Nullomer derived anticancer peptides (NulloPs): Differential lethal effects on normal and cancer cells in vitro

Abdelkrim Alileche, Jayita Goswami, William Bourland, Michael Davis, Greg Hampikian*

Boise State University, Department of Biological Sciences, 1910 University Drive, Boise, ID 83725, USA

ABSTRACT

We demonstrate the first use of the nullomer (absent sequences) approach to drug discovery and development. Nullomers are the shortest absent sequences determined in a species, or group of species. By identifying the shortest absent peptide sequences from the NCBI databases, we screened several potential anti-cancer peptides. In order to improve cell penetration and solubility we added short poly arginine tails (5Rs), and initially solubilized the peptides in 1 M trehalose. The results for one of the absent sequences 9R (RRRRRNWMWC), and its scrambled version 9S1R (RRRRRWCMNW) are reported here. We refer to these peptides derived from nullomers as PolyArgNulloPs. A control PolyArgNulloP, 124R (RRRRRWFMHW), was also included. The lethal effects of 9R and 9S1R are mediated by mitochondrial impairment as demonstrated by increased ROS production, ATP depletion, cell growth inhibition, and ultimately cell death. These effects increase over time for cancer cells with a concomitant drop in IC-50 for breast and prostate cancer cells. This is in sharp contrast to the effects in normal cells, which show a decreased sensitivity to the NulloPs over time.

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1. Introduction

This study investigates the biological properties of the smallest absent peptides, nullomer peptides (NulloPs). These peptides were identified after a search of the NCBI databases, which counted all occurrences of peptide strings, and generated a list of the smallest peptide sequences (currently length-5 amino acids) absent from natural databases. While combinatorial and phage display libraries have been used to generate peptide drugs that were not derived from nature, we have taken a completely new approach to look for effective cancer drugs; we look specifically for what does not exist [21] in nature. Acquisti et al. have dismissed this approach [1], claiming that absent sequences are actually a consequence of mutation and will not be useful for medical or labeling purposes; but others have explored the distribution of absent sequences suggesting possible applications [7,17,31,42]. We have used a new algorithm for this negative *in silico* selection that enables us to identify small epitopes with possible lethal effects [21]. While others have debated whether this approach might yield lethal sequences [5,42], we have now shown that several of these peptides are in fact lethal at micromolar concentrations. Our approach originally generated hundreds of NulloPs (unpublished data), and we have been characterizing their biological effects, seeking to exploit their killing mechanisms to ultimately produce new anticancer peptides. This report focuses on NulloP 9 (NWMWC), its scrambled version 9S1 (WCMNW), and NulloP 124 (WFMHW). To ensure cell penetration we conjugated the NulloPs with PolyArg (RRRRR).

Cancer therapy is a combination of surgery, chemotherapy, radiotherapy and recently growth factors and cytokines, and has not yet met our dreams of curing this disease [2]. In spite of the

* Corresponding author. Tel.: +1 208 426 4992.
  E-mail address: greghampikian@boisestate.edu (G. Hampikian).

application of a wide variety of drugs, anti cancer chemotherapy has always been limited by side effects and chemoresistance [41]. The extreme genetic adaptability of cancer cells in their ultimate quest of immortality and independence gives these cells several survival advantages in harsh conditions; for example they can stop cell division and enter a state of dormancy, or become stem cells capable of regenerating the original tumor [27].

Cancer cells are resistant to apoptosis induction, and a new paradigm in cancer therapy is emerging. Cancer cells survive because their metabolism is altered with significant changes in mitochondrial function, and a lower dependence on pyruvate oxidation, the Warburg effect [6]. The therapeutic approach known as mitochondrial medicine [11,12,16] is justified for several reasons: cancer mitochondria have low oxidative phosphorylation, high $\Delta\psi$m and reactive oxygen species (ROS) output, and a deregulated apoptotic pathway [12]. The mitochondrial outer membrane permeabilization (MOMP) constitutes a "*point of no return*," in that once it begins, cells are destined to die by apoptosis or necrosis [29]. Chemicals targeting the mitochondria have the advantage to initiate the MOMP independently of upstream signals that are frequently impaired in cancer cells, and thus bypass chemoresistance [12,16].

Already many chemicals and peptides (derived from existing proteins involved in cell death) that target the intrinsic mitochondrial death machinery are being used in clinical trials [12,16]. Other natural peptides such as defensins [10,39,53] (endogenous antimicrobial peptides produced by leukocytes and epithelial cells), have demonstrated anticancer potential. Their mechanism of action involves a stimulation of the immune system [10,56], but it is still not clear what makes cancer cells susceptible to these peptides. The general mechanisms suggested are: lysis of cancer cells [23,49], plasma membrane disruption via micellization or pore formation [50], and induction of apoptosis via mitochondrial membrane disruption [48].

One of the characteristics of the NulloPs described here is their high hydrophobicity due to the presence of TRP and MET. Their poor solubility in water becomes a major obstacle for their use. In order to overcome this, we fused them to a cell penetrating peptide (CPP). This is a well established approach that started with the observation that the human immunodeficiency virus 1 (HIV-1) Tat protein can enter cells efficiently in vitro [13]. In 1997, Vives et al. [51] discovered that a 11 amino acids sequence, Tat 48-60 (GRKKRRQRRRPPQ) known as the Tat peptide, can enter cells more efficiently than the full length Tat protein. A PolyArg peptide made from 6 to 9 L-ARG maintains the same cell penetrating capacity as the original Tat peptide [52]. The mechanism(s) of internalization of PolyArg CPP is still a controversial matter. There are two principal routes of internalization: endocytosis (an energy dependent process), and transduction through cellular membrane.

PolyArg CPP [45] and Tat protein [36] interact with the cell membrane by inducing a Gaussian membrane curvature as a prelude to pore formation through which transduction to the cytosol occurs. In spite of the pore formation, after transduction of PolyArg CPP and its cargo inside the cell, the cell membrane integrity is not compromised and the cell remains negative for propidium iodide (PI) staining [37]. The release of the cargo from the PolyArgCPP–Cargo complex has not yet been demonstrated [34].

We have proposed that by identifying the smallest absent DNA sequences, we can find short peptides eliminated by natural selection. These sequences may have unusual properties that can be exploited for drug development [21]. This report demonstrates the anticancer benefits of the nullomer approach, and shows that NulloPs can affect the growth of several cancer cell lines by mitochondrial impairment.

2. Methods

2.1. Cell culture

Cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The human and murine cancer lines are: LnCap (CRL-1740) derived from a human prostate carcinoma, MDA-MB-231 (HTB-26) derived from a human breast adenocarcinoma, B16 (CRL-6322) derived from a C57BL/6J mouse skin melanoma, HUT102 (TIB-162) derived from an ATL (HTLV-1 Adult T cell Leukemia) a gift from Dr. T.A. Waldmann (NIH/NCI), J774A.1 (TIB-67) mouse monocyte/macrophage derived from a BALB/cN reticulum cell sarcoma. Primary normal cell lines were used as well: PCS (PCS-440-010) normal primary prostate epithelial cells, HMEC (ECACC-HMEC 830-05a) normal human mammary cells, WI-38 (CCL-75) human embryonic fibroblasts derived from a 3 months gestation fetus. LnCap, MDA-MB-231, and HUT102 were cultured in RPMI 1640 supplemented with 10% Hyclone fetal bovine serum (FBS) and 1× penicillin streptomycin (100× solution, Invitrogen). B16 and WI-38 were cultured in DMEM supplemented with 10% Hyclone FBS and 1× penicillin streptomycin. PCS cells were cultured in the prostate epithelial cell basal medium (PCS-440-030) supplemented with the prostate epithelial cell growth kit (PCS-440-040). HMEC cells were cultured in the Clonetics MEGM BulletKit (CC-3150). Cell cultures were incubated at 37 °C and 5% CO_2. For PCS and HMEC cells we used passages 2–4. The passaging of cells was done at 80% confluence; after a PBS wash, cells were treated with trypsin–EDTA 0.25% (Invitrogen). Cells were seeded in 96 well plates (Costar) and allowed to adhere to the surface overnight, then the old media was removed and replaced with 90 µl fresh media, with 10 µl containing peptides or other treatment.

2.2. Cell viability assay

Cell viability was measured by the colorimetric MTT assay (Sigma, St. Louis, MO). 3000–5000 cells/well were seeded in 96 well plates and exposed to peptides or other treatments. At each time point, the MTT solution (5 mg/ml in PBS) was added directly to wells and incubated at 37 °C for 4 h, then the formazan crystals solubilized with 100 µl dimethyl sulfoxide (DMSO), and incubated for 1 h at 37 °C. Absorbance at 570 nm was taken by a plate reader (SynergyMx from Biotek, Winooski, VT). Plate readings were exported to Microsoft Excel and GraphPadPrism software. All the wells were analyzed in triplicates. The statistical analysis was done with the GraphPadPRISM*.

2.3. PolyArg-peptides synthesis

The PolyArgNulloPs were made by Elim Biopharmaceuticals (Hayward, CA) and Pierce Biotechnology (Rockford, IL). HPLC purified peptides (purity > 98%) were delivered in 1–2 mg/tube (lyophilized) format and stored at −20 °C. Peptides were solubilized in 1 M trehalose to produce 100 mM stock solutions. Only freshly prepared solutions were used to treat cells. Several concentrations of the peptides were used with a range from 5–10 µM to 100 µM. The trehalose concentration in all reactions was 1 mM (a dose without any effect on cell growth).

2.4. Preparation for scanning electron microscopy

Cells were cultured as described above in 35 mm dishes containing cover slips. Cover slips were prefixed in 4% paraformaldehyde in PBS for 30 min, washed in distilled water 3 times for 5 min each and placed in 2.5% glutaraldehyde for 30 min, followed by washing in distilled water as described. Cover slips were then placed in 0.5%

osmium tetroxide for 30 min and washed again in distilled water. Cover slips were then transferred to 1% tannic acid for 30 min, washed in distilled water and transferred a second time to 0.5% osmium tetroxide, and washed again in distilled water. Cover slips were dehydrated in ethanol (30%, 50%, 70%, 80%, 90% and 100% 3×), 5 min each. The cover slips were transferred to a K850 critical point dryer (Quorum Technologies, Ashford, UK) using 100% ethanol as the transitional fluid. Dried cover slips were sputtered with gold (∼20 nm) in a CrC150 sputter coater (Torr International, New Windsor, NY, USA), and examined in a Hitachi S-3400N scanning electron microscope.

2.5. HCS mitochondrial health assay

The HCS mitochondrial health kit (Invitrogen, Carlsbad CA) uses two dyes: the MitoHealth stain (excitation/emission maxima 550/580 nm) accumulates in the mitochondria of live cells proportional to the mitochondrial membrane potential, Hoechst 33342 (excitation/emission 350/461 nm) stains nuclear DNA in live and dead cells. Cells were seeded in 96 well plates and incubated with peptides for 24 h. Then 50 μl of MitoHealth stain was added to each well and incubated for 30 min. After removing the media, Hoechst 33342 and 3.7% paraformaldehyde were added to cells. After washing the cells twice with PBS, colorimetric reading was taken with the plate reader. Relative fluorescence was measured in BioTek Synergy plate reader. This kit was manufactured for imaging, but we standardized it with SynergyMx plate reader for reading 96-well plates.

2.6. Measurement of total cellular ATP content

Cells were seeded in 96 well plates (white plates from Nunc to block luminescence bleeding between the wells) and allowed to attach for 24 h. Then cells were incubated for 2 h, 24 h and 48 h with peptides (1–50 μM of 9R, 9S1R and 124R), or 100 mM sodium azide as an ATP depletion control [26] At each time point a single reagent, Cell Titer Glow™ (Promega), was added to cells. Complete reagent mixing in 96 wells plates required gentle orbital shaking for 2–10 min. The plate reading was taken by SynergyMx plate reader. Plate readings were exported to Microsoft Excel and GraphPadPrism software. All the wells were analyzed in triplicates. The statistical analysis was done with the GraphPadPRISM*.

2.7. Live/dead assay

Each peptide's effect on cell vitality was measured by the live/dead assay kit from Invitrogen (Carlsbad, CA). This kit uses Hoechst 33342 as a nucleic acid stain (blue fluorescence) and PI (red fluorescence). Since PI is not permeant to live cells, it is commonly used to detect dead cells in a population. The PI concentration used in this study was 6.25 μg/ml. After 2 h incubation with PolyArgNulloPs, cells were stained directly in the reaction media with these two stains, and fluorescent images were taken by Zeiss axiovert 40 microscope (Oberkochen, Germany) using a SPOT imaging camera (Sterling Heights, MI).

2.8. Super oxide assay

After 24 h incubation in 96 wells microplates, cells were treated with peptides or control for 2 h. Then MitoSOX™ red reagent (Invitrogen) was added for 10 min, and washed with PBS. The final plate reading was taken in SnyergyMX plate reader.

2.9. Hemolytic activity

The hemolytic activity of PolyArgNulloPs was determined on human red blood cells (RBCs) from healthy volunteers. Blood samples were centrifuged and RBCs washed twice with PBS. A 10% RBCs suspension was incubated with 10 μM, 25 μM and 100 μM of peptides (9R, 9S1R and 124R) at 37 °C for 24 h with 100 rpm shaking. PBS was used as a negative control (0% lysis: $A_{control}$), and 1% Triton X-100 as a positive control (100% lysis: A_{total}). After incubation the samples were centrifuged and supernatants transferred into a 96-well plate to quantify RBCs lysis by a spectrophotometric reading at $\lambda = 405$ nm [23]. The hemolytic activity [20] in % was calculated using this formula: hemolytic activity in % = {[$A_{sample} - A_{control}$]/[$A_{total} - A_{control}$]} × 100.

2.10. Statistical analysis

All results are expressed as mean ± SE (standard error). The statistical analysis was done with the GraphPadPRISM* version 5.03 using one-way analysis of variance (ANOVA) followed by a Tukey's post test. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. The in vitro effect of PolyArgNulloPs peptides on cell growth

Cells were exposed to different doses (10–100 μM) of PolyArgNulloPs (9R, 9S1R and 124R) for 2 h, 24 h and 48 h. For each time period, the exposure of cells to the peptides was continuous with no change of the culture medium. The 2 h time point was aimed at detecting toxic effects similar those of the control treatment H_2O_2 (0.01%). The 24 h and 48 h time points were used to examine peptide effects related to the cell cycle.

The results show clearly that peptide 124R has no effect on the growth of LnCap prostate cancer (Suppl. R1A) or MDA-MB-231 (Suppl. R1B) cells. Peptide 124R is a PolyArgNulloP with the same number of amino acids, and the same poly Arginine content as 9R, 9S1R, and can be considered a control peptide. The peptide 9R at 100 μM has a toxic effect at 2 h on LnCap cells, similar to the effect of H_2O_2 (0.01%). At 24 h and 48 h, 9R has a moderate effect on LnCap at 20–30 μM, and an almost completely lethal effect at 50–100 μM. However, we do not observe a classic dose dependent effect between 5 and 100 μM with 9R in LnCap cells. With the MDA-MB-231 cells, peptide 9R produces a dose dependent effect (10–100 μM) at 2 h, 24 h and 48 h. The peptide 9S1R shows a dose dependent effect over 10–100 μM at 2 h for both the LnCap (Suppl. R1A), and MDA-MB-231 (Suppl. R1B) cells. This toxic effect is also seen at 24 h and 48 h.

The melanoma B16 cell line was included in this study because skin melanoma is the sixth most prevalent cancer in North America, and its incidence is on the rise [14]. The three peptides 9R, 9S1R and 124R at doses 10–100 μM, and at time points from 2 h to 96 h (Suppl. R1C), have no effect on the growth of B16 cells.

Peptide 124R, at doses 10–100 μM, at time points from 2 h to 96 h, has no effect on the growth of HUT 102 cells. However, peptide 9R and 9S1R have a toxic effect at 100 μM seen from 2 h. In addition, a dose response was observed from 10–100 μM at time points from 48 h to 72 h. In highest range of doses (50–100 μM), a dose dependent effect is only observed at the 96 h time point (Suppl. R1D).

The peptides 9R, 9S1R and 124R have no effect on the growth of J774A.1 cells at doses 10–100 μM, from 2 h to 72 h. At 96 h, peptide 124R has no effect; but there is a dose dependent effect with 9R and 9S1R (although the effect is more pronounced with 9R) (Suppl.

Table 1

IC-50 evolution. The IC-50 (in μM) were calculated from the raw data of MTT readings at 570 nm using the GraphPad PRISM* version 5.03. Dose response curves generated by non linear regression with GraphPad PRISM* were used to determine the IC-50s. Basically the x values from the $y = f(x)$ function were transformed into log 10 scale. The y values normalized between 0% and 100% effects and IC-50 calculated. All results are expressed as mean ± SE (standard error). A p-value of less than 0.05 was considered statistically significant.

Cell line	Time (h)	Peptide (μM)		
		9R	9S1R	124R
LnCap	2	44 ± 2.5	26 ± 1.3	>100
	24	23 ± 1.2	9 ± 0.6	>100
	48	28 ± 1.6	8 ± 0.5	>100
MDA-MB-231	2	39 ± 1.8	18 ± 0.8	>100
	24	29 ± 1.7	12 ± 0.4	>100
	48	16 ± 0.9	10 ± 0.3	>100
PCS	24	28 ± 1.1	26 ± 1.2	>100
	48	35 ± 1.3	22 ± 0.9	88 ± 2.3
HMEC	24	20 ± 0.8	17 ± 0.8	29 ± 1.1
	48	34 ± 1.2	19 ± 0.6	46 ± 2.2
HUT102	2	93 ± 2.3	38 ± 1.4	>100
	24	39 ± 1.1	43 ± 1.4	>100
	48	37 ± 2.5	43 ± 3.2	>100
	72	36 ± 1.8	45 ± 2.2	>100
	96	25 ± 1.6	36 ± 1.4	>100
J774A.1	24	47 ± 2.5	26 ± 1.3	66 ± 3.5
	96	12 ± 0.7	17 ± 1.2	31 ± 1.5

R1E). Microscopically, the cells swell and contain many vacuoles (data not shown).

We also examined the effects of the peptides 9R, 9S1R and 124R on normal cells. Peptide 124R has no effect on the growth of WI-38 (Suppl. R1F), and PCS (Suppl. R1G) cells up to 48 h time point (10–100 μM). However at the highest concentration (100 μM) peptide 124R has an effect on the growth of HMEC cells (Suppl. R1H) at 24 h, and to a lesser extent at 48 h. Peptide 9R and 9S1R have a moderate dose dependent effect (20–100 μM) on WI-38 cells at 24 h, and that effect is restricted to the 50–100 μM treatments at 48 h (Suppl. R1F). Peptide 9R and 9S1R have a moderate effect on the growth of PCS cells at 24 h and 48 h time points (Suppl. R1G). In addition, peptides 9R and 9S1R have a dose dependent effect (10–100 μM) on the growth of HMEC cells at the 24 h and 48 h time points (Suppl. R1H).

Scanning electron micrographs of LnCap and MDA-MB-231 cells treated with 9R (Fig. 1E and F, respectively) and 9S1 (Fig. 1G and H, respectively) for 48 h (100 μM), show greatly reduced cell density compared to untreated LnCap (Fig. 1A) and MDA-MB-231 (Fig. 1B) cultures. Cells treated with the control peptide 124R (Fig. 1C(c) and D(d)) appear similar to untreated cells (Fig. 1A(a) and B(b)). At higher magnification the morphology of 9R and 9S1 treated cells show substantial membrane damage including blebbing and tearing (Fig. 1e, f, g, and h).

3.2. The IC-50 evolution of PolyArgNulloPs peptides in vitro

We calculated the IC-50 of peptides 9R, 9S1R and 124R at every time point where there is an effect on the growth of cells. As shown in Table 1, the evolution of IC-50 for cancer cells is different from that seen in normal cells. For LnCap cells, the 9R IC-50 starts high at the 2 h time point (44 ± 2.5 μM), and drops by 48 h (28 ± 1.6 μM). The same pattern of IC-50 evolution for 9R is seen in MDA-MB-231 cells, where the IC-50 is 39 ± 1.8 μM at 2 h, and falls to 16 ± 0.9 μM at the 48 h time point. The same pattern can be seen with peptide 9S1R; for LnCap cells the IC-50 starts at 26 ± 1.3 μM at 2 h and falls to less than 8 ± 0.5 μM at the 48 h time point. For MDA-MB-231 cells, with 9S1R the IC-50 starts at 18 ± 0.5 μM at the 2 h time point,

and drops to 10 ± 0.3 μM at the 48 h time point. For HUT102 cells, the IC-50 for 9R starts at 93 ± 2.3 μM at the 2 h time point, and falls to 25 ± 1.6 μM at 96 h time point. This observation shows clearly that the cancer cells are more sensitive to 9R and 9S1R over time. Normal cells show an opposite pattern. For PCS and HMEC cells, the IC-50 for 9R, 9S1R and even 124R rises with time. This means that the sensitivity of normal cells to 9R, 9S1R and 124R diminishes with time, while the sensitivity of the cancer cells increases with time. The obvious potential clinical importance of this finding needs to be validated in animal models.

3.3. PolyArgNulloPs effects on cellular ATP level

We studied the effects of peptides 9R, 9S1R and 124R on total cellular ATP content in LnCap and MDA-MB-231 cells. Peptide 124R at doses 1–50 μM, for time points from 2 h to 48 h, has no effect on the cellular level of ATP in LnCap and MDA-MB-231 cells (Fig. 2). This is consistent with the observation that peptide 124R does not affect cell growth. Peptide 9R has a moderate effect at the 2 h time point with 50 μM peptide in both cell lines. This effect persists at the 24 h time point. By 48 h, there is a 50% and 65% reduction with 25 μM and 50 μM 9R, respectively, this latter effect is similar to that seen with our control 100 mM sodium azide. Peptide 9S1R at 50 μM almost completely shuts down ATP production in both cell lines (LnCap and MDA-MB-231) by the 2 h time point, an effect more drastic than the sodium azide control. This is a toxic effect similar to that of anthrax toxin in J774A.1 cells [3,4]. In addition, at 25 μM peptide 9S1R reduces the ATP level by 50% at the 2 h and 24 h time points, and by 85% at the 48 h time point. The effect is even more dramatic in LnCap cells, where 9S1R at 48 h produces a 50% reduction of ATP level at 5–10 μM.

3.4. PolyArgNulloPs effects on mitochondrial function

Mitochondrial membrane potential (MMP) is the driving force behind ATP production by the mitochondrial respiratory chain. As shown in Fig. 3a and b, peptide 124R has no effect on MMP at 24 h in either the LnCap or MDA-MB-231 cell lines. Peptide 9R at 50–100 μM reduces by 40% the MMP in both cell lines. Peptide 9S1R has dramatic effects on MMP, producing a 40% reduction at 30–100 μM in LnCap cells. In MDA-MB-231 the reduction of MMP is even more dramatic: 40% reduction at 20–30 μM, and 95% reduction at 50–100 μM.

Superoxide free radicals production is indicative of mitochondrial damage. We investigated the ROS production 2 h after exposure to the PolyArgNulloPs, before any cellular mechanisms can scavenge these free radicals. As shown in Fig. 4a and b, for both LnCap and MDA-MB-231, peptide 124R at 10–100 μM has no effect on the production of ROS. However, for both cell lines, peptide 9R at 50–100 μM produces more free radicals than the control H_2O_2 (0.01%). Peptide 9S1 at 10–100 μM induces ROS production in a dose dependent manner in LnCap cells, and produces a dramatic increase of ROS production at 100 μM in MDA-MB-231 cells. It is noteworthy that 9R and 9S1R at 100 μM in both cell lines induces more ROS production than the H_2O_2 (0.01%) treated cells.

3.5. Effects of PolyArgNulloPs on RBCs

It is well known that some peptides puncture the cell membrane of RBCs, and this side effect can limit their therapeutic potential. We exposed the PolyArgNulloPs (10 μM, 25 μM and 100 μM) to human RBCs from healthy donors for 24 h at 37 °C. As shown in (Fig. 5), peptides 9R and 124R have no hemolytic effect on human RBCs, and peptide 124R produces only 1% hemolysis at 100 μM. In contrast, peptide 9S1R has significant hemolytic activity: 0.5% at
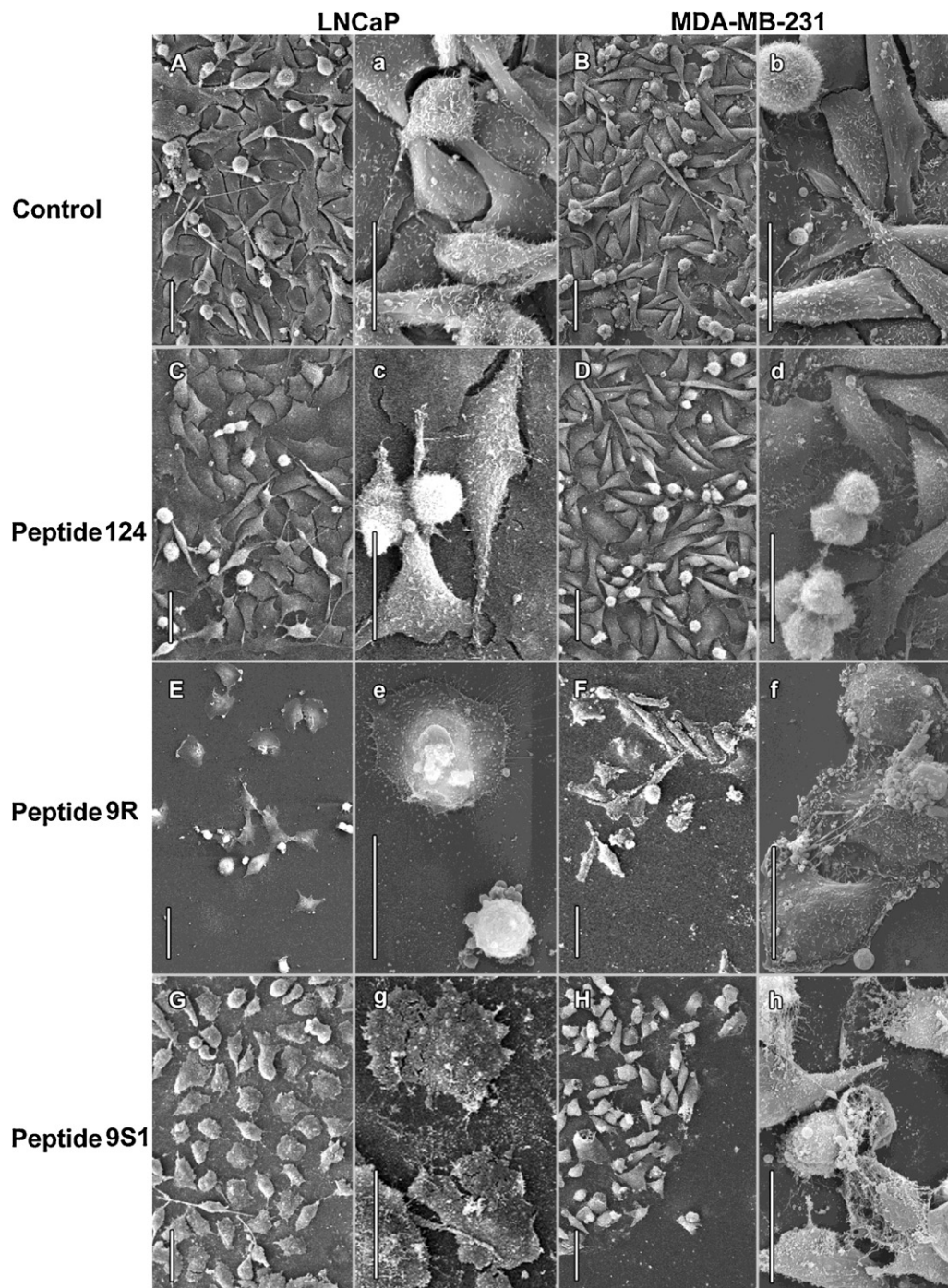
Fig. 1. Scanning electron microscopy of untreated and nullomer (124R, 9R, 9S1R) treated LnCap and MDA-MB-231 cells. (A–H) lower magnification views, (a–h) higher magnification views. (A(a) and B(b)) untreated control LnCap and MDA-MB-231 cells, respectively. (C(c) and D(d)) LnCap and MDA-MB-231 cells, respectively, treated with nullomer 124R (100 μM for 48 h). SEM examination of overall cell growth and membrane integrity after treatment with nullomer 124 is unchanged from untreated control cells. (E(e) and F(f)) LnCap and MDA-MB-231 cells, respectively, treated with peptide 9R (100 μM for 48 h); overall cell growth markedly reduced compared with untreated control cell cultures. Cytopyknosis and membrane blebs appear (e, lower right and F(f)). (G(g) and H(h)) LnCap and MDA-MB-231 cells, respectively, treated with peptide 9S1 (100 μM for 48 h); overall cell numbers less noticeably reduced than in cells treated with peptide 9R, but show widespread severe membrane damage. Scale bars = 50 μm (A–H) and 25 μm (a–h).

10 μM, 2.5% at 25 μM, and 12% at 100 μM. The hemolytic activity of peptide 9S1R may explain its rapid toxic effects at 2 h.

3.6. Live/dead assay

We performed this analysis at the 2 h time point, and used PI as a marker of cell death. Only cells with compromised membrane integrity become PI positive. As shown in (Suppl. R2A) and (Suppl. R2B), both cell lines (LnCap and MDA-MB-231) exposed to H$_2$O$_2$ (0.01%) for 2 h are nearly 100% PI positive. The untreated cells are all PI negative. Cells treated with 100 μM of peptide 124R for 2 h show few PI positive cells (less than 1%). Both cell lines exposed to peptide 9R at 10 μM or 50 μM for 2 h show no effect on PI entry. At 100 μM 9R, both cell lines show significant cell death (30–40% become PI positive). 9S1R has stronger effects on both cell lines, as 30–40% become PI positive with 50 μM 9S1R, and almost 100% are
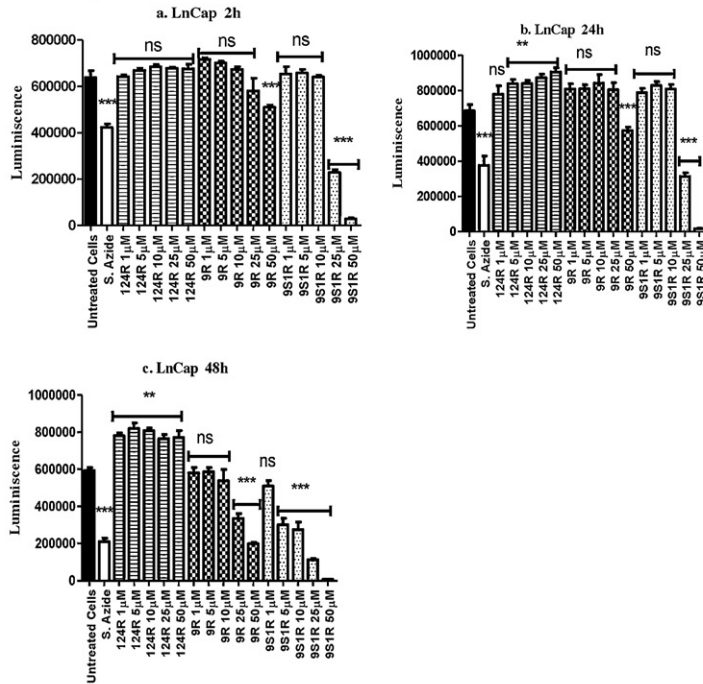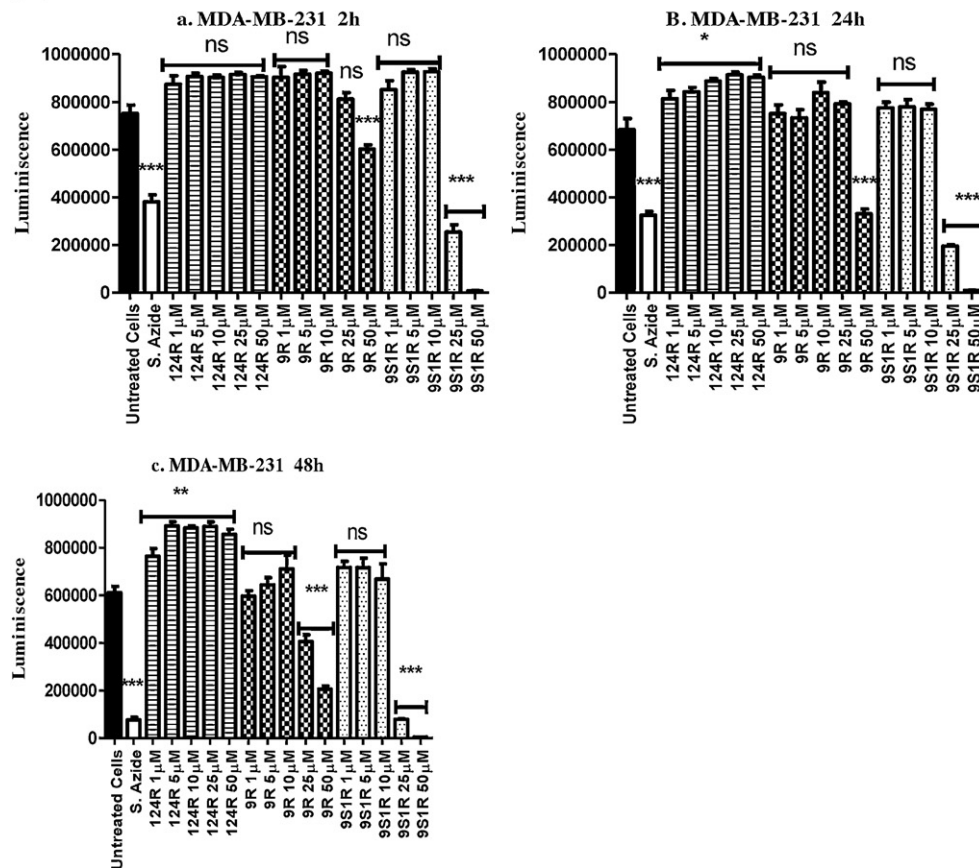
Fig. 2. PolyArgNulloPs effects on cellular ATP level. Cells (3000/well) were seeded in 96 well plates (white plates from Nunc). After 24 h incubation, peptides 9R, 9S1R and 124R were added to the wells. Control conditions included untreated cells, and 100 mM sodium azide. At 2 h, 24 h, and 48 h a single reagent Cell Titer GlowTM (Promega, Madison) was added to the wells (v/v ratio with media), and ATP determined with emission reading using the SynergyMx plate reader. (A) LnCap at (a) 2 h, (b) 24 h, and (c) 48 h. (B) MDA-MB-231 at (a) 2 h, (b) 24 h, and (c) 48 h. The statistical analysis was done with GraphPadPRISM*. Results are as mean ± SE (standard error) of three different experiments. NS, not significant. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.
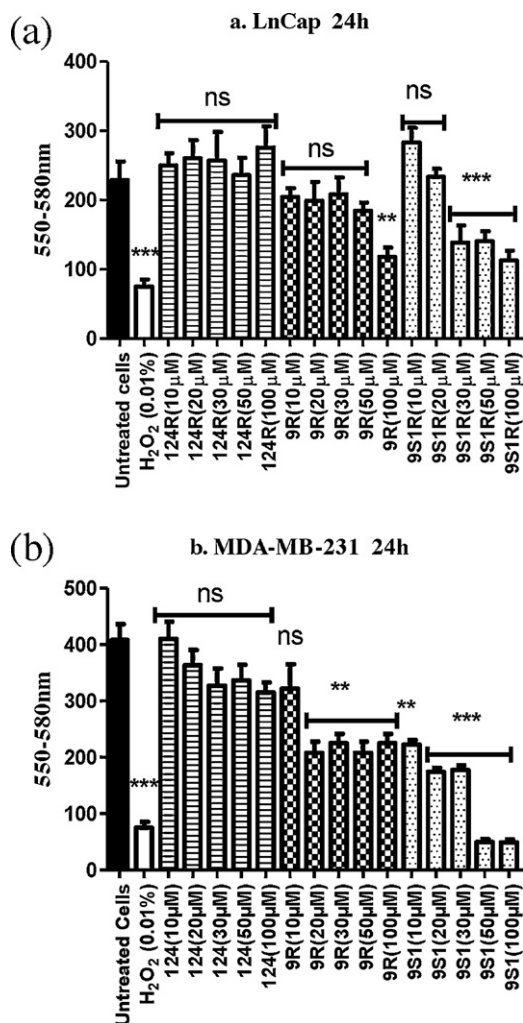
Fig. 3. PolyArgNulloPs effects on MMP. LnCap cells (3000/well) were seeded in 96 well plates (white plates from Nunc). After 24 h incubation peptides 9R, 9S1R and 124R were added to the wells. Control conditions included untreated cells, and H_2O_2 (0.01%) treated cells. At 24 h, 50 μl of mitochondria staining solution was applied per well (following manufacturer's protocol) and incubated for 30 min. Relative fluorescence was measured in BioTek Synergy plate reader. The statistical analysis was done with the GraphPadPRISM*. Results are as mean $\pm$ SE (standard error) of three different experiments. NS, not significant. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. (a) LnCap, at 24 h. (b) MDA-MB-231, at 24 h.

Fig. 4. PolyArgNulloPs effects on ROS production. LnCap cells (3000/well) were seeded in 96 well plates (white plates from Nunc). After 24 h incubation peptides 9R, 9S1R and 124R were added to the wells. Control conditions included untreated, and H_2O_2 (0.01%) treated cells. After 2 h incubation, cells were treated with MitoSox red reagent for 10 min, then washed with PBS, and the plate read in the microplate BioTek Synergy reader. The statistical analysis was done with the GraphPadPRISM*. Results are as mean $\pm$ SE (standard error) of three different experiments. NS, not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (a), LnCap, at 2 h. (b) MDA-MB-231, at 2 h.

PI positive at 100 μM 9S1R. This result is consistent with the effects of 100 μM 9S1R (2 h time point) on cellular ATP and cell survival. But the 2 h time point is too short for an apoptotic process, and the drop in ATP level also contradicts and apoptotic model of cell death. This response is also not typical of necrosis, since cell swelling is not observed.

4. Discussion

4.1. Solubilization of PolyArg peptides in trehalose

The original nullomer peptides 9 (NWMWC), 9S1 (WCMNW) and 124 (WFMHW) are composed of two types of amino acids: MWF (hydrophobic non polar), and CNH (polar but uncharged). They are insoluble in water, and though they are initially soluble in absolute ethanol, after mixing with culture medium they precipitate. We solubilized the PolyArg conjugated peptides in 1 M trehalose (though the NulloPs without PolyArg are insoluble in 1 M trehalose). The solubilization process was instantaneous, and PolyArg conjugated peptides remain stable in solution at room
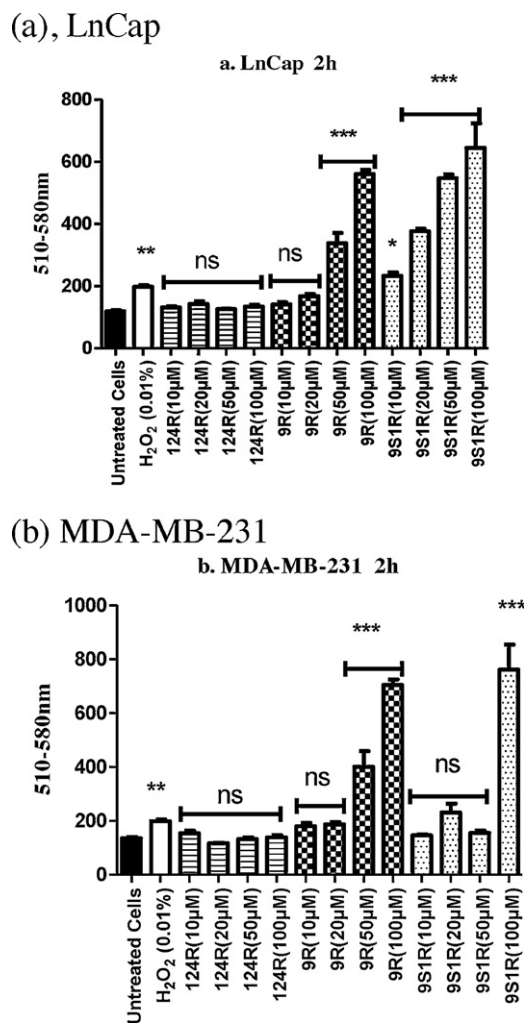
temperature. Trehalose has a water replacing capacity and most likely surrounds the hydrophobic peptides with a carbohydrate coat [30]. It has been used extensively in the formulation of therapeutic proteins, because of its qualities as a protein-stabilizer [25]. This is the first time to our knowledge that a CPP conjugated cargo has been solubilized in trehalose. While others [52] report that an R5 tail is not sufficient for cell penetration, the conjugation of an R5 tail to our peptides 9, 9S1 and 124, facilitates solublization in trehalose.

4.2. PolyArgNulloPs potential for cancer treatment

The treatment of human cancer is not a single drug therapy. A cocktail of drugs aimed at different targets in cancer cells is used to avoid drug resistance. Potential drugs against cancer are characterized by their IC-50, which is the dose that inhibits the growth of 50% of the cell population exposed to the drug. Protocols have been established in the USA by the NCI/NIH [9,40], and in Japan [55], for testing potential new drugs against a panel of cell lines. The current protocols expose cancer cells to drugs for 48 h [57] or
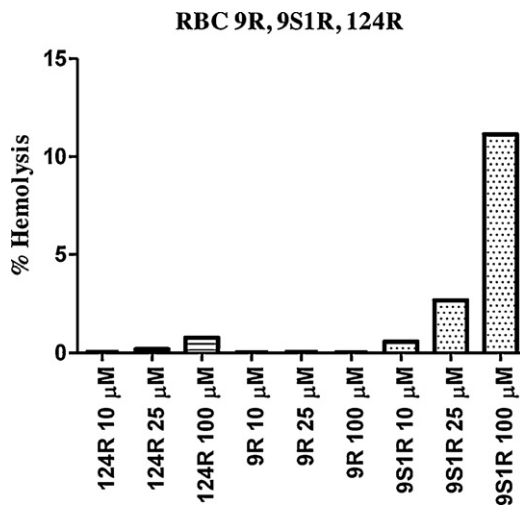
Fig. 5. Hemolytic activity of PolyArgNulloPs. RBCs collected from healthy donors were diluted 1:10 in PBS, and peptides 9R, 9S1R and 124R were added. Control conditions included PBS, and 1%Triton X-100. After 24 h incubation at 37 °C with 100 rpm shaking, RBCs were centrifuged and the supernatant collected in a 96 well plates. Absorbance was determined at 405 nm. The hemolytic activity in % was calculated using the formula: hemolytic activity in % = {[$A_{sample} - A_{control}$]/[$A_{total} - A_{control}$]} × 100.

72 h [40], followed by cell viability assays. It is noteworthy that the screening systems available like the NCI-60 panel of cancer cells [9,40], the JFCR39 panel of 39 cell lines used in Japan [55] or the recently established CMT1000 (Center for Molecular Therapeutics 1000) panel consisting of 1200 cancer cell lines [46], do not include normal cells as controls. The differential sensitivity of cancer cells and normal cells, to cancer drugs, is a complex area. In some studies, normal cells are not sensitive at all to cancer drugs [38]. In other studies, the response of normal cells to cancer drugs depends on the culture protocol: contact inhibited quiescent 3T3 fibroblasts are not sensitive (to cisplatin and etoposide) [24], while exponentially dividing 3T3 fibroblasts [24] and MCF-10A (normal human mammary epithelial cell line) are sensitive [8].

The results presented here include an analysis of the PolyArgNulloPs differential IC-50 evolution in normal and cancer cells. LnCap and MDA-MB-231 cells were included in our study because they represent, respectively, the most frequent cancer in men (prostate cancer) and women (breast cancer). We looked at the killing potential of PolyArgNulloPs at 3 different time points to assess their cytotoxicity and effect on growth. As shown in Table 1, the IC-50 evolution of 9R and 9S1R in LnCap and MDA-MB-231 demonstrates that these cancer lines have an increasing sensitivity to our peptides over time, while the normal cell lines have a decreasing sensitivity. The LnCap IC-50 of 9R starts at $44 \pm 2.5\,\mu M$ at 2 h, and falls to $28 \pm 1.6\,\mu M$ by 48 h. The LnCap IC-50 for 9S1R starts $26 \pm 1.3\,\mu M$ at 2 h, and drops to $8 \pm 0.5\,\mu M$ at 48 h. The same pattern is seen with MDA-MB-231 cells, HUT102 cells and J774A.1 cells. For normal cells like PCS and HMEC, the opposite pattern is seen. The PCS IC-50 of 9R starts at $28 \pm 1.1\,\mu M$ at 2 h, and reaches $35 \pm 1.3\,\mu M$ at 48 h. For HMEC cells, the IC-50 for 9R starts at $20 \pm 0.8\,\mu M$ at 2 h, and reaches $34 \pm 1.2\,\mu M$ at 48 h. This differential pattern of the IC-50 evolution for our peptides shows clearly that normal cells, although inhibited by our peptides can recover, while the sensitivity of cancer cells increases with time. Other researchers have found similar patterns of increased sensitivity for taxol and other drugs [18,32,33,35,44]. Unfortunately, these early observations were not investigated further, and these studies failed to include normal cells [18,32,33,35,44]. The parallel evaluation of cancer cells and normal cells in regard to their sensitivity to cancer drugs is important, because the side effects of cancer drugs on normal cells are a major

clinical issue. To our knowledge the differential evolution of IC-50 in cancer cells versus normal cells is assessed for the first time here. This comparison of normal versus cancer cell sensitivity over time, may be useful to future screening efforts.

4.3. The lack of effects of 9R, 9S1R and 124R on melanoma B16 cells

It is surprising that 9R and 9S1R at 100 µM, even at time points up to 96 h, have no effect on the growth of melanoma B16 cells. The resistance mechanism(s) of B16 cells to PolyArgNulloPs are not known. It has been reported previously that CPPs other than R5 are more efficient at delivering lethal drugs in melanoma cell lines (Bowes melanoma cells [20], and SKMel-37 melanoma cells [15]). Changing the CPP in future studies of the NulloPs may yield better results.

4.4. Comparison of the killing mechanisms of 9R and 9S1R

The analysis of our results support the simple statement that peptides 9R and 9S1R have devastating effects on the mitochondria (as evidenced by their changes in cellular ATP, MMP, and ROS production), and consequently on cell growth. These effects are similar to the effects of amyloid beta peptide on nervous cells in Alzheimer disease, where the amyloid beta peptides are imported into mitochondria [22]. The CPP conjugated peptides do not cross the mitochondrial membrane [43]; however, the mitochondrial outer membrane has a porine protein which is permeable to compounds up to 5000 Da. Our PolyArgNulloPs are in the 1500 Da range, so it is possible that 9R accumulates in the mitochondria, a hypothesis that is under investigation in our lab. Our results, especially the contrast in effects of 9R and 9S1R on cellular ATP, suggest that 9S1R targets non-mitochondrial cytosolic metabolism, whereas 9R attacks the mitochondria. 9S1R (50 µM) almost completely shuts down the ATP production in LnCap and MDA-MB-231 cells. We know that the respiratory chain in the mitochondria is the main source of ATP production in the cells, but glycolysis is important to cancer cell metabolism. The dramatic effect of 9S1R on ATP production (greater than sodium azide) may be caused by an inhibition of cellular glycolysis, which automatically shuts down the respiratory chain in the mitochondria. Therefore, 9S1R is a candidate for the new metabolic strategy of treating cancer through glycolysis inhibition [54] and ATP depletion [28]. Peptide 9R also lowers cellular ATP content by attacking the mitochondria.

4.5. Successful first use of the nullomer approach for drug development

As with any in vitro drug screening, we realize that our results will need to be assessed in animal models. The treatment of cancer is based on the log-kill hypothesis established in 1970 by Skipper et al. [47]. Basically, this hypothesis states that at a certain dose, a cancer drug kills only a fraction of a tumor cell population. This leads to treatment cycles, in which every cycle enriches the proportion of resistant cells. So the results obtained in vitro for a single dose, may not match the results in vivo. Another difference between cell culture and animal studies is that in vitro we start with a small number of cells, and see if a cancer drug can inhibit the growth of that small population; but in vivo studies usually begin with established tumors. As the tumor grows in volume, the fraction of cycling cells diminishes; and as the log-kill hypothesis states, the response to cancer drugs declines. For this reason, cancer therapy uses a cocktail of drugs to attack multiple cellular targets. The relatively high doses used in this paper should not disqualify NulloPs as possible drugs for cancer treatment. Cancer treatment is always a combination of drugs rather than a single drug, and

our next phase of research is looking at possible combination drug treatments. The protocols based on the log-kill hypothesis have been improved recently by a new strategy focusing on the amount of drug per unit time, or dose intensity. This approach is based on high dose drug pulses rather than on a continuous regimen [19]. The first screening of potential anticancer drugs usually focuses a single drug treatment, and we have shown that our PolyArgNulloPs kill cancer cells by targeting mitochondrial health and ATP production. We do not envision their use as single anticancer drugs, but rather combined with other drugs. It is important to note that the difference in sensitivity was measured in rapidly growing, rather than quiescent cell cultures. We are currently studying the differential effects of NulloPs on cancer cells, versus dividing and quiescent normal cells.

5. Conclusion

We present here the first drug candidates produced by nullomer research. The bio-informatic approach, which identified the NulloPs was based on the idea that rare or absent short sequences may be enriched for toxic or problematic peptides. We have shown that the differential effects of PolyArgNulloPs on cancer cells and normal cells indicate that these new peptides should be investigated and optimized for cancer treatment.

Authors' contributions

Abdelkrim Alileche: Devised solubility protocols, designed and performed experiments, analyzed results, and was the lead author in writing the paper. *Jayita Goswami*: Designed and performed of experiments, analyzed results, contributed in writing the paper. *William Bourland*: SEM preparation, imaging and analysis. *Michael Davis*: Contributed to experimental design and analysis of results. *Greg Hampikian*: Devised nullomer approach, designed experiments, analyzed results, contributed in writing paper.

Funding

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.peptides.2012.09.015.

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