A refined cocktailing of pro-apoptotic nanoparticles boosts anti-tumor activity

*Laura Sánchez-García [1, 2, 3] ¥, Rita Sala [2, 4] ¥, Naroa Serna [1,2,3], Patricia Álamo [2, 4], Eloi Parladé [1], Lorena Alba-Castellón [4], Eric Voltà-Durán [1, 2, 3], Alejandro Sánchez-Chardi [5, 6], Ugutz Unzueta [2, 3, 4], Esther Vázquez [1,2, 3] *, Ramón Mangues [2, 4] *, Antonio Villaverde [1, 2, 3] **

[1] Institut de Biotecnologia i de Biomedicina, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain

[2] CIBER de Bioingeniería, Biomateriales y Nanomedicina (CIBER-BBN), C/ Monforte de Lemos 3-5, 28029 Madrid, Spain

[3] Departament de Genètica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain

[4] Institut d'Investigacions Biomèdiques Sant Pau and Josep Carreras Research Institute, Hospital de la Santa Creu i Sant Pau, 08041 Barcelona, Spain

[5] Departament de Biologia Evolutiva, Ecologia i Ciències Ambientals, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain

[6] Servei de Microscòpia, Universitat Autònoma de Barcelona, Bellaterra, 08193, Barcelona, Spain

* Corresponding authors

¥ Equally contributed

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Abstract

A functional 29 amino acid-segment of the helix α5 from the human BAX protein has been engineered for production in recombinant bacteria as self-assembling, GFP-containing fluorescent nanoparticles, which are targeted to the tumoral marker CXCR4. These nanoparticles, of around 34 nm in diameter, show a moderate tumor biodistribution and limited antitumoral effect when systemically administered to mouse models of human $CXCR4^+$ colorectal cancer (at 300 μg dose). However, if such BAX nanoparticles are co-administered in cocktail with equivalent nanoparticulate versions of BAK and PUMA proteins at the same total protein dose (300 μg), protein biodistribution and stability in tumor is largely improved, as determined by fluorescence profiles. This fact leads to a potent and faster destruction of tumor tissues when compared to individual pro-apoptotic factors. The analysis and interpretation of the boosted effect, from both the structural and functional sides, offers clues for the design of more efficient nanomedicines and theragnostic agents in oncology based on precise cocktails of human proteins.

Keywords: Human proteins; nanoparticles; nanomedicine; drug delivery; cancer; pro-apoptotic factors

Highlights

A functional segment of BAX protein can be engineered to self-assemble as fluorescent nanoparticles targeted to the tumoral marker CXCR4.

Despite their CXCR4 selectivity, BAX nanoparticles do not show a tumor biodistribution and accumulate in liver and kidney.

The cocktail administration of BAX nanoparticles with equivalent PUMA and BAK constructs results in high tumor accumulation and potent antitumoral effect.

The combined administration of BAX, PUMA and BAK nanoparticles promote potent tumor cell destruction with a profile different from that of any of the single pro-apoptotic factors.

Introduction

Proteins are promising biopharmaceuticals because of easy biological production, full biocompatibility and high functional versatility that can be further tailored by genetic engineering [1-4]. Considering the different applications of proteins as drugs [5], their ability to promote cell death is of special interest in cancer treatments, which are aimed at the controlled destruction of tumor tissues. Toxins and venom components are among the most explored agents in the catalogue of cytotoxic proteins [6]. However, their non-human origin compromises the applicability in human therapies because of potential severe immunotoxicities that weaken drug safety [7, 8]. In this regard, the use of human proteins would represent a clear advantage over microbial, animal or plant polypeptides. Devoid of straightforward cytotoxic activities, more refined functions such induction of apoptosis have been selected as a critical functionality of antitumor drugs based on human proteins [9]. In this context, human pro-apoptotic factors, that are in general short amino acidic stretches, are suited for engineering and application as antitumoral drugs [10-13].

As for conventional chemical drugs, selective cell delivery [14-20] of pro-apoptotic factors should allow an optimal drug delivery to disease tissues and thus minimize side toxicities linked to off-delivery to healthy tissues. In recent studies, we constructed self-assembling protein-only nanoparticles based on microbial [21], animal [22] and plant [23] toxins in which these agents were selectively delivered to CXCR4-overexpressing cancer cells. Such toxins were engineered to display a cationic peptide at the N-terminus and a histidine-rich peptide at the C-terminus, without losing their cytotoxic potential. Under this modular setting and supported by an unbalanced distribution of electrostatic charges in the protein [24], nanoparticles are spontaneously formed by the coordination of divalent cations in the media (such as Ni^{2+} leaking from the purification columns) with histidine residues from discrete polypeptidic chains [25, 26]. Because of the self-assembling process, nanosized toroid oligomers are generated [27] that result fully stable in the bloodstream [28, 29].
By the systemic administration of such nanostructured toxins, a high therapeutic impact in animal models of human colorectal cancer and acute myeloid leukaemia was determined through the reduction of either tumor volume [21] or dissemination foci [23]. Similar nanostructured CXCR4-targeted constructs were prepared with human pro-apoptotic factors, namely BAK and PUMA, that promoted selective destruction of primary tumor [30]

when administered systemically in mouse animal models of human colorectal cancer. Despite their high selectivity, the tumor destruction capacities of these peptides were globally less potent than those shown by toxins. In an attempt to explore cost-effective strategies to lift antitumor capacities of tumor-targeted pro-apoptotic factors up to clinical requirements, we have designed and produced here a new nanostructured pro-apoptotic factor (the pore forming domain of the human BAX, namely BAXPORO). Acting at levels of the apoptotic cascade different from those of BAK and PUMA, we have tested the therapeutic potential of BAXPORO in colorectal cancer models. We were especially interested in the administration in combination with BAK and PUMA in form of a cocktail of self-assembling humanized protein nanoparticles, looking for enhanced or synergistic activities that might be therapeutically promising.

Materials and methods

Protein design, production, purification and characterization

Synthetic genes encoding for T22-BAXPORO-GFP-H6, T22-PUMA-GFP-H6 and T22-BAK-GFP-H6 were designed in-house fused to pET22b vector and provided by Geneart (ThermoFisher). All the recombinant proteins, which were named according to their modular composition, were produced following the same procedure. First, pET22b plasmid bearing the sequence of interest was transformed by heat shock in *Escherichia coli* Origami B strain (BL21, $OmpT^-$, Lon^-, $TrxB^-$, Gor^-, Novagen), due to the presence of disulphide bonds. Subsequently, cells were grown at 37 ºC and 250 rpm in LB medium supplemented with 100 µg/ml ampicillin (plasmid resistance), 15 µg/ml kanamycin and 12.5 µg/ml tetracycline (bacterial resistances). Protein production was induced upon addition of 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) when an $OD_{550} \sim 0.5$-0.7 was reached. T22-BAXPORO-GFP-H6 and T22-PUMA-GFP-H6 were produced overnight at 20 ºC, whereas T22-BAK-GFP-H6 for 3 h at 37 ºC. Bacterial cells were then centrifuged for 15 min (5,000 g at 4 ºC) and the pellet was kept at -80 ºC until use.

In order to proceed with the protein purification, bacterial cells were thaw and resuspended in Wash buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl and 10 mM imidazole) in presence of the protease inhibitor Complete EDTA-Free (Roche Diagnostics). Bacterial membrane disruption was performed at 1,200 psi using a French Press (Thermo FA-078A), the obtained

lysates were then centrifuged for 45 min (15,000g at 4 ºC) and the soluble fraction was filtered in a pore diameter of 0.22 µm. All the proteins were purified due to the presence of the His-tag by Immobilized Metal Affinity Chromatography (IMAC) using a HiTrap Chelating HP 1ml column (GE Healthcare) in an AKTA purifier FPLC (GE Healthcare). When the sample was already loaded in the column, it was washed using 10 column volumes (CV) of Wash buffer and eluted stablishing a lineal gradient of Elution buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl and 500 mM imidazole). The eluted fractions corresponding to the protein of interest were pooled together and dialysed against sodium carbonate buffer (166 mM NaCO3H pH 8) in the case of T22-BAXPORO-GFP-H6 and sodium carbonate with salt (166 mM NaCO3H and 333 mM NaCl pH 8) in the case of T22-PUMA-GFP-H6 and T22-BAK-GFP-H6. Moreover, all the samples were centrifuged for 15 min (15,000g at 4 ºC) to remove insoluble aggregates. The purity and integrity of the obtained samples was analysed by Western Blot and mass spectrometry (MALDI-TOF). Anti-GFP polyclonal antibody (Santa Cruz) was used for the immunodetection of T22-BAXPORO-GFP-H6. Finally, all the samples were quantified by Bradford's assay.

Dynamic light scattering and fluorescence determination

The volume size distribution of T22-BAXPORO-GFP-H6, T22-PUMA-GFP-H6 and T22-BAK-GFP-H6 was determined by dynamic light scattering (DLS) at 633 nm (Zetasizer Nano ZS, Malvern Instruments Limited, Malvern). Moreover, the GFP fluorescence emission of all the nanoparticles was analysed at a final concentration of 0.1 mg/ml. The measurements were performed by a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) at 510 nm using an excitation wavelength of 488 nm. For comparative analyses, the intensity of fluorescence was corrected by protein amounts to render specific emission values.

Ultrastructural characterization

Ultrastructural characterization of nanoparticle morphometry (size and shape) was determined at nearly native state with two electron microscopy techniques using cryoTEM and FESEM. In a qualitative approach with cryoTEM, microdrops of T22-BAK-GFP-H6, T22-PUMA-GFP-H6, and T22-BAXPORO-GFP-H6 at 0.3 mg/ml in their respective storage buffers were deposited in Holey carbon-coated copper grids (400 mesh), cryofixed in liquid ethane with an EM GP automatic plunge freezer (Leica), placed in a cryo-transfer specimen holder

(Gatan Inc.) and observed in a TEM JEM-2011 (Jeol) operating at 200 kV and equipped with a 895 USC 4000 CCD camera (Gatan Inc.). Representative images of general fields and nanoparticle details were captured at high magnifications (from 40,000x to 150,000x). In qualitative and quantitative approaches with FESEM, drops of the same three samples at the same conditions were directly deposited on silicon wafers (Ted Pella Inc.) for one minute. The excess of liquid was blotted with Whatman filter paper number 1 (GE Healthcare), and samples were air dried for a few minutes and immediately observed without coating with a FESEM Merlin (Zeiss) operating at 1 kV and equipped with a high resolution *in-lens* secondary electron detector. Representative images of general fields and nanoparticle details were captured at high magnifications (from 100,000x to 400,000x).

Cell culture and flow cytometry

$CXCR4^+$ cervical cancer cells (HeLa ATCC-CCL-2) were selected to observe the behaviour of the recombinant proteins *in vitro*. HeLa cells were maintained in Eagle's Minimum Essential Medium (Gibco®), supplemented with 10 % foetal bovine serum (Gibco®) and incubated in a humidified atmosphere at 37 ºC and 5 % of CO_2. In order to study T22-BAXPORO-GFP-H6 internalization, HeLa cells were cultured on 24-well plates at $3 \cdot 10^4$ cells/well for 24 h until reaching 70 % confluence. Proteins were incubated for 24 h at different concentrations (100, 500, 1000 and 2000 nM) in presence of OptiPRO™ SFM supplemented with L-glutamine (Gibco®). Simultaneously, the same conditions were tested with preincubation of AMD3100 (1 h prior to protein incubation). AMD3100 (octahydrochloride hydrate, Sigma) is a T22 antagonist, used to block T22-CXCR4 binding and therefore assess the specificity of the targeted proteins. After protein incubation, cells were washed with DPBS and treated with 1 mg/ml Trypsin-EDTA (Gibco®) for 15 min at 37 °C. This harsh protocol aimed to completely remove the protein that might remain externally attached to the cells. The obtained samples were then analysed by a FACS-Canto system (Becton Dickinson) using a 15mW air-cooled argon ion laser at 488 nm excitation. Experiments were performed in duplicate. For the comparative analysis of protein internalization, intracellular fluorescence was corrected by the specific fluorescence of each protein, rendering values estimative of protein amounts.

Confocal laser scanning microscopy

Confocal microscopy was performed in order to assess protein nanoparticles uptake. HeLa cells were grown on Mat-Tek plates (MatTek Corporation). The day after, cells were washed with DPBS and proteins were added for 24 h at a final concentration of 1 µM. Upon exposure to the nanoparticles, cell nuclei were labelled with 5 µg/ml Hoechst 33342 (ThermoFischer) and the plasma membrane with 2.5 µg/ml CellMask™ Deep Red (ThermoFischer) for 10 min in darkness at room temperature. Confocal images of live cells were collected on an inverted TCS SP5 Leica Spectral confocal microscope (Leica Microsystems,) using 63× (1.4 NA) oil immersion objective lenses. Excitation was reached via a 405 nm blue diode laser (nucleic acids), 488 nm line of an argon ion laser (nanoparticles) and 633 nm line of a HeNe laser (cell membrane). Optimized emission detection bandwidths were configured to avoid inter-channel crosstalk and multitrack sequential acquisition setting were used.

***In vivo* biodistribution assays**

All *in vivo* experiments were approved by the institutional animal Ethics Committee of Hospital Sant Pau. For the biodistribution studies, five-week-old female Swiss nu/nu mice weighing between 18 and 20 g (Charles River, France) were used and maintained in specific-pathogen-free (SPF) conditions. We used a subcutaneous colorectal cancer mouse model derived from the patient sample SP5. This model was generated by implanting 10 mg of SP5 tumor tissue obtained from a donor animal in the subcutis of Swiss nu/nu. When tumors reached a volume of approximately 500 mm^3, the biodistribution assays were initiated by the administration of each of the three different pro-apoptotic nanoparticles alone, namely T22-BAK-GFP-H6, T22-PUMA-GFP-H6 or T22-BAXPORO-GFP-H6, at 5 and 24 h. Each mouse received 300 µg single iv bolus of each nanoparticle. Then we performed biodistribution assays combining in a single iv injection the different pro-apoptotic nanoparticles. We combined either T22-BAK-GFP-H6 or T22-PUMA-GFP-H6 with T22-BAXPORO-GFP-H6, administering 150 µg of each nanoparticle. Then we combined all of them, as a trio, administering 100 µg of each for a total dose of 300 µg. Control animals were iv administered with 150 µl of buffer. At 5 and 24 h after the iv injection, mice were euthanized and subcutaneous tumors, liver and kidneys were collected. Biodistribution of GFP fluorescent nanoparticles was determined measuring *ex vivo* the fluorescence emitted by tumors and normal organs using the IVIS Spectrum equipment (PerkinElmer Inc). The fluorescent signal (FLI) was first digitalized, displayed as a pseudocolor overlay and

expressed as radiant efficiency. FLI values were calculated subtracting the FLI signal from the protein-treated mice by the FLI auto-fluorescent signal of control mice.

Histopathology and Immunohistochemistry analyses

All collected organs were fixed with 4 % formaldehyde in phosphate-buffer solution for 24 hours. Then, the fixed samples were embedded in paraffin for the analyses. 4 µm thick sections were stained with haematoxylin and eosin (H&E). The expression of CXCR4 in tumor and non-tumor organs was assessed by immunohistochemistry using a primary antibody against anti-CXCR4 (1:300; Abcam). Proteolyzed PARP and the active-caspase 3 protein were assessed by immunohistochemistry. A primary antibody against anti-PARP p85 fragment pAb (1:300; Promega) or anti active-caspase 3 antibody (1:300, BD PharMigen) was used to incubate the tumor tissue samples for 25 min and after incubation with the secondary antibody. Two independent blinded counters quantified the number of stained cells per 10 highpower fields (magnification 400x). Representative pictures were taken using CellΛB software (Olympus Soft Imaging v 3.3).

Assessment of mitotic, apoptotic and necrotic rates

Tumor sections stained with DAPI were also used to assess the proliferation capacity by counting the number of mitotic figures per 10 highpower fields (magnification 400x). To assess the apoptotic effect of the nanoparticles in tumors we counted the apoptotic bodies in H&E and DAPI staining per 10 highpower fields (magnification 400x). DAPI staining was performed in Triton X-100 (0.5%) permeabilized sections that were mounted with the ProLong Gold antifade reagent with DAPI mounting media (Thermo Fisher Scientific). Samples were analysed under fluorescence microscope at a wavelength of λex = 334 nm/λem = 465 nm. We also evaluated the percentage of necrotic areas in the H&E stained tumors by using CellΛB software at 15x magnification.

Statistical analysis

Results of the number of mitotic figures, number of apoptotic bodies, tumor necrosis percentage and active-caspase 3 or proteolyzed PARP stained cells between evaluated groups were analysed using both the Student's t-test and the Mann-Whitney U test. Data were reported as mean ± SEM and differences between groups were considered significant

at $p<0.05$. These differences between relevant data were indicated as # or & for statistically significance compared to the buffer treated group at each time or as * for statistically significance between the designated groups. Statistical calculations were performed using SPSS software.

In silico modelling and visualization

Three-dimensional models of each protein building block in aqueous environment were predicted using the Rosetta comparative modelling approach [31] through the Robetta web server (http://robetta.bakerlab.org). Protein sequences were used as query and the green fluorescent protein (PDB ID: 1EMA) [32] as template for comparative modelling. Parameters were set to 10 sampling models, 1 register shift, and a probability of 0.1 of sampling fragments within template regions. For all constructs, secondary structure of residues 8–10 and 13–15 was constrained to a β-sheet, based on its characterized structure [33]. Likewise, α-helix regions from BAK (27–51), PUMA (29–49), and BAXPORO (28–55) were also fixed before modelling to retain essential secondary structure (PDB IDs: 2YV6 (69-93, BAK) [34], 2M04 chain B (68-92, PUMA) [35] and 1F16 (106-134, BAXPORO)) [36]. After the modelling process, candidates with the lowest error estimate were chosen. UCSF Chimera [37] was used to represent three-dimensional structures and to visualize their surface hydropathicity patterns.

Results and discussion

To explore candidates for a synergistic administration of pro-apoptotic factors with antitumoral potential we investigated the components of the Bcl-2 family [38], that are involved in the regulation of apoptotic cell death by the combined activities of anti-apoptotic and pro-apoptotic members (Figure 1). BH3-only pro-apoptotic proteins (BID, BIM, PUMA and NOXA) and multidomain proteins (BAX and BAK) share a similar globular structure with four BCL-2 homology regions (BH1, BH2, BH3 and BH4). BH3-only proteins are activators that convert BAX-BAK monomers into active BH3-exposed BAX-BAK monomers. In the activated form, BAX-BAK insert into the outer mitochondrial membrane (in a process mediated by BH4 domain), oligomerize and induce mitochondrial outer membrane permeabilization (MOMP). Meanwhile, as BH3 activators have a dual role and they also prevent anti-apoptotic BCL-2 proteins from sequestering BH3-exposed monomers of BAX-BAK, leading to homo-oligomerization of BAX-BAK and subsequent pore formation [39]. Regarding the multifactorial complexity of apoptosis, the simultaneous delivery of pro-apoptotic candidates like BAK, BAX or PUMA [40], that stimulate the pro-apoptotic cascade at different levels, appears as a very promising alternative (Figure 1).

In this context, we decided to administer an engineered version of such three proteins targeted to $CXCR4^+$ cells, alone and in simultaneous intravenous injection, in a mouse model of human, $CXCR4^+$ colorectal cancer. For that, we constructed the modular protein T22-BAXPORO-GFP-H6 (Figure 2A) aiming to spontaneous self-assembling, mimicking the modular organization of the previously constructed proteins T22-BAK-GFP-H6 and T22-PUMA-GFP-H6 that formed CXCR4-targeted functional nanoparticles [30] (Figure 2A, B). T22 is a potent peptidic ligand of the tumoral marker CXCR4 [33, 41, 42], useful to drive recombinant proteins to $CXCR4^+$ tumors [23]. For the design of the BAX-based nanoparticles, we selected the 29aa -helix α5 fragment from the whole BAX protein, which is responsible for pore formation [43] (named here BAXPORO). Like the rest of the proteins, T22-BAXPORO-GFP-H6 was well produced in *E. coli* in a full-length form of 34.4 kDa, which showed a tendency to dimerize into about 75 kDa forms (Figure 3A, B). The protein spontaneously assembled as 32 nm-nanoparticles, as determined by DLS (Figure 3C) and confirmed by two independent microscopic techniques, namely cryo-TEM and field emission scanning electron microscopy (FESEM) (Figure 3C, D). The diameter of T22-BAXPORO-GFP-H6 assemblies was

larger than that described for T22-BAK-GFP-H6 (13.5 nm) and T22-PUMA-GFP-H6 (20 nm), and the size of all these oligomers remained constant in physiological buffer at any of the tested dilutions, within the range between 0.05 and 8 mg/ml (not shown). However, 0.1 % SDS promoted an immediate size drop of these constructs [30] (and also of the whole family of related assembles based in C-terminal H6 tails, [26]) indicative of disassembling, until around 7 nm, a size compatible with dimeric building blocks and in agreement with the observed dimerization tendency (Figure 3A). On the other hand, T22-BAXPORO-GFP-H6 showed a specific fluorescence of 509.18 units/mg, slightly lower but comparable to that of the other apoptotic constructs (Figure 2B).

The intrinsic fluorescence of T22-BAXPORO-GFP-H6 nanoparticles allowed their monitoring during the interaction with target $CXCR4^+$ cells, in internalization studies, upon a harsh trypsin treatment specifically designed to remove material attached to the cell surface [44]. T22-BAXPORO-GFP-H6 showed a high cell penetrability, superior to that observed in PUMA- and BAK-derived constructs (Figure 4A). Also, at least at an important extent, T22-BAXPORO-GFP-H6 penetrated cells via CXCR4, as the CXCR4 antagonist AMD3100 [45, 46] largely minimized the uptake of the protein (Figure 4B), as it happens with PUMA- and BAK-derived nanoparticles [30]. CXCR4 is largely overexpressed in metastatic cancer cells in more than 20 human neoplasias, correlating with the prognosis of the diseases [47-50]. Therefore, CXCR4 is an excellent target to very precisely treat metastasis in $CXCR4^+$ cancers such as colorectal cancer in absence of side effects over healthy tissues [51, 52], being highly suitable for precision medicines. When exposed to cultured HeLa cells (Figure 4C, top), T22-BAXPORO-GFP-H6 nanoparticles retained the GFP fluorescence emission and progressively moved from the cell membrane where they were observed at short times, to a perinuclear area where they accumulated upon longer time incubation(Figure 4C, bottom). Orthogonal projections of these confocal images confirmed again the intracellular location of the nanoparticles (Figure 4C). These results fully supported the well performance of the BAXPORO-based construct and prompted us to investigate how the protein would target tumors *in vivo*. The nanostructure of tumor-targeted proteins dramatically favors tumor accumulation [29] by an expected combination of biophysical events [53], including the multivalent, virus-like attachment to target cells [54] and the enhanced permeability and retention effect (EPR) in tumor tissues [55, 56]. Being all these proteins stable in form of assembled and fluorescent

oligomers (Figure 2B, 3C), the biodistribution analyses of such nanostructured pro-apoptotic factors, administered alone or in combination, should be feasible.

As a first step, we tested how stable T22-BAXPORO-GFP-H6 nanoparticles would be in human sera, upon incubation up to 24 h at 37 ºC. As observed, the protein assemblies kept their hydrodynamic size during all the studied period of time (Figure 5A), indicating that the oligomers remained assembled under these conditions. The T22-BAXPOPRO-GFP-H6 correlogram showed a profile, typical in large nanoparticles, in which the correlation coefficient takes some time to decay. This was represented by a plateau at the first timepoints, that confirmed the robustness of the DLS analyses (Figure 5B). In agreement with the volumetric determination of the nanoparticles, T22-BAXPORO-GFP-H6 was observed as a single band in Western blot, of rather constant intensity during the tested period, proving that proteolysis, if any, occurred at very low levels. In addition, 3D models of each protein construct in aqueous environment were generated in ribbon and atomic surface variants, where the functional components and hydropathicity patterns are highlighted (Figure 5C). Such modelling supported the concept that oligomerization based in divalent cation coordination [26, 57] instead mere protein aggregation driven by hydrophobic patches, was the molecular mechanism of nanoparticle formation. This fact accounted for their high stability in complex media and the concentration-independent hydrodynamic volume of the nanoparticles that resulted from a fine assembling process.

Since T22-PUMA-GFP-H6 and T22-BAK-GFP-H6 were already proved to be stable in blood [30], the structural robustness of the new T22-BAXPORO-GFP-H6 proved here pushed us to move to the *in vivo* setting. In particular, we were interested in exploring the antitumoral potential of this construct administered alone or as a drug cocktail, in combination with other pro-apoptotic factors. When systemically administered through the tail vein at a 300 µg dose each, BAK- and PUMA-based nanoparticles accumulated in tumor at 5 h, with a moderate background in liver and kidney (Figure 6A). At 24 h, only residual fluorescence was detected in target and non-target tissues (Figure 6A). In contrast, BAXPORO-based nanoparticles did not biodistribute properly, showing poor tumor targeting and a tendency to accumulate in liver and kidney (Figure 6A, B). The poorly targeted biodistribution of T22-BAXPORO-GFP-H6 versus PUMA and BAK-based nanoparticles could be solely attributed to

the amino acid sequence or conformation of the functional protein, as the rest of the modular construct matched the other ones (Figure 1A). In particular, the incomplete CXCR4-targeting observed in T22-BAXPORO-GFP-H6 (Figure 4B), probably related to a steric defect in the solvent display of T22, might contribute to the protein occurrence in non-target organs (Figure 6A). In addition, the renal accumulation of BAX-based nanoparticles might be additionally accounted by an unstable oligomeric organization of the material, as monomeric forms of related proteins also containing T22 loss their tumor targeting properties and tend to be found in kidney [29]. Interestingly, when nanoparticles, at the same final 300 μg dose, were administered either in pairs (150 μg each) but especially all together in a single injection (TRIO, 100 μg each, Figure 7A, B), the accumulation in tumour of T22-BAXPORO-GFP-H6 increased compared with the protein administered alone (Figure 6A, B, Figure 7A). In the same way, the permanence of the material in such tissue was extended in time, as determined by a strong fluorescence at 24 h (Figure 7A). At the same time, the background occurrence in off-target organs was concomitantly reduced when compared to single drug injections (Figure 7B).

The tumor uptake of T22-BAK-GFP-H6 or T22-PUMA-GFP-H6 administered as single agents (Figure 6A) or when combined with T22-BAXPORO-GFP-H6 as pairs or as a TRIO (Figure 7A) was significantly higher as compared to normal organs such as liver or kidney (Figure 6A, 7B). This was in agreement with our finding of a dramatically higher level of CXCR4 expression, as measured by IHC, in the membrane of tumor cells (Figure 7C), which determines nanoparticle internalization, as compared to a low CXCR4 expression in non-tumor organs, including liver, kidney and spleen (this last organ expresses moderate levels of cytosolic CXCR4 and shows undetectable nanoparticle internalization) (Figure 7). These results support the notion that the used nanoparticles effectively exploit the high CXCR4 expression difference between cancer and normal cells, so that they can achieve a selective delivery of therapeutic domains to cancer cells.

We next studied the antitumor activity of T22-BAK-GFP-H6, T22-PUMA-GFP-H6 or T22-BAXPORO-GFP-H6 nanoparticles administered as single therapeutic agents by measuring active-caspase3 induction and proteolyzed PARP as molecular makers of apoptotic induction in tumor tissues (Figure 8), as well as the presence of apoptotic or cell death bodies in H&E

or DAPI stained tumor tissues (Figure 8). In this regard, we observed a significant increase in all these apoptotic markers 24 h after a single dose administration of each nanoparticle as compared to buffer-treated animals (Figure 8). Considering the biodistribution data that showed a prolonged presence of the TRIO material in tumor tissues, which raised reasonable expectations regarding a potential high therapeutic effect of the drug cocktail, we tested the antitumor effect of the cocktail combinations. It must be noted that in previous studies a modest tumor tissue destruction promoted by PUMA- or BAK-based nanoparticles was described, despite of being highly selective in binding and internalization [30]. Several parameters indicative of antitumoral effect were analyzed upon administration of single, pair and TRIO cocktails of nanoparticles, under the conditions stated above, in colorectal cancer mouse models. As observed, there was a generic and transient reduction of the mitotic figures in the tumor, clearly observed at 5 h but hardly detectable at 24 h (Figure 9A). This was accompanied by a more visible and stable level of apoptosis, peaking at 24 h post administration (Figure 9B), and of DAPI-determined cell death (Figure 9C) and tumor necrosis (Figure 9D). The presence of two or three pro-apoptotic peptides showed a clear non-additive effect, as the pair and TRIO cocktails promoted enhanced apoptotic cell death and necrosis of tumor tissues when compared to single pro-apoptotic factors (Figure 9B, C, D). Importantly, the maximal levels of tumor tissue destruction induced by nanoparticle cocktails were already reached at 5 h, while such maximal therapeutic effect needed 24 h in single protein drugs and in some of the pair mixtures. This finding was observed as a clear tendency that in many cases reached statistical divergences (note the significant differences between 5 h and 24 h time values in panels B and C). The fastest pro-apoptotic activity of protein cocktails was fully confirmed by the analysis of activated caspase-3 and proteolyzed PARP in treated tumors. Both apoptotic markers were activated already at 5 h after administration by all treatments, but levels of activated caspase-3 tended to return to background levels in pair- and TRIO-treated animals (Figure 9E). Proteolyzed Poly(ADP-ribose) polymerase (PARP), an apoptosis indicator, showed an even more acute profile as it was hardly detected in TRIO treatment (Figure 9F), while apoptotic cell death promoted by this cocktail was very high (Figure 9B, C). This was again suggestive of an extremely fast and potent pro-apoptotic effect of the combined PUMA, BAK and BAXPORO-based nanoparticles. This fact which might be partially linked to the improved biodistribution and stable tumor

accumulation of the combined nanoparticle TRIO treatment (Figure 7A) but also to a combined biological effect of the drugs on the target tissues.

Importantly, the achieved antitumor activity occurred in the SP5 colorectal cancer model in the absence of toxicity in any normal organ. We particularly searched for possible histological alterations in kidney and liver, the two non-tumor organs with higher nanoparticle biodistribution (Figure 10). No differences in histology were observed between control buffer-treated mice and T22-BAK-GFP-H6, T22-PUMA-GFP-H6, T22-BAXPORO-GFP-H6 or their pair or TRIO cocktail-mice in these organs. Thus, the glomeruli and surrounding renal tubules were clearly visible and presented no cytoplasmic vacuolation or eosinophilic protein accumulation. Moreover, in liver tissue, the hepatocytes did not lose their architecture, and did not present steatosis or any other histological alteration (Figure 10).

The enhanced targeting of the TRIO mixture that abolished the poor biodistribution of BAXPORO protein (Figure 6A) may be related to a dynamic redistribution of building blocks of the different pro-apoptotic factors in the mixture of nanoparticles, leading to the formation of hybrid nanoparticles. This possibility, that has been experimentally demonstrated in related GFP and BFP-containing protein by FRET [58], would eclipse any defect in CXCR4 binding by T22-BAXPORO-GFP-H6, provided the failing building block results combined with heterologous partners displaying a fully reactive T22. On the other hand, the different level of actuation of the three tested pro-apoptotic drugs in the cell death cascade (Figure 1) would dramatically stimulate the drug functionality. This would occur either by fastening the apoptotic catastrophe or by stimulating a non-apoptotic pathway of cell death. Such a mechanism is observed during the caspase-independent cell death promoted by high doses of the small molecular weight pro-apoptotic factor obatoclax [59], a BH3-only domain which anticancer activity is not affected by the inhibition of caspase-dependent programmed cell death. These findings are consistent with the crosstalk [60] among the different cell death pathways, compatible with data presented in Figure 9, and they would account for the potent therapeutic effect determined here during the combined use of pro-apoptotic factors.

Conclusions

The combined administration of CXCR4-targeted BAXPORO, BAK and PUMA, in form of an equimolar mixture of nanostructured protein drugs, promotes a fast, selective, and efficient destruction of tumoral tissues in mouse models of $CXCR4^+$ human colorectal cancer. The antitumoral effect of the cocktail, at the same protein dose, is much faster and more effective than that observed upon the administration of individual drugs, supporting an enhanced mode of actuation of the mixture. Also, the improved biodistribution of the cocktail compared to that of BAXPORO strongly supports the hypothesis of a structural compensation of the CXCR4 targeting defects of T22-BAXPORO-GFP-H6 in form of hybrid nanoparticles with higher therapeutic action. The proper selection of human pro-apoptotic factors for the combined therapy or theragnosis of cancer, in form of protein drug cocktails, is shown here as an appealing strategy to fully exploit the therapeutic potential and to enhance the therapeutic benefits of human proteins as antitumoral drugs in absence of immunotoxicity-linked concerns of non-human toxic proteins.

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Disclosure

EV, RM and AV are co-founders of NANOLIGENT, devoted to develop antitumoral drugs based on proteins. LSG, NS, UU, RM, EV and AV are con-inventors of the patent application EP17169722.0 on the use of self-structured protein drugs.

Contributions

Laura Sánchez-García, Rita Sala, Naroa Serna, Patricia Álamo, Eloi Parladé, Lorena Alba-Castellón, Eric Voltà-Durán, Alejandro Sánchez-Chardi: Investigation; Methodology; Writing - review & editing

Lorena Alba-Castellón, Ugutz Unzueta: Supervision; Writing - review & editing

Ugutz Unzueta, Esther Vazquez, Ramón Mangues, Antonio Villaverde: Conceptualization; Funding acquisition; Writing - review & editing

Antonio Villaverde; Writing - original draft

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