Figure 1. Schematic representation of the expected roles of pro-apoptotic nanoparticles in the apoptotic cascade. Pro-apoptotic nanoparticles are indicated in coloured modular patterns. Pro-apoptotic proteins are shown in blue boxes while anti-apoptotic proteins are shown in white boxes. Red lines represent inhibition while arrows represent induction of specific processes. T22-BAK-GFP-H6 corresponds to the BH3 domain of BAK protein. BAKBH3 binds to antiapoptotic proteins, inducing caspase-dependent apoptosis without loss of mitochondrial outer membrane (MOM) potential. In detail, BAKBH3 peptide antagonizes Bcl-xL and promote apoptosis by preventing Bcl-xL/Apaf-1 heterodimerization, leaving Apaf-1 free to participate in the fast activation of caspases. T22-PUMA-GFP-H6 corresponds to the PUMABH3 domain that prevents anti-apoptotic BCL-2 from sequestering `BH3-exposed' monomers of BAX-BAK. It also activates the intracellular BAK and BAX leading to the oligomerization of these proteins and MOM permeabilization. T22-BAXPORO-GFP-H6, holding the fragment responsible for pore formation of the full BAX protein, oligomerizes and induces MOM permeabilization.

Figure 2. Design and properties of CXCR4-targeted pro-apoptotic nanoparticles. A. Modular organization of protein building blocks. The pro-apoptotic segments with biological activities are those described in panel B. The N-terminal T22 is a CXCR4-binding peptide and the C-terminal H6 is a polyhistidine tail, necessary for self-assembling. L is a peptide linker (GGSSRSS) to ensure proper steric separation of the modular domains. Relative sizes of boxes are only approximate. B. Molecular mass, origin and other relevant properties are indicated for each building block.

Figure 3. Physical characterization of nanoparticles. A. Immunodetection of purified T22-BAXPORO-GFP-H6 determined through anti-GFP Western blot. M indicates the molecular mass marker in kDa. B. MALDI-TOF profiling of T22-BAXPORO-GFP-H6 oligomers. C. Hydrodynamic size distribution of T22-BAXPORO-GFP-H6 oligomers determined by DLS. D. Representative CryoTEM and FESEM images of T22-BAK-GFP-H6, T22-PUMA-GFP-H6 and T22-BAXPORO-GFP-H6 nanoparticles.

Figure 4. *In vitro* functional characterization of T22-BAXPORO-GFP-H6. A. Analysis of protein internalization at 1000 nM after 24 h of exposure to cultured $CXCR4^+$ HeLa cells. For comparative purposes, intracellular fluorescence units were corrected by the specific fluorescence of each protein, resulting in an estimation of protein amounts (arbitrary units). B. T22-BAXPORO-GFP-H6 internalization in $CXCR4^+$ HeLa cells at 100, 500, 1000 and 2000 nM for 24 h in absence and presence of the CXCR4 antagonist AMD3100. C. Representative confocal micrograph of HeLa cells in culture (top) and incubated with T22-BAXPORO-GFP-H6 at 1000 nM for different times (bottom), to indicate the progressive intracellular accumulation of the material. Cell membranes are visualized in red, nuclei in blue and the GFP protein in green, because of its intrinsic fluorescence. Orthogonal projections confirm the intracellular location of the fluorescent T22-BAXPORO-GFP-H6 nanoparticles.

Figure 5. Stability of nanoparticles *in vitro*. A. Structural stability of T22-BAXPORO-GFP-H6 protein nanoparticles in human serum at 37 ºC at different incubation times, up to 24 h, assessed by DLS. The proteolytic stability was determined by Western blot, using an anti-His monoclonal antibody (inset; numbers indicate incubation time, in hours). HS indicates the DLS plot of human sera without any added protein, and M, the molecular mass marker. B. T22-BAXPORO-GFP-H6 correlogram of DLS data. C. Predicted three-dimensional models of protein constructs containing BAK, PUMA or BAXPORO proapoptotic factors. Color code highlights the T22 peptide (blue), proapoptotic factor (orange), green fluorescent protein (green), and hexa-histidine tag (red) in each ribbon (top) and atomic surface model (middle). The hydropathicity of the models is represented over atomic surface models (bottom) where hydrophobic (grey), polar non-charged (mild blue) and charged residues (deep blue) are differentiated.

Figure 6. Tumor and non-tumor organ biodistribution of protein nanoparticles. A. Representative *ex vivo* fluorescence images (FLI) of tumor and non-tumor mouse organs (liver, kidney, spleen, lung and heart and bone marrow). Images were recorded at 5 or 24 h after the iv administration of 300 μg dose of each protein nanoparticle in mice bearing subcutaneous SP5 $CXCR4^+$ colorectal tumors. B. Quantitative analyses of nanoparticle uptake in tumor and non-tumor organs. Data presented as mean ± SEM (n=3). The name of protein nanoparticles depicted here and in the next figures refers to the functional pro-apoptotic domain carried in them.

Figure 7. Biodistribution of cocktail nanoparticles and CXCR4 expression in tumor and non-tumor tissues. A. *Ex vivo* FLI of tumor mouse organs recorded after iv administration of cocktails of nanoparticles, either in pairs (150 μg each) or as a trio (100 μg each, 300 μg in total). The name of protein nanoparticles depicted here refers to the functional pro-apoptotic domain carried in them. B. Quantitative analyses of cocktail uptake in tumor and non-tumor organs. Data presented as mean ± SEM (n=3). C. High overexpression of the CXCR4 receptor in the membrane of cancer cells in SP5 subcutaneous tumors. In contrast, non-tumor organs showed moderate and mostly cytosolic CXCR4 expression (spleen) or negligible CXCR4 expression (kidney and liver). Scale bars: 100 μm.

Figure 8. Molecular markers of apoptotic cell death induced in tumor tissues by T22-BAK-GFP-H6, T22-PUMA-GFP-H6 and T22-BAXPORO-GFP-H6 nanoparticles. Representative images of mitotic figures and apoptotic bodies (arrows) in H&E and DAPI stained samples, and IHC detection of active-caspase 3 and proteolyzed PARP positive cells, in tumors 24 h after the iv administration of 300 μg of each nanoparticle. Scale bars: 25 μm.

Figure 9. Antitumor effects induced by protein nanoparticles. A. Quantitation of mitotic figures in tumors of treated animals by DAPI staining. B. Quantitation of apoptotic bodies in tumors by DAPI staining. C. Number of dead cells in tumors determined by DAPI staining D. Percentage of necrotic area of tumors by H&E staining. E. Counts of active-caspase 3 stained tumor cells by IHC. F. Proteolyzed PARP stained tumor cells by IHC. All quantified values in panels were obtained by counting 10 high-power fields (×400) per sample. Data were expressed as mean ± SEM. # $p < 0.05$ bars and & $p < 0.05$ bars indicate statistically significant differences compared to buffer-treated group at each time point, or to at least one single nanoparticle, respectively; *$p < 0.05$ bars indicate statistically significant differences between the designated groups. Nomenclature of nanoparticles and cocktails is used as in Figure 6 and 7.

Figure 10. Lack of toxicity in liver and kidney tissues after nanoparticle administration. A complete absence of histological alterations in kidney and liver tissues in confirmed by H&E after T22-BAK-GFP-H6, T22-PUMA-GFP-H6 or T22-BAXPORO-GFP-H6 single administration. It is also confirmed after their cocktail administration in the SP5 colorectal cancer model, as it happened in Control animals administered with buffer. Thus, the glomeruli and renal tubules did not have cytoplasmic vacuolation or eosinophilic protein accumulation. Moreover, the hepatocytes did not lose their architecture and did not present steatosis. Scale bars: 50 μm.