicochemical and functional characterization of T22-DITOX-H6 and T22-DITOX-H6 MMAE conjugates. A. Scheme of T22-DITOX-H6 MMAE conjugation, a one-step process based on an alkyl-amine formation between a maleimide group of the drug and amino groups from protein solvent-exposed Lys. B. MALDI-TOF analysis of T22-DITOX-H6 MMAE

nanoconjugates obtained at four different conjugation ratios (1:5, 1:10, 1:20 and 1:50). Vertical bar in mild yellow refers to T22-DITOX-H6 molecular weight, while conjugated versions are represented in growing-intensity red peaks according to the ratio. Horizontal bar on top indicates the number of MMAE molecules conjugated in each peak. Each conjugation adds 910 Da. C. Z potential of T22-DITOX-H6 and T22-DITOX-H6 MMAE conjugates, expressed as mean \pm SEM. D. Mean hydrodynamic size (in nm) \pm SEM of each protein and nanoconjugate determined by Dynamic Light Scattering. E. THP-1 cells viability (%) upon incubation of T22-DITOX-H6 and T22-DITOX-H6 MMAE nanoconjugates at a range of concentrations (0.1 nM-20 nM). IC₅₀ values and the R² of the fitting are shown for each condition in the table. F. THP-1 cell viability (%) upon incubation at 5 nM of T22-DITOX-H6 and T22-DITOX-H6 MMAE nanoconjugates generated at 1:50 ratio for 48 h. Equimolar incubation of free MMAE and co-incubation of T22-DITOX-H6 and free MMAE are used as control. Data sets are expressed as mean \pm SEM, at least n=3 and statistical significance achieved when (**p<0.001). G. Circular dichroism spectra of T22-DITOX-H6 and 1:50 T22-DITOX-H6-MMAE at pH 8 and pH 5.6. H. Apoptosis of THP-1 cells promoted by T22-DITOX-H6 and T22-DITOX-H6 MMAE nanoconjugates. At left, dot plots of Annexin V test cytometry of T22-DITOX-H6 and 1:50 T22-DITOX-H6 MMAE (at 20 nM) after 24 h of exposure. X axis indicate annexin levels and Y axes propidium iodide levels. Quadrants generated according to control results (Supplementary Figure 2B). At right, annexin test results for each protein at the same conditions. Left bottom in dot plot corresponds to viable cells (mild green), right bottom to early apoptotic cells (mild grey), right top to late apoptosis (dark grey) and left top to non-apoptotic death (black).

Figure 4. Conjugation-induced damage in protein toxins. A. Schematic representation of T22-PE24-H6 and its internalization process (top). Location of lysine residues in T22-PE24-H6 suggests that conjugation with FdU could be impairing its delivery to the cytosol at three different levels (down), affecting its performance. B. Atomic surface representation of T22-DITOX-H6. Lysine residues are shown in pink and the active site of the enzyme in brown. C. Secondary structure insight at the active site with lysine residues and crucial amino acids highlighted. D. Three-dimensional structure of the Green Fluorescent Protein (GFP, PDB ID: 1GFL). The GFP chromophore is highlighted in green and lysines in pink. No lysine residues are projected towards the chromophore.