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Development of HAMLET-like Cytochrome c-Oleic Acid Nanoparticles for Cancer Therapy

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Abstract

In the literature, HAMLET (Human Alpha-lactalbumin Made LEthal to Tumor cells) is described as a tumoricidal complex composed of oleic acid (OA) non-covalently bound to α-lactalbumin. We recently demonstrated that OA is the real drug in this complex and that the protein solely serves as the drug carrier. We hypothesized that by replacing α-lactalbumin with a bioactive protein it should be possible to synergistically increase the efficiency of the complex. Consequently, we developed a HAMLET-like complex composed of OA coupled to the apoptosis-inducing protein cytochrome c (Cyt c). As control we coupled OA to the non-toxic protein bovine serum albumin (BSA). The syntheses of HAMLET-like Cyt c-OA and BSA-OA complexes were performed at pH 8 and 45°C and we loaded 10 and 53 molecules of OA per molecule of Cyt c and BSA, respectively. We found that OA binding promotes protein structural changes characteristic of the protein-OA interactions in HAMLET. Cyt c-OA and BSA-OA complexes had a circular shape and a diameter of 123 and 169 nm, respectively. Cell viability tests showed less than 10% of viability after cancer cell (HeLa and A-549) incubation for 6 h with Cyt c-OA, while normal cells (Cho-K1 and NIH/3T3) showed more than 20% of viability. BSA-OA killed both cell types with less efficiency and no selectivity. The Cyt c-OA complex showed 50% of caspase-3 and caspase-9 activation in a cell-free assay while BSA-OA lacked any caspase activation. Confocal micrographs showed morphological changes indicative of late- and early-apoptosis by the action of Cyt-OA and BSA-OA, respectively. This study demonstrates that using Cyt c increases the potency of OA in HAMLET-like complexes.

Keywords: HAMLET; Apoptosis; Cancer therapy; Oleic acid; Lipid-protein nanoparticle

Abbreviations: ATCC: American Type Culture Collection, BAMLET: Bovine α-Lactalbumin Made Lethal to Tumor Cells, BSA: Bovine Serum Albumin, Cyt c: Cytochrome c, DLS: Dynamic Light Scattering, EPR: Enhanced Permeability and Retention, FA: Fatty Acid, FITC: Fluorescein Isothiocyanate, HAMLET: Human α-Lactalbumin Made Lethal to Tumor Cells, MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, NP: Nanoparticles, OA: Oleic Acid, PMS: Phenazine Methosulfate, SEM: Scanning Electron Microscopy

Introduction

Even with the prominent advances in the development of new cancer treatments, administration of combinations of cytotoxic drugs that produce severe and frequently dangerous side effects are still the most commonly utilized therapies. Unfortunately, all these drugs have low tumor specificity and a frequently narrow therapeutic index [1,2]. To address this, approaches are undertaken to overcome these detrimental circumstances, i.e., development of passive and active targeting delivery systems. For passive targeting, nanosized anticancer drugs and carriers are being developed to promote their tumor accumulation by the enhanced permeability and retention (EPR) effect [3]. In order to design these systems, it is necessary to obtain a particle size that avoids renal filtration (~ >20 nm) but is small enough to specifically accumulate in tumors through fenestrae (100-800 nm) in the irregular tumor vasculature. This phenomenon is also known as the enhanced permeation and retention (EPR) effect [4]. To also enable active targeting, tumor-targeted medicines are being developed that explore hallmarks of cancer, i.e., ligands targeting overexpressed receptors [5]. However, thus far active targeting systems have not shown significant advantages over passive targeting in clinical trials [6,7]. A limitation of targeted therapies is that cancer cells can become resistant by many mechanisms to the approach or the cancer stem cells are not targeted [8]. Another factor contributing to the inefficiency is covering of the targeting ligands by biomolecules in the blood causing function loss [9]. A recent study comparing passively and actively targeted drugs showed larger circulation times and larger amounts accumulated in tumors for the first [10]. Due to these circumstances, EPR-targeted agents (i.e. Doxil®, Myocet®, Abraxane®, DaunoXome®, Oncaspar®) are still regularly administered to patients [11].

During the 90's, Svanborg and co-workers found a lipid-protein complex called HAMLET formed by the milk protein α-lactalbumin and the monounsaturated OA to cause apoptosis exclusively in cancer cells [12]. They observed that the casein fraction of milk affected the viability of the lung cancer cells. However, opposing views on specificity and cytotoxicity of HAMLET/BAMLET (the bovine counterpart) can be found in the literature. For example, while even recent papers still suggest that HAMLET/BAMLET are specifically cytotoxic to cancer cells [13,14], our results demonstrated that BAMLET could kill cancer cells (HeLa and A-549 lines) and normal cells (ChoK-1 and NIH-3T3 lines) almost with the same strength [15] and confirmed the results of Brinkmann on BAMLET cytotoxicity to non-cancer derived primary cells [16]. Similarly, several works established that HAMLET/BAMLET components (α-LA and OA) alone were unable to kill cancer cells [17,18] highlighting the importance of the synergy of the components in the tumor toxicity. Svanborg and co-workers

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conducted experiments to form HAMLET-like complexes using different FAs and showed that only OA (C18:1,9 cis) and vaccenic acid (C18:1:11 cis) coupled to α-LA killed cancer cells. In contrast, trans- or polyunsaturated FAs were unable to do so [19]. However, other articles pointed out that OA prepared without the protein is sufficient for the potent cytotoxicity [20,21]. We demonstrated that the isolated FA OA and the polyunsaturated linoleic acid induced caspase-independent cell death at the same concentration at which BAMLET does [15]. It has also been shown that mono- and polyunsaturated FAs could be internalized by receptor-mediated endocytosis or by diffusion across the cell membranes [22,23] and influenced inflammation, apoptosis, and growth inhibition processes [24,25]. Another important clinical benefit of unsaturated FAs is that they can sensitize multidrug resistant cells to anticancer drugs [26]. In conclusion, data point to the potential of selected FAs in cancer treatment but clearly exclude α-lactalbumin from having any important role in this. It is therefore not surprising that HAMLET-like complexes have been obtained with various proteins, including lysozyme [27], β-lactoglobulin [21], parvalbumin [28], and lactoferrin [28].

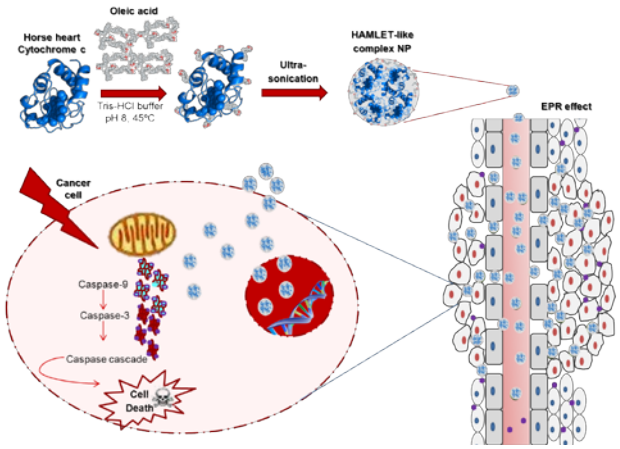
For the past few years our research group has been developing strategies to use proteins as anticancer agents [30,31]. Proteins are a potentially attractive class of drugs because they can be used to target specific cancer hallmarks and the tumor microenvironment. A common cancer hallmark is that apoptosis is disabled [32]. The lack of the apoptotic machinery explains the poor sensitivity of many cancers to most traditional anti-cancer drugs [33]. To restore apoptosis in cancer cells, Griebenow and co-workers have been working on the intracellular delivery of cytochrome c (Cyt c) [30,34]. Cyt c is a small heme protein which activates the intrinsic apoptotic mechanism when the depolarization of the mitochondrial membrane induces its release and other apoptotic factors into the cytoplasm to activate the cascade of caspases [35]. We decided to test whether Cyt c could be employed to create HAMLET-like complexes with OA and whether this would result in synergistic drug action since OA produces cell death via a caspase independent mechanism [15].

Another protein with pharmaceutical potential is serum albumin. Serum albumin is a major blood protein that is a natural FA transporter across endothelial barriers involving the gp60 receptor through a caveolae-mediated mechanism [36]. Serum albumin is used in several FDA-approved drugs (i.e. Abraxane®, Nanocoll®, and Albures®) to treat cancer [37]. The importance that serum albumin brings to these approved and potential drugs is its ability to improve the pharmacokinetic profile, to increase accumulation capacity in the pathological site, and facilitate the tumor uptake of drugs [38,39].

After we demonstrated in our last publication [15] that OA is the cytotoxic agent, we decided to use the apoptosis-inducing protein Cyt c on the development of a cytotoxic formulation. To test whether synergy between Cyt c and OA cytotoxic action can be achieved, we synthetized HAMLET-like complexes and tested their effect on cancer and non-cancer cell lines. In our system, the OA is non-covalently attached to Cyt c to construct an intracellular delivery system (Scheme 1). The complex synthesized with BSA and OA was employed as control in the *in vitro* studies, even though *in vivo* BSA might present some benefits as already introduced. The paper also presents data on uptake and mechanistic details.

Materials and Methods

Cytochrome c from equine heart (≥ 95%; EC 232-700-9), fatty acid free serum albumin from bovine (≥ 98 %; EC 232-936-2), fatty



Scheme 1: Representation of the synthesis of HAMLET-like lipid/protein nanoparticles, possible intracellular delivery, and apoptosis activation.

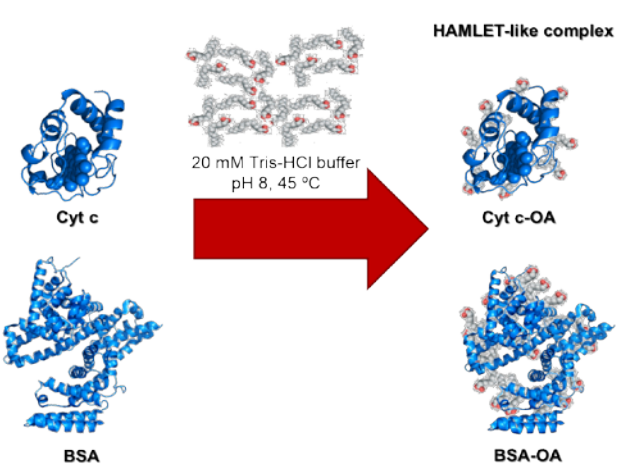


Figure 1: The synthesis conditions for the coupling reactions of BSA and Cyt c to OA to obtain HAMLET-like complexes.

acid quantitation kit (MAK044), Caspase 3 (DEVD-pNA) substrate, Caspase 9 (LEHD-pNA) substrate and oleic acid (OA, >99% purity) were purchased from Sigma Aldrich (St. Louis, MO). The centrifugal filtration system (Millipore Amicon cut off 3-5 kD) was purchased from Thermo Fisher Scientific (Hudson, New Hampshire). HeLa and NIH/3T3 cells, serum, and culture media were purchased from the American Type Culture Collection (ATCC, Manassas, VA). A-549 and Cho-K1 were cordially donated by Dr. Gabriel Barletta, University of Puerto Rico, Humacao Campus. All other chemicals were of analytical grade and from various commercial suppliers and used without further purification.

Synthesis of HAMLET-like complexes

The synthesis (Figure 1) was performed as described by us [15]. Briefly, the proteins (Cyt c and BSA) were dissolved in 20 mM Tris-HCl at pH 8 to a final concentration of 600 μM and the solution heated to 45°C. Then, 40 moles of OA were added to each solution followed by 2 min of sonication (Branson 3510R-MT, 42 kHz, 130 W). The reaction mixture was vigorously stirred at 45°C for 30 min, cooled under tap water, acidified to pH 5, and stirred for 24 h at 4°C. The reactions were neutralized to pH 7 and finally ultrasonicated (Branson 450) for 1 min

at 400 W. Unbound OA was removed by a centrifugal filtration system (Millipore Amicon, cut off 3-5 kD) in 50% ethanol/50% nanopure water (Hermle Labnet Z 323k, 8000 rpm), dialyzed thrice against nanopure water and then lyophilized (Labconco Freezone 6) for 48 h. The HAMLET-like complexes are further on named Cyt-OA and BSA-OA and were stored in amber-glass vials at -20°C.

Physical characterization of the HAMLET-like complexes NP

OA quantification assay: We employed a commercially available FA quantitation kit from Sigma Aldrich according to the manufacturer instructions and as published in detail [15,16]. The complexes were diluted in 50 µL of assay buffer and analyzed in a 96-well plate using palmitic acid standards. 2 µL acyl-CoA synthetase was added to each well to convert FAs to their CoA derivatives. Later, the CoA-FAs are subsequently oxidized with the concomitant generation of color. Octanoate and longer fatty acids can then be quantified. The reaction outcome was quantified by measuring the absorbance at 570 nm using a microplate reader (Thermo Scientific Multiskan FC).

OA coupling confirmation and protein tertiary structure analysis: Fluorescence analysis was used to investigate tertiary structural protein perturbations and performed as described [15,18,19]. Cyt c-OA was adjusted to a protein concentration of 1.5 mg/ml and BSA-OA was adjusted to 0.5 mg/ml in 20 mM Tris-HCl at pH 7.4 with 1% ethanol. Fluorescence emission spectra (λ_{em}=290-400 nm) were obtained using λ_{exc}=280 nm excitation with a Varian Cary Eclipse fluorescence spectrophotometer using a quartz cuvette with 1-cm path length.

Size, polydispersity, zeta potential and morphology: Dynamic light scattering (DLS) and z-potential measurements were performed using a Malvern ZetaSizer Nano-ZS. HAMLET-like complexes (0.5 mg) were dispersed in 2 ml of filtered nanopure water and subjected to ultra-sonication (Branson Ultrasonics 450) for 20 sec at 400 W. The instrument was calibrated using 46 ± 2 nm (Cat. No. 3300A) and 300 ± 6 nm (Cat. No 3050A) nanospheres™ size standards (Thermo Scientific). Scanning electron microscopy (SEM) of HAMLET-like complexes was performed using a JEOL 5800LV scanning electron microscope at 20 kV by dispersing 0.5 mg of the complexes in 500 µL of filtered nanopure water followed by ultra-sonication at 240 watts for 3 min. Then, some small drops of each suspension were put on a carbon tape and air-dried. After 24 h the samples were coated with gold for 10 sec using a Denton Vacuum DV-502A.

In vitro experiments

Cell culture: All cell lines were maintained in accordance with ATCC protocols. Briefly, HeLa, A-549, Chok-1 and NIH/3T3 cells were cultured in 75 cm^2 flasks with minimum essential medium (MEM), Ham's F-12 nutrient mixture, F-12K (Kaighn's Modification of Ham's F-12) and Dulbecco's modified eagle medium (DMEM), respectively, and 1% L-glutamine, 10% fetal bovine serum (FBS), and 1% penicillin in a humidified incubator with 5% CO_2 and 95% air at 37°C. All experiments were conducted before cells reached 15 passages. In each passage, cells were washed twice with PBS, detached using trypsin, and suspended in supplemented medium.

Cell viability assay: Mitochondrial function was measured using the CellTiter 96 aqueous non-radioactive cell proliferation assay. All cell lines (5000 cells/well) were seeded in 96-well plates for 24 h. Cells were incubated with 100 µL of the native proteins (Cyt c and BSA), OA and HAMLET-like complexes (Cyt c-OA and BSA-OA) for 6 h. The concentration of the HAMLET-like complexes added to each well was calculated based on the OA concentration determined in the complex. In other words, cell viability experiments were performed at the same OA concentration (120 µM) to allow for comparison with the free OA. Next, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), and phenazine methosulfate (PMS) was added to each well (333 µg/ml MTS and 25 µM PMS). After 1 h, the absorbance at 492 nm was measured using the microplate reader. HeLa cells treated with 2 µM staurosporin for 6 h were used as positive control and cells without treatment were used as negative control.

Cell-free caspase-3 and -9 activity assay: These assays were adapted as explained by others [40]. HeLa cells were grown to 80% confluency and harvested as explained above. For disruption the cells were suspended in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, 250 mM sucrose, and a protease inhibitor cocktail (serine, cysteine, aspartic acid, and metalloprotease inhibitors). The suspended cells were frozen in liquid N_2 for 2 min and thawed in a 37°C water bath and the freeze/thaw cycle repeated thrice. The lysate was centrifuged at 10,000 x g for 20 min to remove the mitochondrial and nucleus fraction. The protein concentration in the lysate was determined by Bradford assay [41]. The cell-free reactions were performed in homogenizing buffer in a total volume of 100 µL. The reaction was initiated by adding 100 µg/ml of native proteins (Cyt c and BSA), OA and HAMLET-like complexes (Cyt c-OA and BSA-OA) to freshly purified cytosol (3 mg/ml of proteins). The reaction was incubated at 37°C for 150 min. Later, 20 µl of the reaction mixtures was withdrawn and added to 78 µl of a mixture containing 100 mM HEPES at pH 7.5, 10% w/v sucrose, 0.1% w/v CHAPS (3-[(3-cholamido-propyl)-dimethylammonio]-1-propane-sulfonate), 10 mM DTT, and 2% v/v DMSO. This mixture was prepared for caspase-3 and caspase-9 assay in two different 96-well plates. Afterwards, 2 µl of the caspase-3 substrate DEVD-pNA (10 mM) and 2 µl of the caspase-9 substrate LEHD-pNa (4 mM) were added to the samples in the respective 96-well plate. The plates were incubated overnight at room temperature and the absorbance at 405 nm was measured for both assays using a microplate reader (*Thermo* Scientific *Multiscan* FC).

Apoptosis induction determined by confocal microscopy: HeLa cells (25,000 cells) were seeded and incubated in Lab-tek chambered coverglass (4-wells) as described by us [15,34,42]. The cells were incubated with native proteins (Cyt c and BSA), and HAMLET-like complexes (Cyt c-OA and BSA-OA) at 80 µM OA concentration at 37°C for 6 h. For detection of apoptosis-dependent nuclear fragmentation, the cells were washed with PBS (1×) and incubated initially with DAPI (300 nM) and thereafter with PI (75 µM) for 5 min each. HeLa cells were then fixed using 3.7% formaldehyde. The coverslips were examined under a Zeiss laser-scanning microscope 510 using a 67× objective. Co-localization of DAPI and PI upon internalization into HeLa cells was determined, which is representative of highly condensed and fragmented chromatin in apoptotic cells. DAPI was excited at 405 nm and its emission detected at 420-480 nm. PI was excited at 488 nm and its emission detected above 505 nm.

Cellular uptake determined by confocal microscopy: The internalization of HAMLET-like complexes (Cyt c-OA and BSA-OA) was determined by confocal laser scanning microscopy. To execute these experiments, the protein components (Cyt c and BSA) of the HAMLET-like complexes were labeled with FITC through the lysines residues. HeLa cells (25,000 cells) were seeded in Lab-tek chambered

coverglass (4-wells) as described above. The cells were incubated with FITC-Cyt c-OA and FITC-BSA-OA at 80 μM OA concentration, DAPI (300 nM) and a membrane marker (FM-4-64; 10 mg/mL) at 37°C for 6 h. Afterwards, the medium was removed and the cells were washed with PBS thrice followed by fixation of the cells with 3.7% formaldehyde. The coverslips were examined under a Zeiss laser-scanning microscope 510 using a 100× oil immersion objective and excitation at 488 nm for FITC and FM-4-64. FITC-Cyt c-OA and FITC-BSA-OA fluorescence was detected at wavelengths between 513-588 nm and the FM-4-64 between 598-738 nm. DAPI was excited at 405 nm and its emission detected at 420-480 nm.

Statistical analysis

All measurements and samples were analyzed in quadruplicate of two independent experiments and results are expressed as mean values ± SD. The analysis was performed using SigmaPlot (SyStat Software) followed by Student t test to obtain p-values. Statistical significance was established at $p<0.05$. The following notation was used throughout: *: $p<0.05$; **: $p<0.01$; and ***: $p<0.001$.

Results and Discussion

Characterization of HAMLET-like complexes

Cyt c and BSA were incorporated in our experiments to determine if biologically relevant proteins could form HAMLET-like complexes. In this study, Cyt c, a pro-apoptotic protein, and BSA, an intrinsic tumor-targeting protein [38], were used as OA-carrier. In our case, BSA was used as a control for the *in vitro* assays since its tumor accumulation capabilities are only relevant in *in vivo* experiments. Cyt c has the capability to be both, nanosized protein-based carrier and the therapeutic agent.

It is known that excessive heat and pH extremes can perturb the protein structure and consequently lead to loss of function. This is not desirable in our case because the apoptotic activity of Cyt c is necessary for the suggested synergistic effect of protein drug and lipid drug. In our last paper some BAMLET complexes were synthetized using different treatment methods [15]. Based on our past results, we chose a set of mild conditions, which promote OA binding but avoid detrimental protein structural perturbations, namely 20 mM Tris-HCl buffer at pH 8 heated to 45°C and ultra-sonicated (Figure 1). The resulting complexes were purified using a centrifugal filtration system (Millipore Amicon) and freeze-dried.

Coupling of OA to Cyt c and BSA was qualitatively confirmed by fluorescence emission spectroscopy (Figure 2). BAMLET complexes typically show an increase in the fluorescence intensity and a red shift in the maximum upon OA binding to the protein [15,18]. However, the magnitude of spectral changes is protein dependent and varies significantly depending on the reaction conditions employed [29,43]. The binding of OA to the protein is based on hydrophobic and electrostatic interactions [44,45] and these interactions can perturb protein structure. The increase in fluorescence intensity observed in our cases (Figure 2) was less remarkable than in previous studies [46,47], likely because we employed mild conditions. The fluorescence intensity increase can be due to some loss in tertiary structure because protein aromatic residues are inhibited from self-quenching upon OA binding [48,49]. It was therefore important to test the functionality of Cyt c in the complex by measuring the caspase activation by the Cyt c-OA complex which we present later in this paper.

To determine the amount of OA bound to Cyt c and BSA (mol/mol), the FA content was measured using a commercial colorimetric kit (Table 1). The particles were completely dissolved by the detergent in the buffer and the amount of OA moieties coupled to the protein in the particles was determined. The results show that 12 and 53 OA molecules were attached to Cyt c and BSA, respectively. This was expected because BSA has a molecular weight of 66 kD and is roughly four times larger than Cyt c.

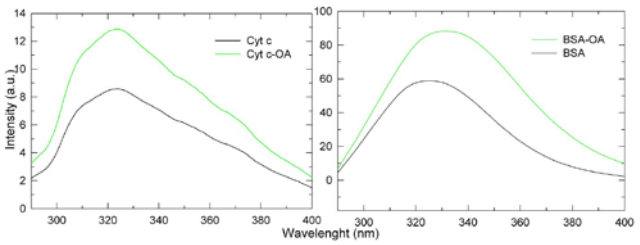


Figure 2: Fluorescence emission spectra (α_{exc}=280 nm) of the HAMLET-like complexes synthesized in this work.

Samples	OA loading (OA/protein molar ratio)[2]	Diameter (nm)[3,5]	Polydispersity index[4]	z-Potential (mV)[5]
Cyt c-OA[1]	12 ± 2	122.9 ± 5.5	0.174 ± 0.013	-0.489 ± 3.2
Cyt c				18.4 ± 4.0
BSA-OA[1]	53 ± 5	169.0 ± 4.6	0.416 ± 0.019	-81.3 ± 13.8
BSA				-27.4 ± 4.9

[1]Syntheses were performed in Tris-HCl buffer at pH 8 and 45°C. [2]Number of OA attached on average to each protein molecule determined by FA quantification assay. [3]This size was considered the upper limit for particle size determined by DLS. [4]Polydispersity is used as a measure of the homogeneity of the NP. [5]For these analyses, native Cyt c and FA-depleted BSA were dissolved and HAMLET-like complexes were suspended in filtered nanopure water

Table 1: Characterization of HAMLET-like complexes.

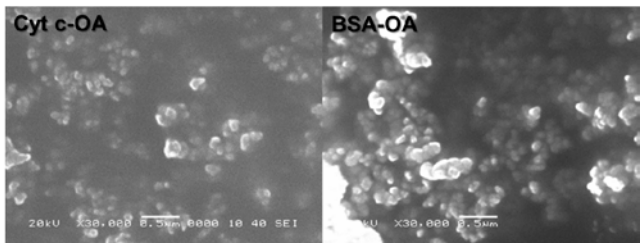


Figure 3: SEM images ofthe HAMLET-like complexes synthesized. In agreement with DLS measurements (Table 1) the images display spherical nanoparticles.

Another important property of drug delivery systems is their size. Tumor areas are often richly vascularized by copious angiogenesis but the blood vessels are "leaky" because of large fenestrae. Consequently, drug delivery systems can accumulate in the tumor interstitial space by the EPR effect if they have a suitable size [7] (Scheme 1). The gap size of the aberrant vasculature ranges from 100-800 nm depending of the tumor type [3]. Our previous study was the first that evaluated the size and morphology of BAMLET complexes using DLS and SEM. Those BAMLET complexes had a diameter of 200 nm and spherical shape [15]. The HAMLET-like complexes obtained herein were also spherical NP as demonstrated by SEM (Figure 3). (Zoom of the SEM images included in the Supplementary Information.) The particle size of the hydrated micellar system was determined by DLS and was 122.9 nm for

Cyt c-OA and 169.0 nm for BSA-OA (Table 1). These data demonstrate that the HAMLET-like complexes are protein aggregates with OA associated with them. In addition, we performed a NP stability assay determining the activity of the drug in the HAMLET-like complexes after exposure to stress conditions, i.e., 75% relative humidity and 50°C (for more details see the Supplementary Information).

Cytotoxicity against cancer and normal cell lines

It has been claimed previously that HAMLET-like complexes are tumor-selective [13,50]. Nevertheless, none of our previous data support the notion that the α-LA is essential for the anti-tumor activity or produces significant selectivity. So, in essence data obtained with the constructs in this work can be compared to free OA because free OA has the same toxicity as HAMLET has. Consequently, next we compared the cytotoxicity of free OA versus OA in the Cyt c complex to investigate whether or not Cyt c would increase the toxicity of the complex (Figure 4). Our results indeed show a statistically significant increase in cell death when HeLa cells where incubated for 6 h with Cyt c-OA at the same concentrations than with OA alone. Thus, cytotoxicity was enhanced synergistically by binding to the pro-apoptotic Cyt c.

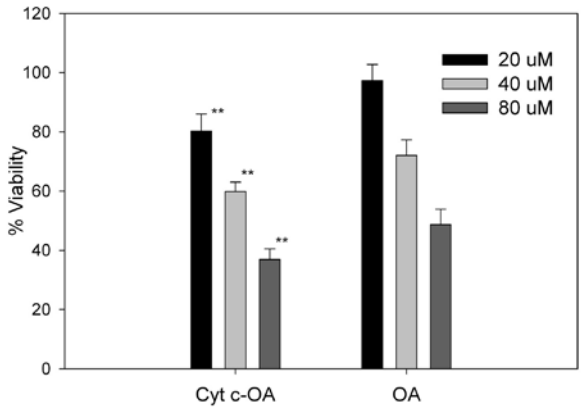


Figure 4: LC_{50} determination after incubation of HeLa cells with Cyt c-OA and OA at different concentrations. The **indicate statistically significant differences compared with OA alone as a control; p<0.01.

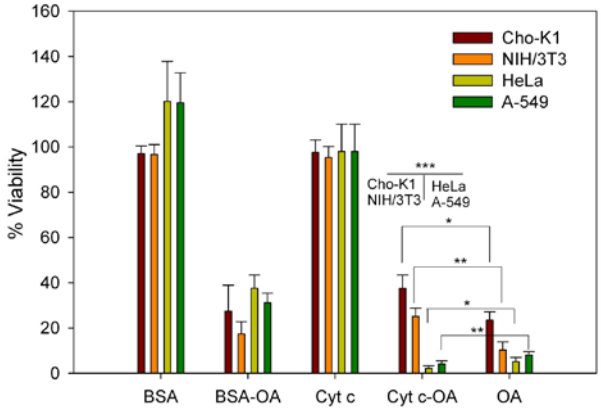


Figure 5: Non-specific cytotoxicity of 120 μM of HAMLET-like complexes and OA towards normal cells (Cho-K1 and NIH/3T3) and cancer cells (HeLa and A-549) after 6 h of incubation.The asterisks indicate significant differences: *: p<0.05; **: p<0.01; and ***: p<0.001.

FAs formulation	Relative caspase-3 activation[1] (%)	Relative caspase-9 activation[1] (%)
OA	<1 ± 1	<1 ± 1
Cyt c-OA[2]	48 ± 4	56 ± 5
Cyt c-OA[3]	26 ± 2	30 ± 3
Cyt c[4]	100 ± 9	100 ± 3
Cyt c[2]	73 ± 3	81 ± 4
BSA-OA[2]	<1 ± 1	<1 ± 2
BSA[2]	<1 ± 2	<1 ± 1

[1]The relative activities were calculated from the absorbance changes caused by the caspase-3 and caspase-9 hydrolysis of pNA-substrates, 10 mM DEVD-pNA and 4 mM LEHD-pNA, respectively. pNA formation was monitored colorimetrically at ~ 405 nm. [2]These samples were prepared using the original conditions mentioned before in Tris-HCl, pH 8 and 45°C. [3]This complex was prepared using the original conditions except by heating at 65°C. [4]Native Cyt c. The values in the table are the averages of triplicate measurements and the error values are the calculated S.D.

Table 2: Capability of various preparations to activate caspase-3 and -9 in cell-free assays.

We next proceeded to test all constructs and controls by viability assay using normal cells and cancer cells (Figure 5). Cho-K1 and NIH/3T3 were selected as normal cell lines and HeLa and A-549 as cancer cell lines. All OA containing preparations were adjusted to a concentration of 120 μM OA and the protein controls to the same Cyt c and BSA concentration as in the complexes. This means that even though the actual loading of OA in each complex was different, the same concentration of OA was added to the cells with both HAMLET-like complexes. HAMLET-like complexes and OA showed cellular toxicity for all cell lines after 6 h while the protein alone controls Cyt c and BSA did not show any toxicity. We anticipated the latter because Cyt c cannot enter the cell without OA attached. In contrast, some cancer cells accumulate serum albumin and metabolize it; in consequence they grow even faster in the presence of BSA. Interestingly, Cyt c-OA was much more aggressive statistically to the cancer cells than to normal cells. OA alone was significantly more cytotoxic to normal cells than Cyt c-OA and less toxic towards cancer cells. In other words, Cyt c-OA should have a better therapeutic index or window than the isolated OA. The BSA-OA complex was similarly toxic to normal cells than Cyt c-OA or OA but the viability of cancer cells was substantially increased. It is possible that instead of a synergistic effect we find in the case of Cyt c-OA we find an antagonistic effect of the drugs because serum albumin has the inherent ability to serve as a growth nutrient.

Cellular uptake and cell death pathway

As stated before, HAMLET can kill cancer cells by activating different cell death pathways [51]. After internalization, the proposed apoptotic pathway induced by HAMLET includes lysosomal leakage and activation of BAX. Furthermore, HAMLET induces mitochondrial membrane permeabilization, BAX oligomerization, and cytochrome c release [52,53]. Contradictory results were reported on the caspase activation by the apoptotic machinery. Caspase inhibitors and P_{53} mutations have shown to have no effect on BAMLET-induced cytotoxicity indicating existence of caspase-independent cell death [54]. For these reasons, we carried experiments to investigate the mechanisms of cell death induced by HAMLET-like complexes. We performed cell-free caspase-3 and -9 activation colorimetric assays. Protein-enriched cell lysates were obtained by freeze-thaw disruption cycles and centrifugation to remove any mitochondria to eliminate any caspase activation due to inherently released Cyt c. The results (Table 2)

show that both caspases were not activated by OA, BSA, and the BSA-OA complex, in agreement with the literature [54].

Cyt c had the expected effect in activating both caspases, but when in the Cyt c-OA complex the activation was reduced. This was likely due to some preparation-induced Cyt c denaturation because temperature-treated Cyt c also had a somewhat lower activity. In addition are OA moieties bound to lysine residues on the protein surface [15], which could interfere with its capability to bind to Apaf-1. Nevertheless, Cyt c-OA was able to activate the caspases, which potentially can explain why it works synergistically with OA. Overall we confirmed that OA induces a caspase-independent cell death pathway. However, when it is coupled to Cyt c the cascade of caspases is activated too.

Previous experiments have shown that HAMLET causes chromatin condensation and cell shrinkage [47,51]. These cell morphological changes are due to the activation of apoptosis or autophagy-like cell death by the action of HAMLET [17]. Apoptosis induced by our HAMLET-like complexes was qualitatively investigated using DAPI (blue) and PI (red) co-localization analysis by confocal microscopy (Figure 6). HeLa cells were incubated with BSA-OA (A), Cyt c-OA (B) for 6 h and stained with DAPI and PI. Cells treated with the HAMLET-like complexes showed co-localization of DAPI and PI (Figure 6). Cells treated with BSA-OA demonstrated chromatin perturbation, pyknosis, and PI signal increment in the cells indicative of late apoptosis (Figure 6A). Cyt c-OA-treated cells also showed cell shrinkage due to ongoing apoptosis but with some different morphological changes, which might be due to the caspase activation induced by Cyt c (Figure 6B).

Next, we sought to investigate the cellular fate of the HAMLET-like complexes. Previously, the cellular distribution of HAMLET was studied and after internalization HAMLET mostly accumulated in the nucleus [55]. In this study, the localization of HAMLET-like complexes (Cyt c-OA and BSA-OA) was determined in HeLa cells after 6 h of incubation (Figure 7). We coupled fluorescein isothiocyanate (FITC) to the primary amines of the proteins Cyt c and BSA in the HAMLET-like complexes. The HeLa cells were incubated with the FITC-Cyt c-OA and FITC-BSA-OA and with the phospholipid marker FM-4-64. After 6 h, the treated HeLa cells were stained with DAPI and thereafter examined by confocal microscopy. Under the chosen conditions, green fluorescence displayed in the micrographs is due to the internalization of the HAMLET-like complexes, red fluorescence shows phospholipids (membrane, endosome, lysosome, and nuclear envelope) and blue fluorescence shows the labeled nucleus. Red FM-4-64 dye was used to understand the uptake through the membrane but due to the already advanced stage of cell death, this marker actually only showed the

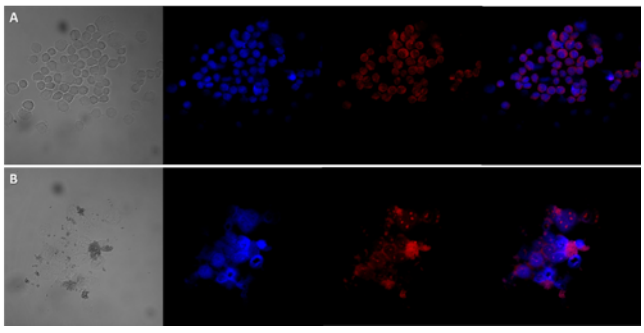


Figure 6: Confocal microscopy of HeLa cells after 6 h of incubation with HAMLET-like complexes. (A-B) were incubated with DAPI (blue) and PI (red); (A) 80 μM BSA-OA complex; (B) 80 μM Cyt-OA complex.

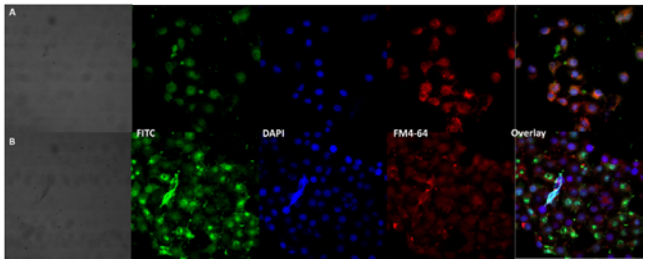


Figure 7: Cellular-uptake and nucleus co-localization: (A-B) were incubated with FITC-HAMLET-like complex (green), DAPI (blue), and FM4-64 (red); (A) 80 μM BSA-OA complex; (B) 80 μM Cyt-OA complex.

lipids of the nuclear envelope [56]. The confocal micrographs confirm the presence of apoptosis and demonstrate that OA in HAMLET destabilizes the composition of the membrane. Co-localization of the green and blue markers resulting in violet fluorescence demonstrates that the complexes were internalized into the nuclei. HAMLET-like complexes were also found in the cytoplasm after 6 h of incubation and thus Cyt c could also interact with Apaf-1 to induce apoptosis.

Conclusion

In summary, lipid-protein NP as described in this work are an inexpensive and effective technique for the intracellular delivery of proteins. We coupled cytotoxic OA to two proteins, i.e. pro-apoptotic Cyt c and BSA as a control. We demonstrated that Cyt c-OA and BSA-OA NP were able to induce cell death in cancer and normal cell lines due to the expected action of OA. Cyt c-OA complexes were more efficient towards cancer cells but it is likely that the complex should be modified with targeting ligands to avoid cell death in non-cancer cells. In difference to OA alone, Cyt c-OA complexes induce apoptosis via the activation of caspase-3 and -9 in the cytoplasm of the target cells. Future studies should be geared towards investigating in how far EPR effect accumulation in tumors occurs for the complexes. Passive accumulation could in particular make the Cyt c-OA complex an interesting drug candidate. In how far active delivery strategies have to be incorporated as well remains to be seen.

Author's Contribution

YD carried out all the experimental studies, designed the experimental strategy, analyzed data and the statistics, and drafted the manuscript. MMC helped to carry out the *in vitro* experiments, the statistics and the confocal microscopy studies. JHR and GH helped in the experimental part. KG conceived the study, participated in its design and coordination, and finalized the manuscript. All authors read and approved the final manuscript.

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