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Multifunctional liposomes for enhanced anti-cancer therapy

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MULTIFUNCTIONAL LIPOSOMES FOR ENHANCED ANTI-CANCER THERAPY

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ABSTRACT

Macromolecular drugs have great promises for cancer treatment, such as the pro-apoptotic peptide D-$(KLAKLAK)_2$ and the bcl-2 antisense oligodeoxynucleotide G3139. However, these macromolecules require efficient drug carriers, like liposomes, to deliver them inside cells. Also, if these macromolecules can be combined in a single liposome, the cancer cell killing will be greater than using just one. With this possibility in mind, cationic liposomes (CLs) were elaborated to encapsulate both macromolecules and deliver them inside cells. Later, surface modification of CLs was investigated through the addition of polyethylene glycol (PEG) to obtain long-circulating liposomes.

CLs were prepared through charge alternation among D-$(KLAKLAK)_2$, G3139 and DOTAP. These liposomes were characterized with particle size and zeta-potential measurements, antisense entrapment and peptide loading efficiency. The *in vitro* effects of CL formulations were tested with B16(F10) cells through viability studies, uptake assay and detection of apoptosis. CL formulations were also applied *in vivo* in B16(F10) tumor-bearing mice through intratumoral injections, and tumor growth inhibition and detection of apoptosis were evaluated. Next, the mechanism of action of the CL formulations was investigated by Western blotting. Later, PEG was incorporated at increasing amounts to the liposomes to determine which concentration can better prevent interactions between PEG-cationic liposomes (PCL) and B16(F10) cells. Next, pH-cleavable PEG was prepared and then added to the liposomes in the same amount that PEG in PCL could decrease interaction with cells. Finally, cell viability studies were

performed with CL, PCL and pH-sensitive PCL (pH-PCL) formulations after pre-incubation at pH 7.4 or at pH 5.0.

Positively charged CL particles were obtained after encapsulation of negatively charged D-$(KLAKLAK)_2$/G3139 complexes. *In vitro*, CLs containing D-$(KLAKLAK)_2$/G3139 complexes could reduce B16(F10) cell viability with half of the concentration needed for G3139 alone in CL to reduce the cell viability by 40%. Also, it was found greater apoptotic signal in cells treated with CLs containing D-$(KLAKLAK)_2$/G3139 complexes than CLs with G3139 only. *In vivo*, D-$(KLAKLAK)_2$/G3139 complexes in CL significantly inhibited tumor growth compared to the saline treated group, through apoptosis induction. However, the mechanism involved in cell death by apoptosis seems to be independent of reduction of bcl-2 protein levels. PEG2000 at 1% mol could significantly reduce activity of PCL formulation towards B16(F10) cells compared to CLs. After pre-incubation at pH 7.4, PCL and pH-PCL had decreased activity compared to CL towards B16(F10) cells. After pre-incubation at pH 5.0, while CL and PCL had the same activity with the cells as in neutral pH, pH-PCL formulation had its PEG cleaved and its cytotoxicity was restored against the melanoma cells.

Thus, D-$(KLAKLAK)_2$/G3139 complexes in CL had enhanced anti-cancer therapy, through apoptosis, than G3139 alone in CL *in vitro* and *in vivo*. *In vitro*, PCL and pH-PCL particles obtained can have a prolonged blood residence time, and, once a tumor tissue is reached, pH-PCL can have its cytotoxicity restored because of hydrolysis of cleavable PEG at a lowered pH.

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ANOVA	Analysis of variance
CL(s)	Cationic Liposome(s)
DOPE	l,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine
DOTAP	dioleoyl-1,2-diacyl-3-trimethylammonium-propane
EPR	Enhanced Permeability and Retention
HBG	Hepes Buffered Glucose
HPLC	High performance liquid chromatography
MOMP	Mitochondrial Outer Membrane Permeability
ODN(s)	Oligodeoxynucleotide(s)
PBS	Phosphate Buffered Saline
PCL(s)	PEG– Cationic Liposome(s)
PEG	Polyethylene Glycol
PEG2000-DSPE	(1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]
PEG5000-DSPE	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000]
PEG-Hz-PE	PEG-Hydrazone-PE
pH-PCL	pH-sensitive PCL
RES	Reticulo Endothelial System
SCCA2	Squamous cell carcinoma-specific antigen 2
SEC	Size Exclusion Chromatography

SEM	Standard error of measurements
TBS	Tris Buffered Saline
TEA	Triethylamine
TLC	Thin Layer Chromatography
TLR9	Toll-like receptor 9
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VDAC	Voltage-Dependent Anion Channel

1) INTRODUCTION

1.1. Statement of the problem and hypotheses.

The use of therapeutic macromolecules, such as proteins, peptides and nucleic-acid based drugs, has great promises for cancer treatment [1-4]. The anti-cancer therapy promoted by some of these macromolecules is achieved through induction of apoptosis, which can be initiated after disturbance of the mitochondrion (intrinsic pathway) [5]. Some macromolecules are pro-apoptotic agents that can directly interfere with the mitochondrial electrochemical potential, such as the pro-apoptotic peptide D-$(KLAKLAK)_2$ [6]. Other macromolecules can also act like pro-apoptotic drugs by reducing the levels of anti-apoptotic proteins present on the mitochondrion‘s surface, such as the bcl-2 protein. Consequently, cancer cells can become more susceptible to other chemotherapeutic agents, because there will be less anti-apoptotic protein to resist the apoptosis induction by a chemotherapeutic. A macromolecule which promotes such a decrease in bcl-2 protein levels is the antisense oligodeoxynucleotide G3139, which prevents the translation of BCL-2 mRNA into the protein [7].

Since the pharmacological targets of D-$(KLAKLAK)_2$ and G3139 are within cells, these macromolecules need to cross the cancer cell membranes to exert their therapeutic effects. However, their cell entrance is minimal because these macromolecules are highly hydrophilic while cell membranes are hydrophobic. Therefore, a drug delivery system, such as liposomes, is necessary to incorporate/encapsulate these macromolecules and help them pass through cell plasma

membranes. Once inside the cells, these carriers should release the macromolecules to the cytosol.

Liposomes are vesicles composed of phospholipids self-assembled in bilayers. They have been studied as drug delivery systems for four decades. They are biocompatible and cause few toxic or immunogenic reactions [8, 9]. The phospholipids that form these carriers can be positively charged (cationic) to help encapsulate macromolecules as well as deliver these drugs inside cancer cells [10, 11].

Nonetheless, the systemic use of liposomes is limited because these particles can bind to opsonizing proteins in the blood, be recognized as foreign particles by organs such as liver and spleen and eliminated before they can reach their target organ, such as a tumor tissue. A known molecule that can help liposomes overcome these barriers is the polyethylene glycol (PEG) polymer. PEG on the surface of liposomes prevents the binding of opsonins, increases blood residence time of the liposomal particles and helps them reach the tumor tissue [9, 12, 13].

The disadvantage of having PEG molecules on the surface of liposomes is that the anti-tumor activity is reduced inside the tumor. Ideally, once inside a tumor, PEG should be removed so that liposomes can have their anti-tumor activity restored. The PEG molecules can be removed by taking advantage of the unique features of a tumor tissue, such as a relatively low pH in the extracellular environment, to design PEG molecules that are hydrolysable due to a decrease in the pH from the systemic blood to the tumor tissue [14].

In this study, the pro-apoptotic peptide D-$(KLAKLAK)_2$ and the antisense oligodeoxynucleotide G3139 were used. The first hypothesis was that both

macromolecules, when combined in a single cationic liposomal (CL) carrier and delivered within cancer cells, would enhance cancer cell killing *in vitro* and *in vivo* more than either of them alone.

The second hypothesis was that PEG molecules incorporated at the surface of these CLs would decrease their cytotoxic activity towards cancer cells *in vitro*. However, this activity would be restored when low pH hydrolysable PEG molecules were incorporated instead at the surface of liposomes, followed by a pre-incubation in a lowered pH medium before testing on cells. The importance of having such modified liposomes is the obtainment of carriers that are able to have a prolonged blood residence time and that do less harm in healthy tissues. However, when a tumor site is reached, they can have their full cytotoxic activity restored against the cancer cells.

1.2. Review of the literature.

This section contains background information concerning therapeutic macromolecules and their inherent challenges for clinical use, followed by how many of these macromolecules act to promote apoptosis through disturbance of the mitochondrion. Next, the macromolecules used in this study, G3139 and D-$(KLAKLAK)_2$, are introduced. Finally, a brief overview of liposomal drug carriers is presented.

1.2.1. Macromolecular therapeutics

The use of macromolecular drugs including nucleic acid-based therapeutics, proteins and peptides holds great promises for the treatment of many pathologies, including cancer, cardiovascular and neurodegenerative diseases [1], and for biomedical applications such as tissue engineering and regenerative medicine [15]. The advantages of the use of macromolecules, such as the nucleic acids, over conventional small molecule drugs include their ability to act in a sequence-specific manner on defined molecular targets [1]. Recently, however, there have been increasing concerns that even very carefully designed macromolecules may have unwanted off-target effects [2].

Moreover, an inherent challenge for their development as drugs available for clinical use is their poor delivery efficiency. High molecular weight and hydrophilic macromolecules are unlikely to be absorbed through the gut and airway mucosa, drastically reducing their bioavailability. When these macromolecules are injected intravenously, they can be rapidly captured by circulating proteins in the plasma, or metabolized and excreted before reaching their site of action. Even when they can reach their target organ, cell plasma membranes can be a very difficult barrier due to their hydrophobicity. Additionally, most of these macromolecules have their sites of action inside cells. Thus, efficient delivery systems that are able to encapsulate/incorporate the desired macromolecule(s), protect them from protein binding and escape rapid metabolism and excretion, as well as have the ability to cross cell membranes to deliver these macromolecules inside cells are required [1-4].

1.2.2. Cancer treatment, apoptosis and mitochondria

In cancer treatment, some macromolecules act to stimulate programmed cell death (apoptosis) that reduces tumor tissue mass and/or prevents progression to angiogenesis and metastasis. One of the major targets for induction of apoptosis is the mitochondrion. Programmed cell death in mitochondria can be induced by disrupting the Mitochondrial Outer Membrane Permeability (MOMP). This disruption can occur through use of agents that directly interfere with the mitochondrial transmembrane potential ($\Delta\Psi m$) with consequent mitochondrial swelling and release of soluble pro-apoptotic factors (e.g. cytochrome c) to the cytosol [5, 16-19].

Another strategy for disrupting the MOMP is to target specific proteins in the outer membrane that regulate the apoptotic process, such as proteins of the bcl-2 family (examples: bad, bax, bcl-2, bcl-xl). Bad and bax are pro-apoptotic proteins that function by opening pores in the outer membrane, such as the Voltage-Dependent Anion Channel (VDAC), that promote the release of soluble transmembrane pro-apoptotic factors. On the other hand, bcl-2 and bcl-xl are anti-apoptotic proteins which prevent the opening of these pores. Thus, apoptosis can be induced by stimulating bad and bax or inhibiting the action of bcl-2 protein [5, 16-19].

The soluble transmembrane proteins, such as cytochrome c, that are released after disruption of the MOMP by either method act as pro-apoptotic factors by forming complexes (e.g. cytochrome c/Apaf-1/caspase 9-apoptosome containing complex) that ultimately lead to activation of effector caspases, like caspase 3. These effector caspases

promote the nuclear fragmentation and membrane blebbing which are characteristic features of apoptotic cells [5, 16-19].

1.2.3. Antisense oligodeoxynucleotide G3139

Cancer cells can overexpress bcl-2 protein and escape the apoptotic pathway even when these cells are treated with chemotherapeutics that induce programmed cell death. One macromolecular therapeutic agent able to reduce levels of bcl-2 protein, and thus increase cancer susceptibility to apoptosis-inducing agents, is G3139. Also called Genasense® or Oblimersen®, G3139 is an 18-mer chemically single-stranded oligodeoxynucleotide (ODN) sequence designed to be complimentary to the first six codons of bcl-2 mRNA. It prevents expression of bcl-2 protein by binding to the initiation region of the bcl-2 mRNA and consequently blocking the translation of that mRNA, which is later degraded by RNAse H. G3139 escapes degradation by nucleases, because its nucleotides are covalently linked by phosphothioester bonds, instead of their phosphodiester analogs [7, 20].

Nevertheless, G3139 still has the same delivery issues as other therapeutic macromolecules. Ongoing developmental and pre-clinical studies have focused on improving G3139 stability and increasing its delivery efficiency. Most of the *in vitro* studies have used G3139 complexed with cationic liposomes to transfect cells. Some of the cell types used were breast [7, 20], melanoma [21-23], and leukemia [24, 25] cancer cells. In other studies, G3139 was conjugated to polymers, like PEG [26], or linkers, such as disulfide bonds [27], to prolong its half-life *in vivo*. Although these studies have shown

improved stability and better delivery efficiencies than the free antisense, most of the clinical studies have made use of the "naked" G3139.

Since G3139 can decrease bcl-2 levels, it has been evaluated in several clinical trials with conventional cytotoxic drugs to increase their effectiveness. Examples of phase I trials include the association of G3139 with doxorubicin and docetaxel for breast cancer [28] and with cisplatin plus 5-fluorouracil for gastro-esophageal malignancies [29]. There were also reported phase II clinical trials which combined Oblimersen® with docetaxel for prostate cancer [30] and with rituximab for non-Hodgkin's lymphoma [31]. Phase III clinical trials have also been reported, such as the use of G3139 for advanced melanoma with dacarbazine [32], for chronic lymphocytic leukemia with fludarabine plus cyclophosphamide [33], and for multiple myeloma with dexamethasone [34].

Even though G3139 has been involved in all these clinical trials, there have been discussions about its efficacy. For example, it was found that G3139 plus dacarbazine does not significantly improve the overall survival rate, the primary endpoint needed for the FDA marketing approval, of advanced melanoma cancer patients compared to dacarbazine alone. Nonetheless, G3139 with dacarbazine did increase progression-free survival, overall response, complete and durable responses versus only dacarbazine [35, 36].

Furthermore, concerns have been raised about the pharmacological action of G3139, because non-antisense (off-target) effects have been found. In 518A2 human melanoma cells which cause advanced melanoma, a study indicated that, although G3139 promoted down regulation of bcl-2 protein, it did not make these cells more susceptible to chemotherapy. Moreover, G3139 alone induced apoptosis through the release of

cytochrome c [37]. Later, it was found that the release occurred after interaction of G3139 with the mitochondrial VDAC [38], which locked the channel in an open conformation, allowing the escape of cytochrome c to the cytosol [39]. In addition, proteomic studies with 607B human melanoma cells [40] revealed down-regulation of some glycolytic enzymes by G3139, and consequent reversion of the Warburg effect, the increased rate of glycolyis in the presence of oxygen [41]. Lastly, the anti-tumor effects of G3139 may also be related to the presence of unmethylated Cytosine-phosphate-Guanine (CpG) motifs in its molecule, which can activate Toll-like receptor 9 (TLR9). Once activated, TLR9 initiates a signaling cascade that induces the production and release of pro-inflammatory cytokines, which can have a strong anti-tumor response [42, 43].

1.2.4. Pro-apoptotic peptide D-$(KLAKLAK)_2$

D-$(KLAKLAK)_2$ is a 14-mer *de novo* positively charged peptide initially developed as an antimicrobial agent. It is composed of three amino acids: lysine (K), leucine (L), and alanine (A). Their sequence causes the peptide to adopt an α-helical conformation, with lysine (hydrophilic and positively charged) facing one side of the molecule and leucine and alanine (hydrophobic and neutral) facing the opposite side [44]. Its mechanism of action, which is chiral-independent, is the disruption of the negatively charged bacterial cell membrane by electrostatic interaction; the "D" enantiomer makes it resistant to proteases [44, 45]. It can kill bacteria at 3μM, 1% of the dose required to kill 50% of eukaryotic cells (LC_{50} = 300μM) [6]. This preferential killing activity towards

prokaryotic microorganisms is due to the anionic phospholipids present in the bacterial membrane, while eukaryotic cells have a relatively neutral cell membrane [6, 44].

Since bacteria and mitochondria share a common evolutionary ancestry, Ellerby and co-workers tested D-$(KLAKLAK)_2$ and found that it promoted apoptosis in a mitochondria-dependent cell-free system. Thus, it was hypothesized that the delivery of this peptide to cancer and cancer endothelial cells represented a new molecule for anti-cancer therapy [6]. In fact, it was found later on in Jurkat cells that $(KLAKLAK)_2$ directly interfered with the negative mitochondrial transmembrane potential ($\Delta\Psi m$). The organelle swelled and released cytochrome c which catalyzed peroxidation of the cell membrane through increased amounts of reactive oxygen species, including H_2O_2, with consequent cell death (apoptosis) [46].

However, since D-$(KLAKLAK)_2$ is relatively non-toxic to eukaryotic cells, including cancer cells, this peptide needs to be delivered inside cells to be able to reach mitochondria and exert its pro-apoptotic effect. In most attempts, groups have chemically conjugated D-$(KLAKLAK)_2$ to another moiety able to be internalized by cells. Some used specific peptides or proteins targeting cell surface receptors, such as cancer endothelial "homing" domains, like RGD-4C and CNGRC, which bind to integrins [6, 47], a peptide targeting HER-2 receptor in breast cancer cells [48], and monoclonal antibodies against CD19 and CD33 in malignant B lymphoid and myeloid cells, respectively [49]. Other research teams used non-specific motifs that are internalized in a receptor-independent manner, such as the protein transduction domains PTD-5 [50], TAT [51], octarginine and PFVYLI peptides [52].

Meanwhile, Oshikiri *et al* [53] constructed plasmids containing a gene sequence for $(KLAKLAK)_2$ linked to the squamous cell carcinoma-specific antigen 2 (SCCA2) promoter region and incorporated them in adenovirus vectors. From all infected cell lines with those vectors, only the ones that expressed the SCCA2 antigen had their growth inhibited due to the expression of $(KLAKLAK)_2$ peptide. Presently, there are very few studies involving the use of non-viral vectors. In one, a glycol chitosan conjugated to 2,3-dimethylmaleic acid was used to encapsulate D-$(KLAKLAK)_2$ at pH 7.4, through formation of a ionic complex. When pH was dropped to 6.8, there was a switch in the polymer charge and the peptide was released to cells [54]. In another study, the peptide was conjugated to a β-sheet-forming peptide containing a hydrophobic alkyl moiety. The molecules obtained were able to self-assemble in nanofibers, which stabilized the α-helical conformation of the KLAK peptide, and made it cell-permeable [55]. Thus, there are few studies involving the use of D-$(KLAKLAK)_2$ with other nanocarriers, such as liposomes.

1.2.5. Liposomes

Liposomes are vesicles composed of phospholipids assembled in bilayers, either formed naturally or produced artificially [8]. They have been studied for drug delivery purposes for approximately 40 years with liposomal formulations approved for clinical use and many others in clinical trials. Liposomes are biocompatible and cause few toxic or immunogenic reactions. They can incorporate either water-soluble drugs in their

hydrophilic core or water-insoluble drugs in the hydrophobic compartment in the phospholipid bilayers [9].

The phospholipids that compose liposomes can be electrically neutral, positively or negatively charged. The most commonly used lipids for macromolecular drug delivery systems, including nucleic acid-based therapeutics, are the positively charged phospholipids (examples: DOTAP, DOTMA) that form positively charged CLs. CLs form stable complexes with the negative charges from the nucleic acids, and protect them from nucleases of biological fluids. Also, positive complexes adsorb better to the relatively negative cell membranes (by electrostatic interaction) than the negative free nucleic acids, and are internalized by endocytosis in endosomes [10, 11]. Most cationic liposomes also contain a zwitterionic neutral phospholipid, like DOPE. Once the cationic liposome is internalized, DOPE aids liposomal escape from endosomes and, consequently, prevents degradation of the vectors by lysosomes. DOPE acts by disrupting the endosomal membrane because it undergoes a phase transition change from lamellar to an inverted hexagonal H_{II} phase when in a low pH characteristic of the endosomal compartment. This change in phase transition allows the fusion of liposomal and endosomal membranes, and releases the nucleic acid cargo into the cytosol [56, 57]. DOPE also decreases interactions between cationic liposomes and negatively charged glycosaminoglycans present in the interstitial space, allowing better transfection efficiencies [58].

Depending on the intended tissue that needs to be reached, liposomes (cationic or not) may need to circulate in the blood for some time to reach the desired tissue at therapeutic levels. On the other hand, liposomes tend to be rapidly cleared from the body

because organs of the Reticulo Endothelial System (RES), such as the liver and the spleen, can recognize foreign substances (i.e. liposomes) bound to serum proteins (opsonins) and eliminate them. The most extensively studied molecule that increases blood circulation time of liposomes is the polymer PEG. Generally conjugated to phospholipids anchored in the liposomal bilayer, PEG on the surface of liposomes can prevent binding of opsonins, by steric hindrance, and consequently be uptake by the RES. With increased circulation time, liposomes are able to reach pathological sites, like tumor tissues. There, the leaky vasculature associated with large pores between endothelial cells allows for tissue passage and accumulation of PEG-liposomes in the parenchyma, a phenomenon known as the Enhanced Permeability and Retention (EPR) effect [9, 12, 13, 59].

An increasing area of research is the development of PEG conjugated to linkers able to respond to certain environmental stimuli, like differences in pH or temperature. When conjugated to liposomes, they should have prolonged circulation properties, similar to regular PEG-phospholipid chains and the ability to accumulate in tumors through the EPR effect. In addition, due to a decreased pH and/or elevated temperature in pathological areas, such as tumors, these linkers should release PEG moieties and expose hidden moieties or functions, like positive charges, that will make it easier for liposomes to interact with and bind to cancer cells [14].

These liposomes containing more than one functionality on their surface (e.g. stimuli-sensitive PEG and positive charges) and that act in an orchestrated manner are termed multifunctional liposomes [14]. This terminology can also be applied to liposomes containing more than one component loaded in the same formulation, such as

the carriers containing siRNAs against MRP1 and BCL2 mRNAs to prevent drug efflux pumps and escape from apoptosis, respectively, along with doxorubicin, a cell death inducer [60].

1.3. Study outline.

The multifunctional liposomes developed in this study are composed mainly of CLs, containing D-$(KLAKLAK)_2$ peptide and antisense G3139 in a single liposomal carrier. This study is divided in two major parts. In the first part, the core of these CLs was elaborated to contain both macromolecules. After formulations were obtained, the *in vitro* effects of these CLs containing the molecules were examined through cell viability studies, uptake of drugs and apoptosis detection through caspase signaling. Next, tumor growth inhibition studies and TUNEL assay for detection of apoptosis were assessed after *in vivo* intratumoral injections. Later, the mechanism by which the macromolecules worked to promote an increased apoptotic activity and, thus, enhanced anti-cancer therapy was investigated *in vitro*.

In the second part, the effects of surface modification of CLs were evaluated by the addition of PEG molecules. They were added to the CLs in a sufficient amount to decrease the liposomal activity *in vitro*, and this decrease was examined by cell viability studies. Next, pH-sensitive PEG molecules were prepared, according to previously developed protocols, and added to the liposomes. Finally, new cell viability studies were conducted to check whether the multifunctional liposomes had differential cytotoxic activity after pre-incubation in normal or lowered pH.

2) SPECIFIC AIMS

The specific aims of this study were as follows:

Aim 1: Prepare and characterize CLs loaded with the pro-apoptotic peptide D-$(KLAKLAK)_2$ and antisense ODN G3139. Evaluate size and zeta-potential of the particles obtained, ODN entrapment, and D-$(KLAKLAK)_2$ loading efficiency.

Aim 2: Study the interaction of prepared CL formulations with cells, using cell viability studies, uptake assay using fluorescence microscopy, and by detection of apoptosis.

Aim 3: Assess the effect of the double-loaded CL formulations *in vivo* after intratumoral injections, on tumor growth inhibition and detection of apoptosis after isolation and staining of tumor sections using a TUNEL assay.

Aim 4: Investigate the mechanism by which G3139 and D-$(KLAKLAK)_2$ macromolecules act that will contribute to an enhanced anti-cancer therapy when combined in a single formulation *in vitro*, through the use of biochemical techniques such as Western blotting to detect bcl-2 protein down-regulation;

Aim 5: Prepare and evaluate PEGylated CLs with non-cleavable and cleavable PEG-PE chains. With this in mind:

a) Prepare and characterize the effects of cationic liposomes containing amphiphilic polymer PEG2000-DSPE (non-cleavable) chains on their surfaces in sufficient amount to shield cationic charges, minimizing their ability to interact with cells, through cell viability studies.

b) Prepare and characterize cationic liposomes, containing the pH-hydrolysable polymer PEG-Hz-PE (cleavable) chains on their surfaces in the same amount as determined for non-cleavable PEG2000-DSPE. Evaluate the *in vitro* differential activity of these liposomal formulations through cell viability studies after pre-treatment at neutral or at acidic pHs.

3) MATERIALS AND METHODS

3.1. Development and evaluation of CLs loaded with D-(KLAKLAK)$_2$/G3139 complexes [61].

3.1.1. Materials

Phosphorothioate bcl-2 antisense ODN G3139 (5'- TCT CCC AGC GTG CGC CAT – 3') and control ODN G3622 (5'- TAC CGC GTG CGA CCC TCT – 3') were synthesized by Invitrogen (Carlsbad, CA). Pro-apoptotic peptide D-(KLAKLAK)$_2$ was synthesized at Tufts Core Facility (Boston, MA). Fluorescent labeled 5-FAM-D-(KLAKLAK)$_2$ was purchased from AnaSpec, Inc (San Jose, CA). The lipids l,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), dioleoyl-1,2-diacyl-3-trimethyl-ammonium-propane (DOTAP), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Rhodamine-PE), and amphiphilic polymers (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (PEG2000-DSPE), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-5000] (PEG5000-DSPE), were purchased from Avanti Polar Lipids (Alabaster, AL). All other chemicals were of the reagent grade.

3.1.2. Preparation of D-(KLAKLAK)$_2$/G3139 complexes

Pro-apoptotic peptide D-(KLAKLAK)$_2$ and the antisense ODN G3139 were co-loaded in CLs, based on the charge ratio among the peptide D-(KLAKLAK)$_2$, the antisense G3139 and DOTAP, one of the phospholipids used to prepare CLs (3.1.3). It was assumed that each D-(KLAKLAK)$_2$ peptide contained 6 (six) positive charges from the lysine residues, each G3139 antisense molecule contained 18 negative charges from phophorothioates, and each DOTAP phospholipid had one positive charge from the amino group when at pH 7.4. Initially, D-(KLAKLAK)$_2$ peptide and G3139 were diluted separately with Hepes (10mM) Buffered Glucose 5% (w/v) (HBG) solution (pH 7.4) [62], and then mixed to produce a 250µl solution of D-(KLAKLAK)$_2$ and G3139 complexes at a +/- charge ratio of 1:2, respectively.

3.1.3. Preparation of CL loaded with D-(KLAKLAK)$_2$/ G3139 complexes

CLs were prepared according to the rehydration/extrusion method [63]. Chloroform solutions of the phospholipids DOPE and DOTAP were mixed at 1/1 mol ratio. Next, the chloroform was evaporated using a nitrogen stream and a lyophilizer (Freezone 4.5, Labconco, Kansas City, MO), to produce a dried lipid film. Then, the lipid film was rehydrated with 250µl of HBG. The solution containing negative complexes of D-(KLAKLAK)$_2$/G3139 (prepared as in 3.1.2) was then added to the hydrated lipid film of DOPE and DOTAP, to produce a suspension with D-(KLAKLAK)$_2$, G3139 and DOTAP at 1:2:6 (+/-/+) charge ratio, respectively, i.e. positive charges in excess. The

500uL suspension was immediately passed through a stack of two 200nm pore size polycarbonate membranes eleven times with a hand held extruder (Avanti) to produce cationic liposomes. A CL formulation loaded with only G3139, prepared in a similar manner as above, was prepared as a control for *in vitro* and *in vivo* studies. CL formulations loaded with only D-$(KLAKLAK)_2$ peptide were also attempted.

3.1.4. Characterization of CL loaded with D-$(KLAKLAK)_2$/G3139 complexes

The resulting formulations were characterized with respect to particle size, zeta-potential, complex formation, and loading efficiency. Particle size and zeta-potential were determined by dynamic light scattering with a Zeta Plus analyzer (Brookhaven Instruments Corporation, Brookhaven, NY). The measurements were carried out in triplicate for each formulation. Complex formation was verified by agarose gel electrophoresis, using an E-gel® electrophoresis system (Invitrogen, Carlsbad, CA). A precast 0.8% E-gel® cartridge was pre-run for 2min at 60V and 500mA followed by the loading of 1μg of G3139 and samples were run for five minutes. Peptide loading efficiency inside CL was analyzed by size exclusion chromatography (SEC), using 5-FAM-D-$(KLAKLAK)_2$ as a tracer. Formulations containing the fluorescent peptide were prepared as above and eluted separately using a Sepharose CL-4B (Sigma, St. Louis, MO) column bed. One milliliter fractions were collected and checked for fluorescence intensity (485nm excitation/528nm emission) using a fluorescent plate reader (Synergy HT, BioTek Instruments, Winooski, VT). 5-FAM-D-$(KLAKLAK)_2$ entrapment efficiency was calculated as the percentage of total area found in the first peak. A

formulation without G3139 was prepared as a control to demonstrate the effect of complexation of D-$(KLAKLAK)_2$/G3139 on the peptide encapsulation efficiency.

3.1.5. Cell lines

B16(F10) murine melanoma and MCF-7 human breast cancer cells were chosen for this study. B16(F10) cells are derived from the parent B16 cell line, which causes spontaneous melanoma in C57BL/6 mice. It was selected for its capacity to form lung colonies *in vivo*, and later established *in vitro* after 10 cycles of lung colony formation [64, 65]. Also, it was assumed that this cell line has elevated levels of bcl-2 based on the fact that the corresponding human melanoma 518A2 has high levels of bcl-2 protein [66]. MCF-7 cells were chosen because they also have very elevated levels of bcl-2 [7]. They were grown in Dubelcco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) at 37^0C and 5% CO_2 in T-flasks.

3.1.6. Cell viability studies

Cells were transferred to 96-well plates (5,000/well) after being harvested with 0.25% trypsin and 2.21mM EDTA solution. After 24 hours, cells had their medium removed and 100μL of serum-free medium was added containing serial dilutions of each liposomal formulation up to 1μM of ODNs (1.52μM of D-$(KLAKLAK)_2$ and 54 μM of DOTAP). After 24 hours of incubation, the medium was removed and a new serum-containing medium (complete) was added to each well. Then, 20uL of CellTiter-Blue®

cell viability reagent (Promega, Madison, WI) was added to each well, and plates were re-incubated for two hours. Fluorescence intensity was measured with 590nm emission after 530nm excitation wavelengths, using a Synergy® HT fluorescent plate reader (BioTek Instruments). Relative cell viability was calculated with cells treated with serum-free medium only as controls, and the assay was carried out in triplicate.

3.1.7. Cell uptake studies

B16(F10) cells were grown as above. Later, these cells were transferred to a 6-well plate (8 x 10^4/well) containing a sterilized glass coverslip at each well. After 24 hours, cells had their medium removed and 1mL of serum-free medium was added containing FAM-D-$(KLAKLAK)_2$/G3139 complexes in CL (3μM of G3139) and 300nM MitoTracker® Red (Invitrogen). After one hour incubation at 37^0C and 5% CO_2, medium-containing formulation was removed and cells were washed once with serum-free medium. Coverslips were attached to glass slides cell side down with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL) mounting medium and visualized under an epifluorescent microscope (Olympus BX51, Olympus, Center Valley, PA).

3.1.8. Caspase 3/7 activity assay

B16(F10) cells were grown and treated in a 96-well plate as in the cell viability studies (3.1.6) for 4 hours. After removing medium-containing formulations and adding 100μL of fresh complete medium, 100μL of Apo-ONE® caspase 3/7 reagent (Promega)

was added to each well and the plate was re-incubated for 18 hours. Then, fluorescence intensity (485nm excitation/528nm emission) was measured with a fluorescent plate reader (Synergy® HT, BioTek Instruments). Percentage increase in caspase (3 and 7) activity was calculated by comparing the results obtained with the cells treated with serum-free medium only as controls. The assay was carried out in triplicate.

3.1.9. Antitumor activity in tumor-bearing mice

Male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were inoculated with 10^6 B16(F10) melanoma cells injected subcutaneously in the left flank and divided into three groups (n = 4/group). Fourteen days later, when tumors had reached approximately $200mm^3$([length x $width^2$/2), doses of 20µL of CL formulation containing peptide/ODN or ODN alone (corresponding to 4µg of G3139 and 1.5µg of D-$(KLAKLAK)_2$) were administered daily by intra-tumoral injections. Also, the volume of each tumor was measured every day with a vernier caliper. Four days later, when the average tumor volume of the control (saline) group reached $1130mm^3$, all mice were sacrificed by CO_2 asphyxiation, and tumors were excised. Tumor growth inhibition was determined by comparison of the mean (± SEM) tumor volumes among the treatment and control groups.

3.1.10. TUNEL assay

Tumor-bearing mice were administered a single dose of 60μL of CL formulation containing D-$(KLAKLAK)_2$/G3139 complexes by intra-tumoral injection when tumors reached approximately $600mm^3$. Saline-treated mice with similar-sized tumors were used as controls. After 24 hours, the mice were sacrificed by CO_2 asphyxiation and excised tumors were frozen immediately in Tissue Tek OCT 4583 compound (Sakura Finetek, Torrance, CA) at -80^0C without fixation. Tumor sections (8μm thick) were cut with a cryotome (Triangle Biomedical Sciences, Durham, NC) and fixed with 4% (w/v) paraformaldehyde on glass slides. A terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed with a TdT-FragEL® DNA fragmentation detection kit (Calbiochem, San Diego, CA) and the sections were examined by fluorescence microscopy (Olympus BX51, Olympus). All animal studies (see sections 3.1.9 and 3.1.10) were performed according to a protocol approved by the Institutional Animal Care and Use Committee at Northeastern University.

3.2. *In vitro* investigation of the mechanism of action of multifunctional liposomes.

3.2.1. New cell viability studies

B16(F10) cells were grown as previously described. They were seeded in two different 96-well plates (~5,000/well) after harvest with 0.25% trypsin and 2.21mM EDTA solution. After 24 hours, the medium was removed and 100μL of serum-free

medium was added containing serial dilutions of each liposomal formulation up to 400nM of ODNs (600nM of D-$(KLAKLAK)_2$ and 21.6µM of DOTAP). One plate was incubated for 24 hours and the other for 48 hours. After their respective incubation times, their media were removed and 100µL of new serum-containing medium (complete) was added to each well in both plates. Then, 20uL of CellTiter-Blue® cell viability reagent (Promega) was added to each well, and plates were re-incubated for two hours. Fluorescence intensity was measured with 590nm emission after 530nm excitation wavelengths with a fluorescent plate reader (Synergy® HT, BioTek Instruments). Relative cell viability was calculated with cells treated with serum-free medium only as controls. The assay was carried out in triplicate

3.2.2. Evaluation of bcl-2 protein expression by Western blotting analysis

For the determination of bcl-2 protein levels *in vitro*, B16(F10) cells were grown in a 6-well plate until they reached $5x10^5$ cells/well. The medium was removed and 2mL of serum-free medium was added containing CLs, prepared as described earlier, with 200nM of ODNs (300nM of D-$(KLAKLAK)_2$ and 10.8µM of DOTAP). After 24 hours, cells were washed once with serum-free medium and harvested with 0.25% trypsin and 2.21mM EDTA solution. Cell pellets obtained were washed with cold PBS, and kept on ice for 30min with 50µL of Radio-ImmunoPrecipitation Assay (RIPA) lysis buffer (Thermo Fischer Scientific, Rockford, IL) containing Halt® protease inhibitor cocktail without EDTA (Thermo Fisher Scientific). Cell lysates were centrifuged at 3000rpm for 5 minutes and supernatants were stored at -80^0C. Total protein concentrations were

determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) for each lysate. Fifty micrograms of total protein per sample were loaded on a 4-20% SDS-polyacrylamide gel (Expedeon, San Diego, CA), and immersed in a Tris/Tricine/SDS buffer (Expedeon), and run at 125V for 90 minutes. The gel was transferred to an Immobilon-P PVDF membrane (Millipore, Bedford, MA, USA) in a Tris/Tricine blotting buffer (Expedeon) for 90 minutes at 25V. The membrane was blocked for one hour at 37^0C on a rotating shaker with a Tris Buffered Saline (TBS) T20 Blocking Buffer (Thermo Fischer), and, later, washed three times with TBS containing 0.1% Tween 20. The membrane was then incubated with mouse monoclonal antibody (mAb) 10C4 (Invitrogen) against murine bcl-2 protein and with rabbit polyclonal antibody (pAb) against β-actin (Cell Signaling Technology, Danvers, MA) in T20 Blocking Buffer overnight at 4^0C under constant shaking. Following further washing, the membrane was immersed in a goat anti-mouse or goat anti-rabbit IgG-horseradish peroxidade (HRP) antibody (Cell Signaling Technology) solutions in T20 Blocking Buffer at room temperature for one hour on a rotating shaker. Bands were then developed with Pierce Super Signal West Dura Extended Duration Substrate (Pierce) and imaged with the Kodak Image Station *In Vivo* FX (Carestestream Health, Rochester, NY).

Bands obtained after development had their pixel density determined using the National Institute of Health Image J software (Bethesda, MD) after background subtraction. Bcl-2 band densities for each treatment group were normalized to their respective β-actin bands (bcl-2/β-actin ratio). The assay was performed in triplicate and control (not treated) cells were considered to have bcl-2 levels at 100%.

3.3. Surface modification of CLs, containing D-$(KLAKLAK)_2$/G3139 complexes: development and initial evaluations.

3.3.1. Preparation of CLs, containing D-$(KLAKLAK)_2$/G3139 complexes

CL formulations, loaded with D-$(KLAKLAK)_2$/G3139 complexes, were prepared as previously described.

3.3.2. Preparation of PCL

PEG2000-DSPE was added at increasing molar percentages (1%, 3%, 5%, etc) to a lipid film composed of DOPE and DOTAP. Meanwhile, molar percentages of the later phospholipids were reduced to keep the same total amount of lipids (in moles), but their molar ratio (1/1, mol/mol) was maintained. D-$(KLAKLAK)_2$/G3139 complexes were prepared as previously described, but the amounts of the peptide and antisense were adjusted to maintain the same (+/-/+) charge ratio of 1:2:6, among D-$(KLAKLAK)_2$, G3139 and DOTAP, respectively. The 250µL solution of HBG 5% containing peptide/antisense complexes was added to 250µL of hydrated lipid film. The 500µL suspension was immediately passed through a stack of two 200nm pore size membranes eleven times to produce the PCL formulations.

3.3.3. Determination of the amount of PEG in PCL needed to shield liposomes

PCLs at increased molar percentages of PEG, prepared as above, were tested in B16(F10) cells to verify at what concentration PEG chains prevented interactions between the liposomes and cells. Cells were seeded in 96-well plates at approximately 5,000 cells/well, and treatment was done through serial dilutions up to 1μM of G3139 (1.5μM of D-$(KLAKLAK)_2$ and 54μM of DOTAP). After 24 hours, B16(F10) cell viability, through Cell Titer-Blue® (Promega) assay, was determined relative to cells treated with serum-free medium only as controls. The assay was carried out in triplicate. The PCL formulation with the molar percentage of PEG that least reduced cell viability, compared to non-pegylated CL, was assumed to be the one that could better shield the liposomes from being taken up by the cells.

3.3.4. Determination of which PEG molecule can better shield PCL

PCL formulation with PEG5000-DSPE was also prepared with the same molar percentage of PEG2000-DSPE in PCL that could better prevent interactions between liposomes and B16(F10) cells. Then, PCL formulations, one with PEG2000 and the other with PEG 5000, with the same molar percentage of PEG were tested to check which PEG molecule could better shield the liposomes from interacting with cells. The assay was done through viability studies with B16(F10) cells in triplicate, and compared to CL formulations, as above.

3.3.5. Preparation of hydrolysable PEG-Hz-PE

The synthesis of pH-cleavable polymer PEG-Hz-PE was done in three steps, according to previously developed methods [67-69].

In the first step, 19μmol (38mg) of mPEG2000-SH (Nektar Therapeutics, Huntsville, AL) and 45μmol (14mg) of 4-(4-N-maleimidophenyl) butyric acid hydrazide hydrochloride (MPBH) (Pierce) were separately diluted in 0.5mL of Phosphate Buffered Saline (PBS). The two solutions were then mixed by constant stirring with 20μL (7-fold molar excess over PEG) of triethylamine (TEA) (Sigma-Aldrich, St Louis, MO) as a catalyst overnight at room temperature. The solution was then dialyzed against PBS using a 1KDa MWCO membrane (Spectra/Por 6, Spectrum Labs, Rancho Dominguez, CA) for two hours at room temperature to remove impurities. The solution obtained after dialysis was lyophilized with the Freezone 4.5 freeze dryer (Labconco). The powder obtained, a PEG-hydrazide derivative, was weighted and dissolved in chloroform (**Figure 1**).

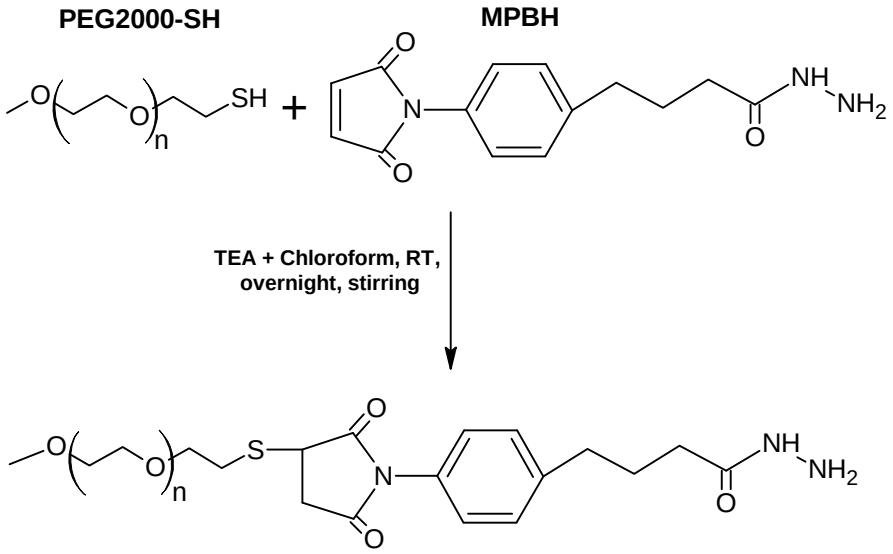


Figure 1. Reaction between PEG2000-SH and MPBH to produce a PEG-hydrazide derivative.

In the second step, 68.4µmol (50mg) of 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanolamine (DPPE-SH) (Avanti) was reacted with 139.4µmol (30mg) of 4-acetyl phenyl maleimide (Sigma-Aldrich) and 20µL of TEA (2-fold molar excess over lipid). The chloroform solution containing the three reactants was left overnight with constant stirring at room temperature. The next day, chloroform was removed with a rotary evaporator (Labconco), and the product was then purified through silica gel chromatography, using a chloroform: methanol (80:20 v/v) mobile phase. Thin Layer Chromatography (TLC) was used to identify the fractions containing the product. These fractions were pooled and concentrated using the rotary evaporator and the activated phospholipid was obtained (**Figure 2**).

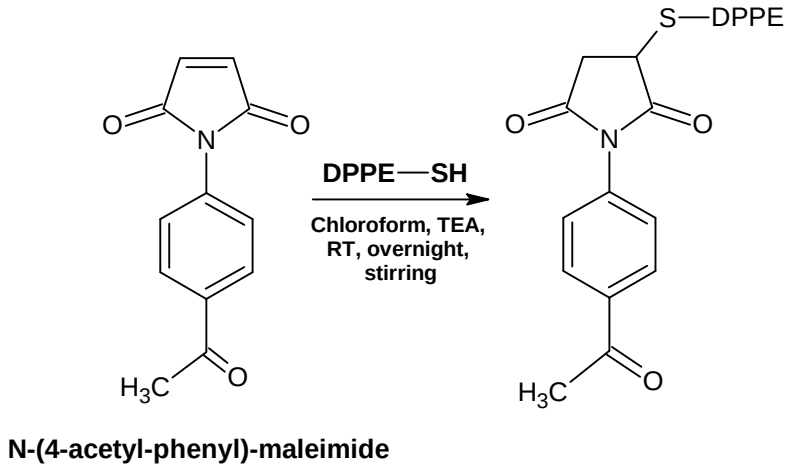


Figure 2. Reaction between N-(4-acetyl-phenyl)-maleimide and DPPE-SH for activation of the phospholipid.

Finally, in the third step, 6.92µmol (16mg) of the PEG-hydrazide derivative was reacted with 6.13µmol (5.8mg) of the activated phospholipid in chloroform overnight with constant stirring and at room temperature. On the next day, the chloroform solution was evaporated under reduced pressure and the PEG-Hydrazone-PE (PEG-Hz-PE) product was freeze dried (Labconco). Later, the obtained conjugate was weighed and dissolved in chloroform (**Figure 3**).

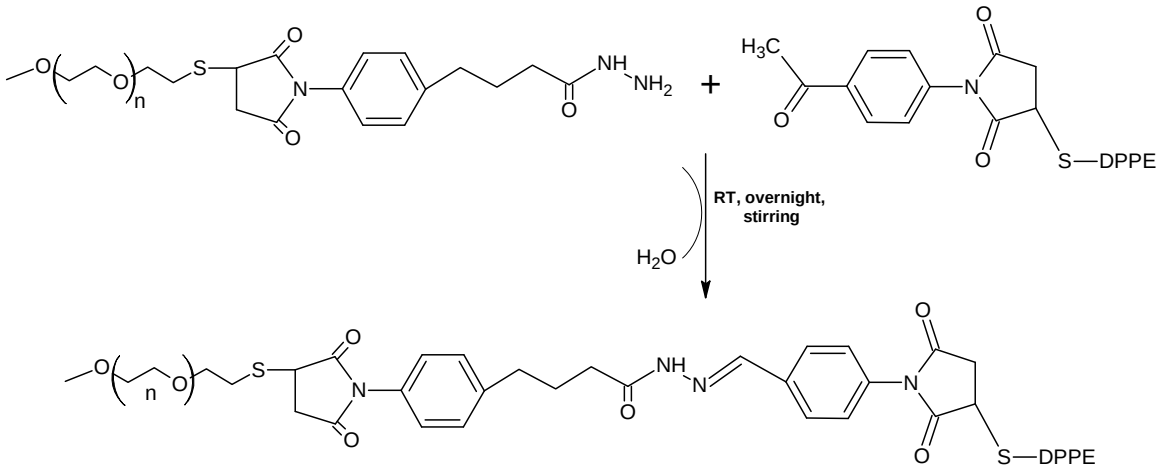


Figure 3. Generation of PEG-Hz-PE after addition of PEG-hydrazide to activated phospholipid.

The formation of the products was monitored by TLC. Dragendorff reagent was used to detect PEG and derivatives, while molybdenum blue was used to identify the lipids. The products obtained from each step were also analyzed by proton Nuclear Magnetic Resonance (^{1}H-NMR) to confirm the structures obtained.

3.3.6. Cleavability of PEG-Hz-PE

To confirm that PEG-Hz-PE can be hydrolyzed at a lowered pH, pH-dependent degradation of PEG-Hz-PE micelles was observed by high performance liquid chromatography (HPLC), using a size exclusion column (Shodex KW-804, Waters, Milford, MA). PEG-Hz-PE micelles were prepared by the evaporation of a chloroform solution of PEG-Hz-PE with 0.5% mol of Rhodamine-PE to form lipid films. Then, these lipid films were hydrated with 50µL of PBS (1.2mM solution of PEG-Hz-PE micelles) either at pH 7.4 or pH 5 and incubated for 3 hours at 37^{0}C. Next, these micelles were

eluted in phosphate buffer (100mM phosphate, 150mM sodium sulfate) at 1mL/min and detected through ultraviolet (UV) and fluorescence (550nm excitation/590nm emission) [67-69].

3.3.7. Preparation of pH-sensitive PCL (pH-PCL)

PEG-Hz-PE was added to a lipid film composed of DOPE and DOTAP at the same molar percentage of PEG2000-DSPE that could better shield PCL from interactions with B16(F10) cells. The pH-PCL formulation was prepared in the same manner as the PCL formulation.

3.3.8. Characterization of the multifunctional liposomes

The multifunctional liposomal formulations CL, PCL and pH-PCL, with PEG2000-DSPE and PEG-Hz-PE at 1% mol, had their particle sizes and zeta-potential measured by dynamic light scattering (ZetaPlus, Brookhaven Instruments Corporation). These measurements were performed in triplicate immediately after the formulations were prepared and after three hours incubation at pH 7.4 or at pH 5.0 at 37^0C. Encapsulation efficiency studies were done through SEC using a Sepharose CL-4B column bed to verify the amount of FAM-D-$(KLAKLAK)_2$ encapsulated in the liposomes. One milliliter fractions were collected and checked for fluorescence intensity (485nm excitation/528nm emission) using a plate reader (Synergy® HT, Biotek

Instruments). The 5-FAM-D-$(KLAKLAK)_2$ peptide entrapment efficiency was calculated as the percentage of total area found in the first peak.

3.3.9. Differential activity of pH-PCL

CL, PCL and pH-PCL formulations were aliquoted and pre-incubated with HBG 5% at pH 7.4 or at pH 5.0 each for 3 hours at 37^0C. After the pre-incubation, all formulations had their pH raised back to 7.4 with an appropriate volume of DMEM serum-free (determined empirically) [69]. Then, the medium containing formulations was added to B16(F10) cells in serial dilutions up to 1μM of G3139 (1.5 μM of D-$(KLAKLAK)_2$ and 54 μM of DOTAP) and incubated for 24 hours at 37^0C. Next, B16(F10) cell viability was determined as previously described.

3.4. Statistical analyses.

In all the experiments (*in vitro* and *in vivo*), results presented are an average (mean) of values ± standard errors of measurement (SEM). One-way analysis of variance (ANOVA) was used to compare these averages in the *in vitro* experiments (cell viabilities, increase in caspase activity and bcl-2 levels) at selected concentrations and in the *in vivo* experiments (tumor volumes), with Tukey post-hoc test. Statistical significance was considered achieved if $p < 0.05$ [70]. These statistical tests were done with the SPSS® software version 17 (IBM Corporation, Somers, NY).

4) RESULTS

4.1. Development and evaluation of CL loaded with D-(KLAKLAK)$_2$/G3139 complexes [61].

Charge alternation was used to encapsulate the cationic peptide D-(KLAKLAK)$_2$ inside CLs. The peptide was first complexed through electrostatic interaction with anionic antisense G3139 in excess to achieve overall D-(KLAKLAK)$_2$/G3139 negatively charged complexes. Then, these negative complexes were entrapped inside CL when added to a hydrated lipid film composed of DOTAP and DOPE phospholipids, and the obtained suspension was extruded. **Figure 4** shows an idealized representation of the CL particle.

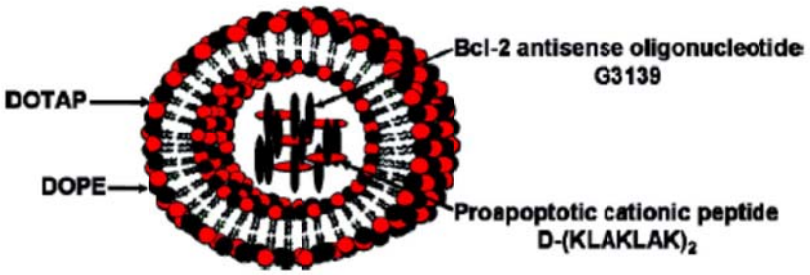


Figure 4. Schematic representation of a CL loaded with the pro-apoptotic peptide D-(KLAKLAK)$_2$ and bcl-2 antisense oligodeoxynucleotide G3139 complexes.

The initial complexation between D-(KLAKLAK)$_2$ and G3139 at a (+/-) charge ratio of 1:2, respectively, gave large particles (235 ± 20nm) with a negative zeta-potential (-29 ± 3.5mV), as expected from the negative charges in excess from G3139. When these negative complexes were added to the lipid film containing DOTAP and DOPE and

extruded, cationic liposomes, loaded with the complexes, were produced with a mean diameter of 195nm and charge of + 43mV, due to the excess of positive charges from DOTAP (+/-/+ charge ratio of 1:2:6 D-$(KLAKLAK)_2$/G3139/DOTAP, respectively) (**Table 1**). These liposomal particles remained colloidally stable in Hepes Buffered Saline (10mM Hepes, 150mM NaCl, pH 7.4) for at least three weeks. Formulations with a (+/-/+) charge ratio of 1:2:2 (D-$(KLAKLAK)_2$/G3139/DOTAP) were also tried, but during the addition of the complex to the lipid film precipitation occurred , which might indicate neutralization between the oppositely charged particles.

Table 1. Particle size* and zeta potential* of negative $(KLAKLAK)_2$/G3139 complexes and CL loaded with negative complexes.

	Particle size (nm)	Zeta potential (mV)
Negative complexes ($(KLAKLAK)_2$/G3139)	235 ± 20.6	-28.9 ± 3.5
Cationic Liposomes ($(KLAKLAK)_2$/G3139/DOTAP)	194.7 ± 2.6	43.15 ± 3.6

* mean ± SEM, with n = 3.

An indication of the complex formation between D-$(KLAKLAK)_2$ peptide and G3139 was observed (**Figure 5**) when the green fluorescent labeled peptide FAM-D-$(KLAKLAK)_2$ solution (left) had its fluorescence quenched when added to the G3139 solution, producing a gold color solution (right). The quenching of fluorescence may have been caused by the close proximity, through electrostatic interaction, between the fluorophore linked to the peptide and the nonfluorescent antisense G3139. This phenomenon is known as contact quenching [71].

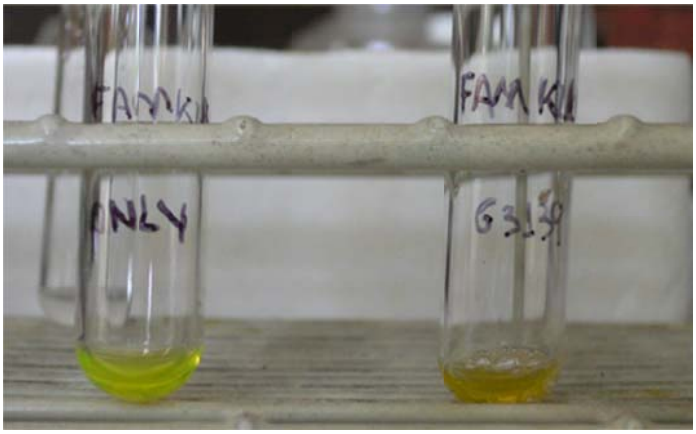
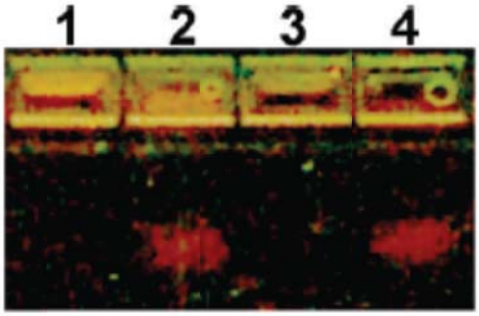


Figure 5. Green fluorescent labeled FAM-D-(KLAKLAK)$_2$ peptide solution (left) when added to G3139 solution had its color changed (right).

Later, the complex formation and its entrapment inside cationic liposomes were confirmed by electrophoresis on a 0.8% agarose gel (**Figure 6**). The gel retardation assay showed that G3139, when complexed with the peptide could not enter the gel. It was retained in the well because of the size restriction (**lane 1**). The complex was disrupted when treatment with heparin displaced G3139 to the gel (**lane 2**). CL formulations containing encapsulated complexes also did not allow migration of ODN through the gel (**lane 3**), and they released G3139 to the gel after CL formulations were treated with Triton X-100 at 1% followed by heparin (**lane 4**).



(1) "Naked" complex, non-treated.
(2) "Naked" complex, treated with heparin.
(3) CL (peptide/ODN), non-treated.
(4) CL (peptide/ODN), with Triton X-100 and heparin.

Figure 6. Gel retardation assay of negative "naked" complexes (lanes 1 and 2) and cationic liposomes containing negative complexes (lanes 3 and 4).

The peptide encapsulation efficiency studies were done with the fluorescent labeled peptide FAM-D-$(KLAKLAK)_2$ either free in solution or in two different CL formulations. Size exclusion chromatography (SEC) (**Figure 7**) showed that about 50% of FAM-D-$(KLAKLAK)_2$ peptide was encapsulated in CLs, which eluted in the void volume, only when first complexed with G3139. Attempts to encapsulate the peptide without ODN resulted in negligible encapsulation with a similar elution pattern as the free FAM-D-$(KLAKLAK)_2$. Thus, the pre-complexation of the peptide with the ODN is an absolute requirement for the preparation of CLs loaded with D-$(KLAKLAK)_2$ and G3139.

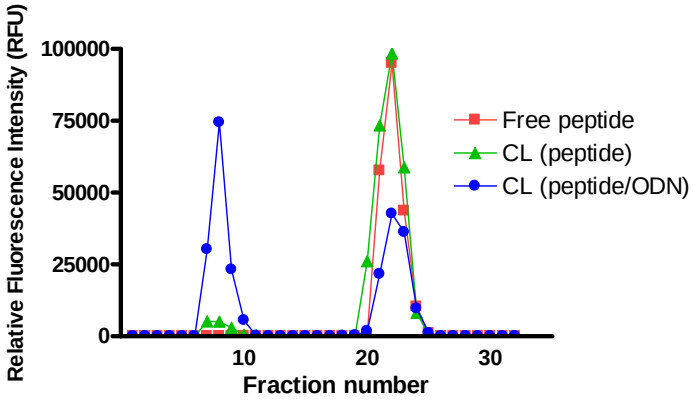


Figure 7. FAM-D-(KLAKLAK)$_2$ encapsulation efficiency in three different formulations after SEC.

4.1.1. *In vitro* studies

After preparation and characterization of cationic liposomes loaded with D-(KLAKLAK)$_2$ and G3139 (Aim 1), studies were performed with B16(F10) and MCF-7 cells to test the efficacy of this combined therapy *in vitro* (Aim 2). Four different cationic liposomal formulations were prepared, using G3139, or its reverse sequence, G3622, which lacks antisense activity [20, 23]. The oligonucleotides were incorporated inside DOPE/DOTAP cationic liposomes either alone or complexed with D-(KLAKLAK)$_2$ as described (3.1.2 and 3.1.3). After 24 hours of treatment, the viability assay was performed using a dye (CellTiter-Blue®) that is only reduced by live cells, giving a fluorescent product. Relative viability (%) was determined with cells treated only with serum-free medium as controls.

As **Figure 8** indicates, although the formulations and their concentrations used were the same, viability patterns obtained were quite different for the cell lines tested. In **Figure 8a**, treatment for 24 hours with CL formulations caused almost no toxicity to

MCF-7 cells (≤ 10% reduction in viability). The fact that this cell line over expresses bcl-2 protein suggests that a higher concentration of G3139 and, consequently D-(KLAKLAK)$_2$, is needed to decrease its viability. In fact, Chi and co-workers [7] needed to treat MCF-7 cells for two days with 800nM of G3139 each day to reduce the bcl-2 protein levels, followed by a reduction in the breast cancer cell viability.

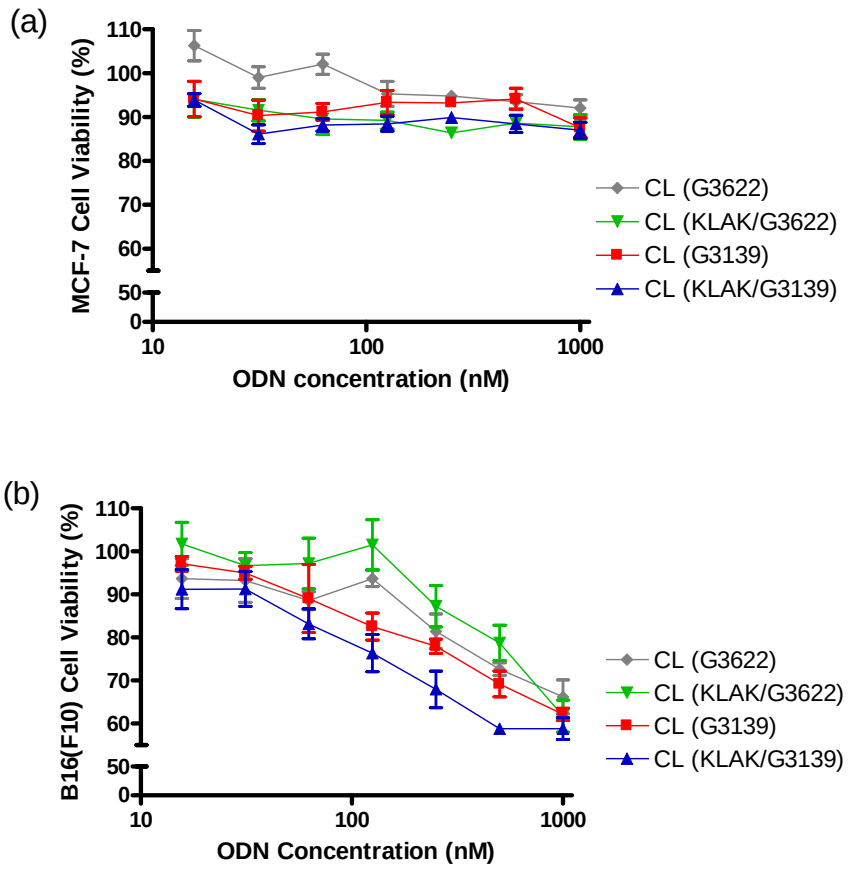


Figure 8. Percentage MCF-7 (a) and B16(F10) (b) cell viabilities (mean ± SEM, with n=3) after treatment with four different CL formulations at increasing concentrations.

On the other hand, in **Figure 8b**, there was a dose-dependent reduction in B16(F10) cell viability when D-(KLAKLAK)$_2$ and G3139 complexes were encapsulated

inside CLs. Their complexation reduced by half the concentration of G3139 (125nM) needed to significantly decrease viability (by 30%) compared to the non treated cells ($p < 0.02$) when the antisense ODN is used alone (250nM) ($p < 0.01$). **The decrease in the G3139 concentration needed to significantly reduce viability in B16(F10) cells is an indication of the advantages of combining this antisense ODN with D-(KLAKLAK)$_2$ peptide in a single formulation**. At the highest concentrations (500 and 1000nM), G3139 complexed with D-(KLAKLAK)$_2$ reduced B16(F10) cell viability by 40%. However, at these highest concentrations, CL formulations containing G3622 also showed some toxicity, especially at 1μM, which can be correlated to a non-specific sequence mediated cell death. Thus, adjustments in the concentration range of the macromolecules in CLs might be necessary for B16(F10) cell line in future studies to separate cell toxicity by G3139 from toxic effects by the non-specific sequence G3622. Meanwhile, 125 and 250nM concentrations should be emphasized more in subsequent experiments.

The next step was to verify whether the decrease in cell viabilities was caused solely by the drug carriers used. Since cationic liposomes alone can be very toxic to cells, empty DOPE/DOTAP liposomes were prepared and tested with dilutions up to the same concentrations used as with the macromolecules. CL formulations with no drugs reduced B16(F10) and MCF-7 cell viabilities by a maximum of 20% at the highest concentration (**Figure 9)**. It appears that at this concentration range (up to 54μM of DOTAP), DOPE/DOTAP cationic liposomes could bind, escape endosomes and deliver their cargo (both macromolecules) inside the cells without causing major toxicity.

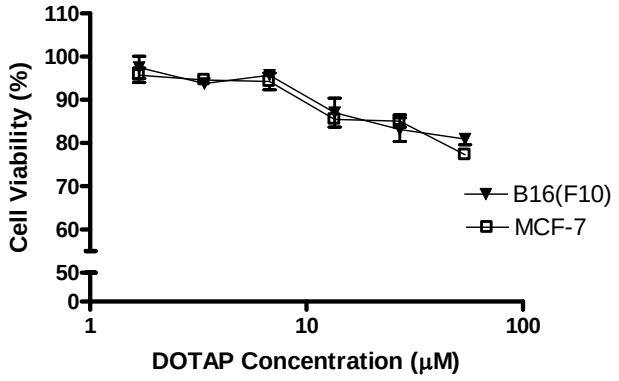


Figure 9. Percentage viability of B16(F10) and MCF-7 cells (mean ± SEM, with n=3) treated with empty CLs at increasing amount of lipids.

Other formulations tried that caused almost no toxicity to cells were the naked macromolecules, either alone or in the negative (D-(KLAKLAK)$_2$/G3139) complexes. These macromolecules could not cross cell membranes due to their large size and hydrophilicity, confirming the need for a drug carrier to deliver the macromolecules inside cancer cells. CL formulations with only D-(KLAKLAK)$_2$ were also tried. However, because of the insignificant encapsulation of the peptide without G3139, the cytotoxic profile of this formulation was the same as the empty cationic liposomes.

The uptake of CL formulations, containing the green fluorescently labeled peptide FAM-D-(KLAKLAK)$_2$ complexed with G3139, was evaluated with fluorescence microscopy. Cells were viewed after one hour incubation with serum-free medium containing CLs and the dye MitoTracker® Red. **Figure 10** shows representative pictures of cells visualized under bright field (**a**) and under epifluorescence (**b-d**). In the bright field (**Figure10a**), the intact morphology of B16(F10) cells is shown. After setting microscope parameters so that no green and red signals are noticed from the not-treated cells (data not shown), green and red fluorescence emissions were observed in **Figures**

10b and **10c**, respectively. When compared to the cells viewed in **Figure 10a**, both green fluorescence signal, which comes from the peptide FAM-D-$(KLAKLAK)_2$, and red fluorescence signal, from MitoTracker® Red, seem to be associated with cells. Since MitoTracker® Red, which is taken up by cells through diffusion, had a similar fluorescence association pattern with cells as the fluorescent labeled peptide (**Figure 10d**), which was encapsulated in CL, it appears that CL formulations were also taken up by B16(F10) cells.

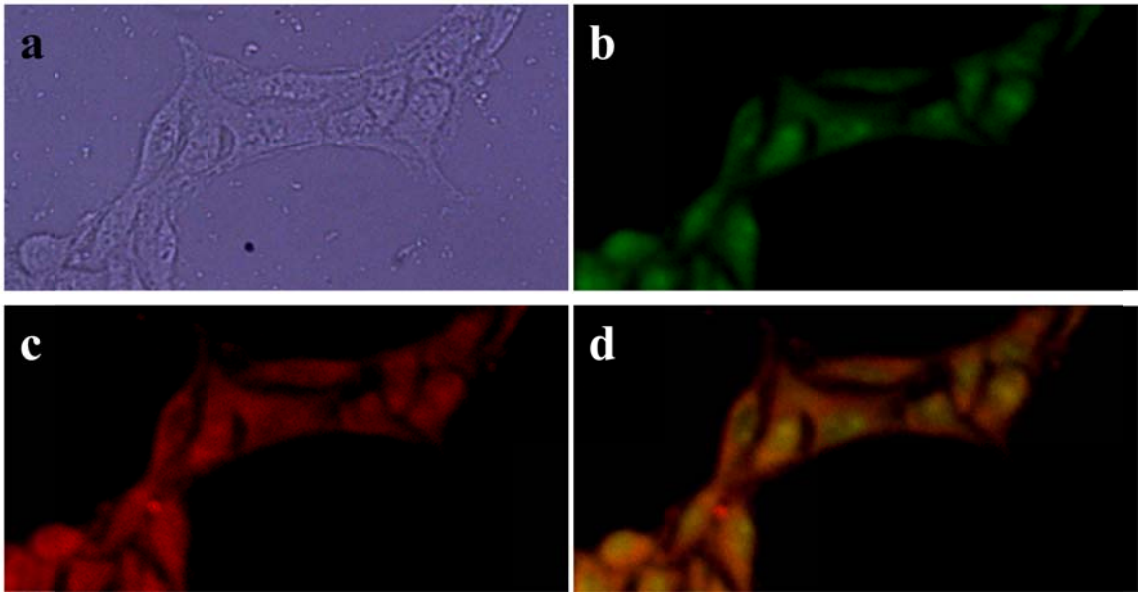


Figure 10. B16(F10) cells visualized under bright field (a) and under epifluorescence (b-d).

A previous study reported cell uptake of fluorescent labeled G3139 when complexed with DODAC/DOPE liposomes [22]. Since the study indicated that the peptide D-$(KLAKLAK)_2$ could not be encapsulated inside CL without pre-complexation with G3139, a fluorescently labeled peptide was used instead for the experiment.

Moreover, according to the green fluorescence associated with cells, it appears that both D-$(KLAKLAK)_2$ and G3139 were taken up by B16(F10) cells due to the complex formation.

Since both macromolecules have the ultimate pharmacological effect of promoting apoptosis, an experiment was conducted to verify whether the complexed macromolecules would enhance programmed cell death. Apoptosis assessment was done measuring caspases 3 and 7, which are the effector caspases that execute some common features of the apoptotic pathway, like DNA fragmentation [72]. This measurement was done indirectly through cleavage of a substrate, by the caspases, which gave a fluorescent product.

At the ODN concentration range of 15-500nM, there was a 25-35% increase in caspase activity compared to the control group when CLs had G3139 alone. On the other hand, the same G3139 concentration range complexed with KLAK peptide at a concentration range of 23-750nM in CLs had a 40-55% increase in caspase activity compared to non-treated B16(F10) cells (**Figure 11**). At 125nM ODN concentration, G3139 alone in CL had significantly greater caspase activity than the non-treatment group ($p < 0.006$), and at 250nM the significance level increased ($p < 0.001$). Meanwhile, CL formulations containing D-$(KLAKLAK)_2$/G3139 complexes not only had a higher caspase activity than control ($p < 0.001$ at 125 and at 250nM concentrations) but also had significantly greater caspase signal than CL with G3139 only ($p < 0.03$ at 125nM, and $p < 0.006$ at 250nM). Moreover, D-$(KLAKLAK)_2$/G3139 complexes in CL could achieve a higher caspase activity (up to 55%) than cisplatin used at 20μM (up to 40%) in a melanoma cell line in a previous study [73]. **These results demonstrated an enhanced**

apoptotic effect when D-(KLAKLAK)$_2$ and G3139 macromolecules were loaded in the same CL formulation.

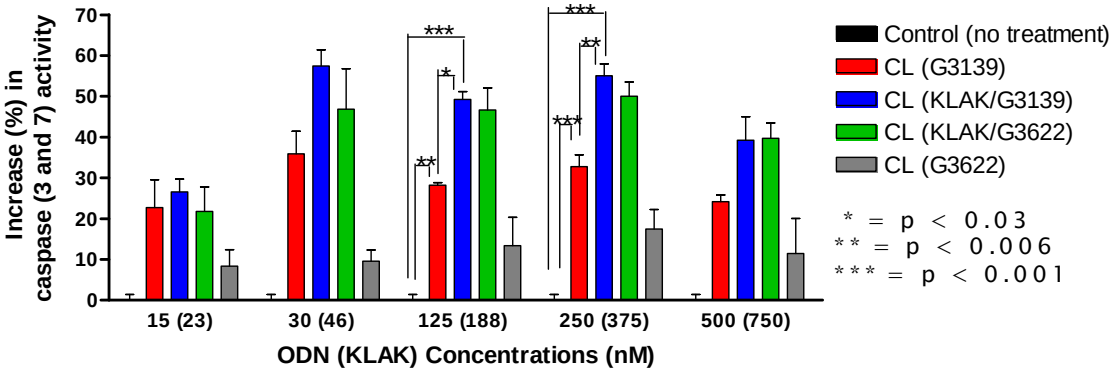


Figure 11. Percentage increase of caspase (3 and 7) activity in B16(F10) cells (mean ± SEM, with n=3) after treatment with different CL formulations at increasing concentrations.

Apparently reduced caspase activity of CL containing KLAK/G3139 complexes after the maximum reached at 30nM ODN concentration could be related to quenching of fluorescence from the product. Higher concentrations will induce apoptosis, with increased fluorescent product, earlier than lower concentrations. Subsequently, fluorescent signal quenched over time. Then, after 18 hours of incubation, while the fluorescence from elevated concentrations already quenched, the product from lower concentrations was just formed and it still had high fluorescent signal after that incubation time when the measurements were taken.

4.1.2. *In vivo* studies

The results obtained in the *in vitro* studies encouraged tests of the feasibility of combined anti-tumor therapy in mice bearing B16(F10) melanoma tumors (Aim 3). At the beginning of treatment, the average tumor volume was 218 ± 72mm^3. Mice received either CL formulations containing G3139 alone, or CL formulations containing D-(KLAKLAK)$_2$/G3139 complexes or saline, through daily intratumoral injections. Also, the volume of each tumor was measured every day. Four days after the start of the treatments, tumor volumes of the control (saline) group reached 1130mm^3 (1129 ± 123mm^3, n=3), mice were sacrificed, and tumor growth inhibition was determined by comparison of the mean (± SEM) tumor volumes among the treatment and control groups (**Figure 12**). G3139 alone in CL formulations did not significantly inhibit tumor growth compared to the saline group. Tumors reached 816mm^3 (816 ± 115mm^3, n=4). However, treatment with D-(KLAKLAK)$_2$/G3139 complexes in CLs was associated with a tumor size of 500mm^3 (500 ± 350mm^3, n=4), which was significantly lower compared to the control group ($p < 0.05$) (**Figure 12a**). In **Figure 12b**, excised tumors from the treatment groups were placed on a 12-well plate and photographed.

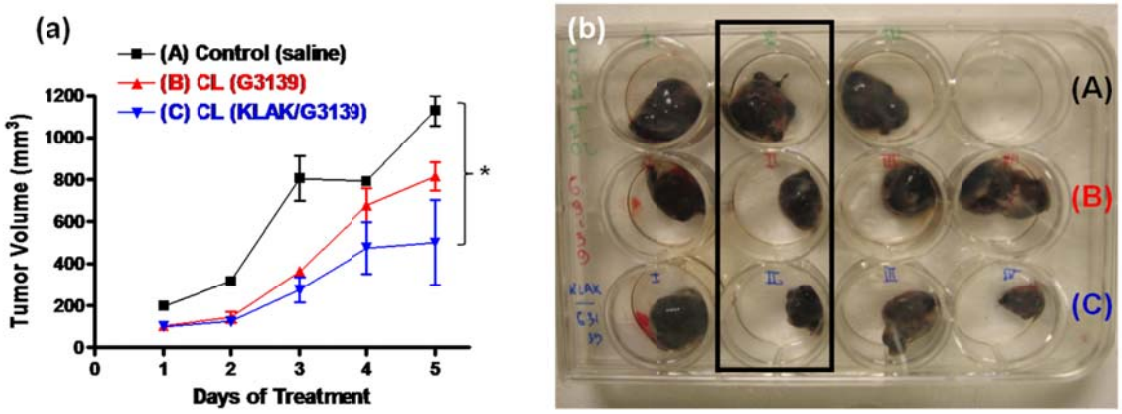


Figure 12. *In vivo* treatment of mice bearing B16(F10) tumors: (a) tumor measurements (mean ± SEM, with n= 3-4), and (b) their images after treatment with (A) saline (control), or (B) CL (G3139), or (C) CL (KLAK/G3139) for 5 days (*p < 0.05, one-way ANOVA with Tukey post hoc test).

The following experiment assessed whether apoptosis *in vivo* was the mechanism responsible for significant growth inhibition between tumors treated with saline and the ones treated with CL containing D-$(KLAKLAK)_2$/G3139 complexes. For this study, a single dose of CL containing D-$(KLAKLAK)_2$/G3139 complexes or saline was applied to mice bearing B16(F10) tumor, through intratumoral injection. Tumor tissue sections were examined under epifluorescence microscopy after TUNEL staining. **Figure 13** shows representative pictures of tumors treated with saline (**a**) and tumors treated with CL formulations loaded with D-$(KLAKLAK)_2$/G3139 complexes (**b**) visualized with a dual channel fluorescence filter. The green nuclei, which represent DNA fragmentation from apoptotic cells, was found only in tissue sections from tumors treated with CL formulations containing D-$(KLAKLAK)_2$/G3139 complexes.

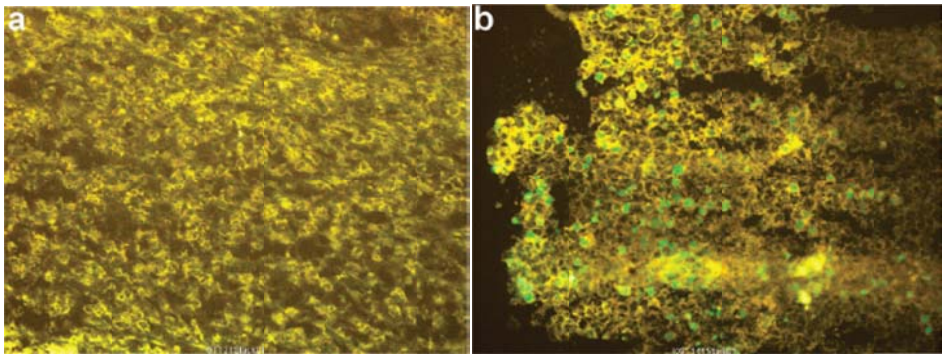


Figure 13. Detection of apoptosis (green nuclei) after TUNEL assay of (a) control (saline) and (b) CL (KLAK/G3139) treated B16(F10) tumor-bearing mice.

The results obtained in both experiments *in vivo* further demonstrated the importance of combining D-(KLAKLAK)$_2$ and G3139 in a single liposomal formulation to enhance anti-cancer therapy of B16(F10) melanoma, through increased apoptotic activity.

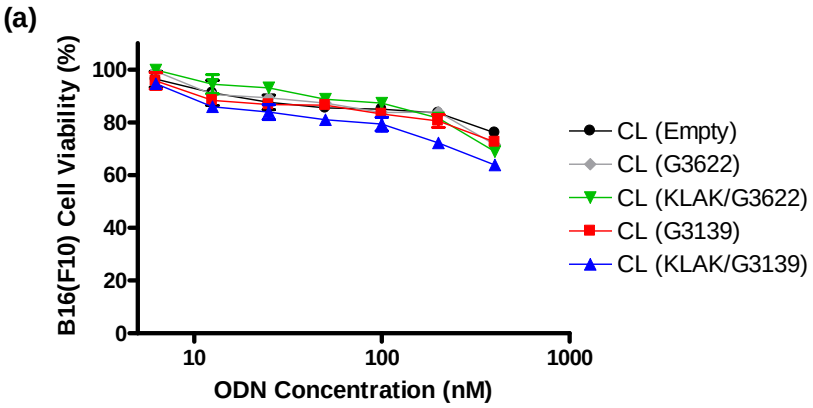
4.2. *In vitro* investigation of the mechanism of action of multifunctional liposomes.

The CLs loaded with D-(KLAKLAK)$_2$/G3139 complexes had enhanced anti-cancer therapy, through increased apoptotic activity, against B16(F10) murine melanoma compared with CL containing only G3139 *in vitro* and *in vivo*. However, it was unclear how the macromolecules worked to enhance the apoptotic effect. Thus, the next steps investigated the mechanism of action of the multifunctional liposomes (Aim 4).

The first step was to separate G3139 concentrations that could reduce viability of B16(F10) cells from concentrations that elicit a non-specific sequence mediated cell death as noted earlier by the toxicity of G3622, the reverse sequence of G3139, at higher concentrations. Once these concentrations were found, studies could move on to detect

whether the apoptotic effects of $(KLAKLAK)_2$ and G3139 molecules were due to down-regulation of the bcl-2 protein levels.

CL formulations were then prepared in a similar manner as before. Formulations were diluted in serum-free medium up to 400nM of ODNs (600nM of D-$(KLAKLAK)_2$ and 21.6μM of DOTAP). This new concentration range was chosen based on the fact that previously 500nM of ODNs showed some toxicity of G3622. Next, B16(F10) cells were incubated for 24 and 48 hours in different plates and their viability determined as previously described. According to **Figure 14**, B16(F10) cells lost viability at these lower concentrations.



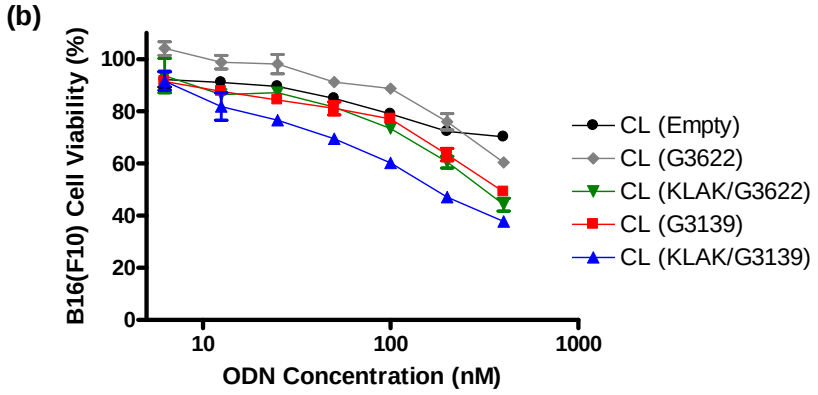


Figure 14. Percentage B16(F10) cell viability (mean ± SEM, with n = 3) after CLs treatment for 24 hours (a) or 48 hours (b) at increasing concentrations.

After 24 hours (**Figure 14a**), there was 30% reduction in viability when B16(F10) cells were treated with 200nM of G3139 complexed with 300nM of D-$(KLAKLAK)_2$ in CLs. This reduction was not only significant different from the non-treatment group ($p < 0.001$), but also significant different from the other CL formulations ($p < 0.03$ and lower at 200nM of ODN). Meanwhile, the same 200nM of G3139 alone in CL reduced B16(F10) viability by only 20%. At 400nM, although there was more reduction in viability than at 200nM, toxicity of CL formulations containing G3622 was also increased.

After 48 hours (**Figure 14b**), the variation in cytotoxicity among the different CL formulations became more pronounced. An additive effect was observed when 100nM of G3139 complexed with 150nM of D-$(KLAKLAK)_2$ reduced B16(F10) viability by 40%. Meanwhile, 100nM of G3139 alone and D-$(KLAKLAK)_2$ at 150nM, with G3622, in CLs reduced viability by only 20% each. The same 40% reduction in viability was observed with G3139 alone in CL only when the ODN concentration was doubled from 100nM to 200nM. This amount of G3139 complexed with 300nM of KLAK peptide reduced

B16(F10) viability even further to 50%. Interestingly, there was some toxicity promoted by D-$(KLAKLAK)_2$ when complexed with G3622, the reverse sequence of G3139, compared to G3622 alone in CLs. In this case, toxicity was probably due to the presence of the peptide. At 400nM, the appearance of a non-specific sequence mediated cell death promoted by G3622 alone in CLs (40% reduction in viability) was perceptible. Other formulations tested that showed minimal toxicity included: "naked" D-$(KLAKLAK)_2$/G3139 complexes, free D-$(KLAKLAK)_2$, free G3139, and CL formulations containing only D-$(KLAKLAK)_2$ peptide.

Based on the cytotoxicity results obtained, the highest concentration of ODNs that could be used without eliciting toxicity by a non-specific sequence was 200nM. At this concentration, the reduction in viability was more evident after 48 hours. Since down-regulation of the bcl-2 protein, promoted by G3139, should occur before the cell viability decreases, B16(F10) cells were treated for 24 hours with the CL formulations for the protein studies. Later, the obtained cell lysates were analyzed through western blotting, with β-actin as a loading control.

A representative western blotting analysis of bcl-2 protein is shown in **Figure 15**. The reverse sequence G3622, either complexed with D-$(KLAKLAK)_2$ (**D**) or alone (**E**) in CLs, did not reduce the levels of bcl-2 protein compared to not treated cells (**A**) as expected (118.5 ± 3.46% and 100.7 ± 3.69%, respectively). Interestingly, G3139, alone (**B**) or complexed with KLAK peptide (**C**), in CLs also did not reduce bcl-2 protein levels compared to non-treated cells (129.7 ± 25.42% and 124.5 ± 18.02%, respectively). **It appears that the apoptotic events promoted by CLs loaded with D-**

$(KLAKLAK)_2$/G3139 complexes in B16(F10) cells were independent of bcl-2 protein reduction (a non-antisense effect).

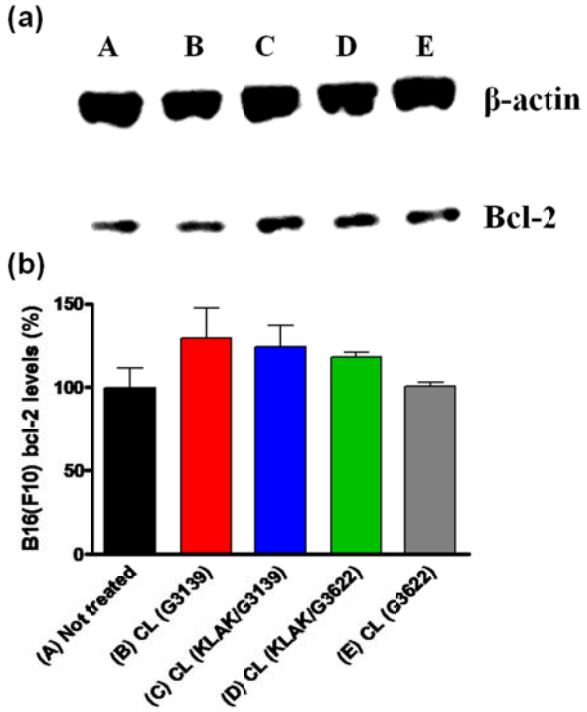


Figure 15. B16(F10) bcl-2 protein analysis after 24 hours treatment with different CL formulations: (a) Representative western blotting and (b) Percentage levels of bcl-2 (mean ± SEM, with n=3) normalized to β-actin levels (bcl-2/β-actin ratio).

Although it was reported that 200nM of G3139 significantly reduced bcl-2 expression in human melanoma cells [66] and led to clinical trials of G3139 against 518A2 melanoma, similar results could not be found for the B16(F10) murine melanoma cells. Other research groups found bcl-2 protein down-regulation in other cell lines, like K562 leukemia (by 70%) [24] and oral carcinoma KB cells (by 85%) [74, 75].

A possible explanation for the absence of down-regulation of bcl-2 protein in these murine melanoma cells is that G3139 was developed as an antisense molecule for

human bcl-2 mRNA and may not be as effective as for murine bcl-2 mRNA. However, it is very unlikely that G3139 did not bind the mRNA and did not block the translation of murine bcl-2 protein due to the high homology (80% similarity) between the human and mouse bcl-2 mRNAs [76, 77].

The non significant bcl-2 protein down-regulation in B16(F10) cells corresponds more with recent findings that indicate toxicity induced by G3139 in 518A2 human melanoma is independent of bcl-2 protein down-regulation [40, 42, 78]. Lai and co-workers suggested that G3139 in these 518A2 melanoma cells may target the mitochondrial VDAC channels and the subsequent release of cytochrome c from mitochondria [38]. Since D-$(KLAKLAK)_2$ promotes disruption of the mitochondrial membrane potential with also release of cytochrome c [46], the detection of cytochrome c in cytosolic fractions might be a better approach to understand the mechanism of increased apoptotic activity by the multifunctional liposomes.

4.3. Surface modification of CLs, containing D-$(KLAKLAK)_2$/G3139 complexes: development and initial evaluations.

In order to obtain multifunctional liposomes better fit to escape capture by the Reticulo Endothelial System (RES) and have prolonged blood residence time (Aim 5), CLs were modified with polyethylene glycol (PEG) by incorporating amphiphilic polymer PEG2000-DSPE into the liposomal membrane. PEG-Cationic Liposomes (PCLs) were prepared with increasing molar percentages of PEG2000-DSPE to find out which concentration of PEG is better able to shield the positive charges from the PCLs

and to decrease their interaction with cells, which should make such liposomes less toxic to non-cancer cells, protecting healthy tissues (Aim 5a).

The prepared PCLs and CLs without PEG were then used for cytotoxic studies. These formulations were incubated for 24 hours with B16(F10) cells in serial dilutions up to 1μM of G3139 (1.5 μM of D-$(KLAKLAK)_2$, and 54 μM of DOTAP). The only component that had its molar concentration changed among the formulations was PEG2000-DSPE due to the different molar percentages among the PCLs produced. After trying several different concentrations of PEG in PCLs, a pronounced decrease in toxicity of PCL with 1% mol of PEG2000-DSPE was found compared to the CL formulation ($p < 0.05$ at 125nM and at 250nM) (**Figure 16**).

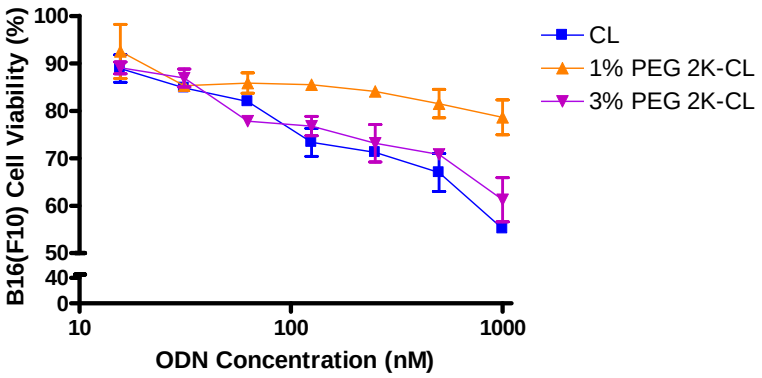


Figure 16. Percentage B16(F10) cell viability (mean ± SEM, with n=3) after treatment with PCLs containing PEG2000-DSPE at increasing molar percentages.

The above finding can be related to the spatial conformation assumed by PEG chains when grafted on the surface of liposomes, which depends on the molecular weight of the used PEG and its concentration [79]. It is hypothesized that at 1% mol of PEG2000-DSPE, PEG assumes a mushroom conformation, where the relatively negative

PEG is able to interact with the positive charges of the liposomes, and, thus, reduce the interaction of PCLs with cells. As the concentration of PEG is increased, the PEG chains assume a brush conformation due to the increase in PEG density [79-81].

It was also investigated whether the increase in the size of the PEG molecule could also increase the degree of shielding of PCLs. For this purpose, PCL formulations were prepared containing either PEG2000-DSPE or PEG5000-DSPE at 1% mol and tested with the melanoma cells. **Figure 17** indicates that PEG5000-DSPE at 1% mol also significantly decreased the toxicity of PCL compared to plain CL (at 125nM, $p < 0.05$; at 250nM, $p < 0.003$). However, the degree of shielding was about the same for both PEG molecules in their respective PCLs.

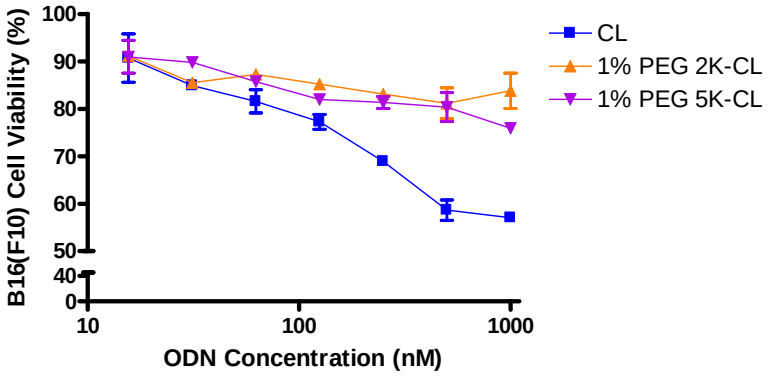


Figure 17. Percentage B16(F10) cell viability (mean ± SEM, with n=3) after treatment with PCLs containing 1% mol of PEG with different molecular weights.

The PCLs should have long-circulating properties because of PEG and avoid capture by organs of the RES. In addition, due to the expected prolonged blood residence time, they can accumulate in tumors through the Enhanced Permeability and Retention (EPR) effect [12, 59]. Nevertheless, their interaction with tumor cells should be reduced

due to shielding of the positive charges of PCL by PEG. To restore the toxic activity of PCL, it would be desirable to remove PEG from the surface of liposomes once inside the tumors.

Thus, further studies were conducted on the development of pH-sensitive PEG-PE chains that could be cleaved under a lowered pH environment, characteristic of a tumor tissue, to reveal the hidden positive charges from PCL that would be able to interact with and deliver the macromolecules to the melanoma cells (Aim 5b).

The pH-sensitive PEG-PE polymers were obtained as in previous studies [67-69]. In the first step, PEG2000 with a thiol group (PEG2000-SH) on one of its distal ends was reacted with a maleimide group-containing hydrazide (MPBH) compound to link the thiol group of PEG to MPBH (see Figure 1). Next a phospholipid, phosphothioethanolamine (PE-SH), was conjugated to another maleimide-containing compound, 4-acetyl phenyl maleimide (see Figure 2). Finally, the hydrazide group of the PEG compound reacted with the acyl group of the phospholipid to link PEG and phospholipid molecules through a hydrazone bond and the polymer PEG-Hydrazone-PE (PEG-Hz-PE) was obtained (see Figure 3).

The above reactions were followed with Thin Layer Chromatography (TLC). In the first step, the PEG-hydrazide product was stained by Dragendorff reagent and glowed under UV light, while the precursors PEG2000-SH and MPBH stained for Dragendorff and glowed under UV light, respectively. Next, the acyl activated phospholipid reacted with molybdenum blue and glowed under UV light, while the precursors PE-SH and 4-acetyl phenyl maleimide stained with molybdenum blue and glowed under UV light, respectively. Finally, the obtained PEG-Hz-PE was stained by both Dragendorff and

molybdenum blue reagents, while the precursors PEG-hydrazide and the acyl activated phospholipid stained with Dragendorff reagent and molybdenum blue, respectively.

After obtaining the pH-cleavable polymer PEG-Hz-PE, it was necessary to verify if it could really be hydrolyzed under acidic pH. The cleavage of the prepared pH-sensitive PEG was examined through a previously developed methodology [67-69]. Micelles made of the pH-cleavable polymer PEG-Hz-PE and trace amounts of Rhodamine-PE (Rh-PE) were incubated in pH 7.4 or pH 5.0 at 37^0C for three hours, and then analyzed through high performance liquid chromatography (HPLC) using a size exclusion column. **Figure 18** shows representative chromatograms of the micelles with (**a**) no incubation; (**b**) after three hours incubation in pH 7.4 and (**c**) after three hours incubation in pH 5. The peak corresponding to intact micelles was indicated after 9-10 minutes [67-69] in all three chromatograms. At 12-13 minutes, there was also a small peak noticeable in (**a**) and (**b**), which represented the unreacted PEG-hydrazide in trace amounts. In (**c**), however, this latter signal was drastically increased after pre-incubation in pH 5, which suggested the breakage of the hydrazone bond and the release of PEG-hydrazide. The presence of the micelle peak in (**c**) was an indication that not all hydrazone bonds from PEG-Hz-PE were broken and micelles still remained.

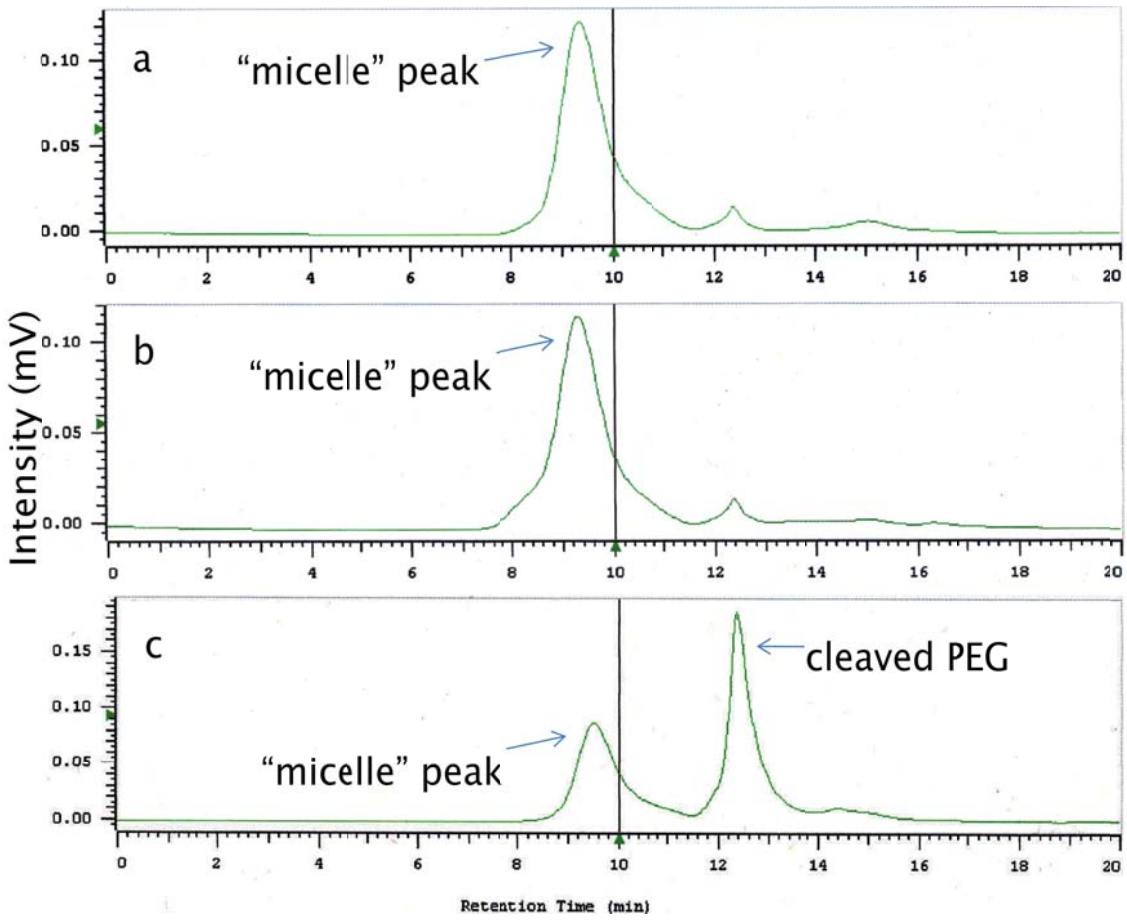


Figure 18. Representative chromatograms of PEG-Hz-PE micelles after (a) no pre-incubation, (b) after 3 hours pre-incubation at 37^{0}C in pH 7.4, or (c) after 3 hours pre-incubation at 37^{0}C in pH 5.0.

Following the proof-of-principle experiment that the polymer is able to be hydrolyzed when in a lowered pH environment, multifunctional liposomal formulations were prepared containing D-(KLAKLAK)$_2$/G3139 complexes. Three formulations were prepared in neutral HBG 5%: CLs, PCLs with 1% mol of PEG2000-DSPE, as previously prepared and pH-sensitive PCL (pH-PCL), with 1% mol of PEG-Hz-PE. The approximate sizes of the particles produced, which did not change after three hours incubation at 37^{0}C in pH 7.4, were: 195 nanometers (nm) for CL, 230nm for PCL and 245nm for pH-PCL. The difference in sizes may be attributed to the presence of the PEG

chains that increased the diameter of the liposomes. After 3 hours incubation at 37^0C in pH 5.0, there was no change in the sizes of CL and PCL. However, pH-PCL size was reduced to 215nm because of the possible hydrolysis of PEG-Hz-PE.

The hydrolysis of PEG-Hz-PE in pH-PCL was also confirmed by measuring the charge of the multifunctional liposomes. The CL and PCL had zeta-potentials of 47mV and 35mV, respectively, which did not change when pre-incubated either in neutral or acid pHs. However, pH-PCL had its charge slightly increased from 39 to 45mV in acid pH. A feasible explanation for the charge increase was that, with the release of PEG-hydrazide, there were less PEG molecules to interact with and neutralize the positive charges. A summary of the particle sizes and zeta-potential of the multifunctional liposomes after being produced and after incubation for three hours at 37^0C in pH 7.4 or pH 5 are presented in **Tables 2** and **3**.

Table 2 –Particle size* of three types of multifunctional liposomes

Liposomes	pH 7.4; zero hour	pH 7.4; 37^0C; 3 hours	pH 5; 37^0C; 3 hours
CL	194.5 ± 6.36	206.8 ± 2.78	199.7 ± 4.10
PCL	230.6 ± 5.73	229.7 ± 2.32	232.4 ± 5.65
pH-PCL	245.3 ± 5.30	248.7 ± 1.97	214.3 ± 4.35

* mean ± SEM, with n=3.

Table 3 – Zeta potential* of three types of multifunctional liposomes

Liposomes	pH 7.4; zero hour	pH 7.4; 37^0C; 3 hours	pH 5; 37^0C; 3 hours
CL	47.7 ± 2.68	46.5 ± 4.12	45.3 ± 2.64
PCL	36.2 ± 2.41	30.6 ± 1.86	32.6 ± 3.42
pH-PCL	39.2 ± 0.97	35.6 ± 3.18	44.7 ± 2.94

*mean ± SEM, with n=3.

Later, encapsulation efficiency of FAM-D-$(KLAKLAK)_2$ was verified in all three formulations. CL had encapsulation of 45%, close to the previous determination, but PCL and pH-PCL had encapsulation of approximately 30% **(Figure 19)**. The difference in encapsulation efficiency may be correlated to the orientation of PEG molecules during preparation of the liposomes. In the rehydration and extrusion processes, some PEG molecules can be oriented towards inside the aqueous core of the liposomes [80, 82]. The presence of the PEG chains decreased the free volume in the core and repelled the negatively charged complexes and, thus, decreased the amount of macromolecules encapsulated inside the liposomes

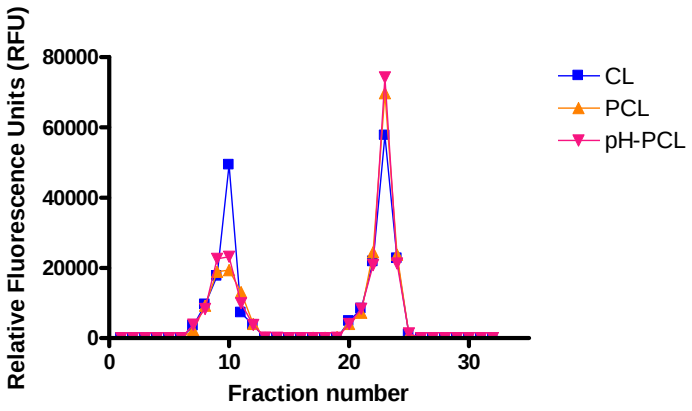


Figure 19. Encapsulation efficiency of FAM-D-$(KLAKLAK)_2$ in three multifunctional liposomal formulations.

The next step was to test the multifunctional liposomes with cells. Even though it has been previously demonstrated that CL formulations can reduce viability of B16(F10) cells and that PCL with 1% mol of PEG2000-DSPE can reduce the interaction of the positive charges from the liposomes with the murine melanoma cells, it was necessary to verify if the pH-PCL formulations would have differential activities when pre-incubated at neutral (pH 7.4) or acid (pH 5) buffers. For this purpose, formulations were prepared and pre-incubated for three hours in neutral or acid buffers as previously described. Formulations were then diluted with serum-free medium, where the pH was increased to 7.4, and added to cells up to 1μM of G3139. After 24 hours incubation with the formulations, viability was determined.

Figure 20 indicates that at pH 7.4 (**Figure 20a**), pH-PCL with 1% mol of PEG-Hz-PE had similar activity as PCL with 1% mol of PEG2000-DSPE. Both PEG molecules, cleavable and non-cleavable, could in their respective formulations prevent the liposomes from interacting with and decreasing the viability of B16(F10) cells compared to CL. In addition, CL formulations significantly reduced viability compared to the PEG-liposomal formulations from 250nM ($p < 0.05$, for PCL and for pH-PCL) of G3139.

On the other hand, when formulations were pre-incubated at pH 5.0 (**Figure 20b**), pH-PCL reacted differently towards B16(F10) cells. The liposomes had their PEG chains hydrolyzed during the pre-incubation in acid, and were able to interact with the cells and be internalized. After the pre-incubation, which simulates the lowered pH of a tumor tissue, the cytotoxic activity of pH-PCL was similar (not significant different) to CL formulations. Nevertheless, the cytotoxicity of pH-PCL was only significantly different

from PCL at high concentrations (500 and 1000nM, $p < 0.05$). This suggests that, after the pre-incubation at pH 5.0 for 3 hours at 37^0C, not all PEG-Hz-PE was hydrolyzed, as suggested by the results obtained with the respective micelle formulation (see **Figure 18**) and an increase in pre-incubation time might be necessary to cleave all PEG-Hz-PE.

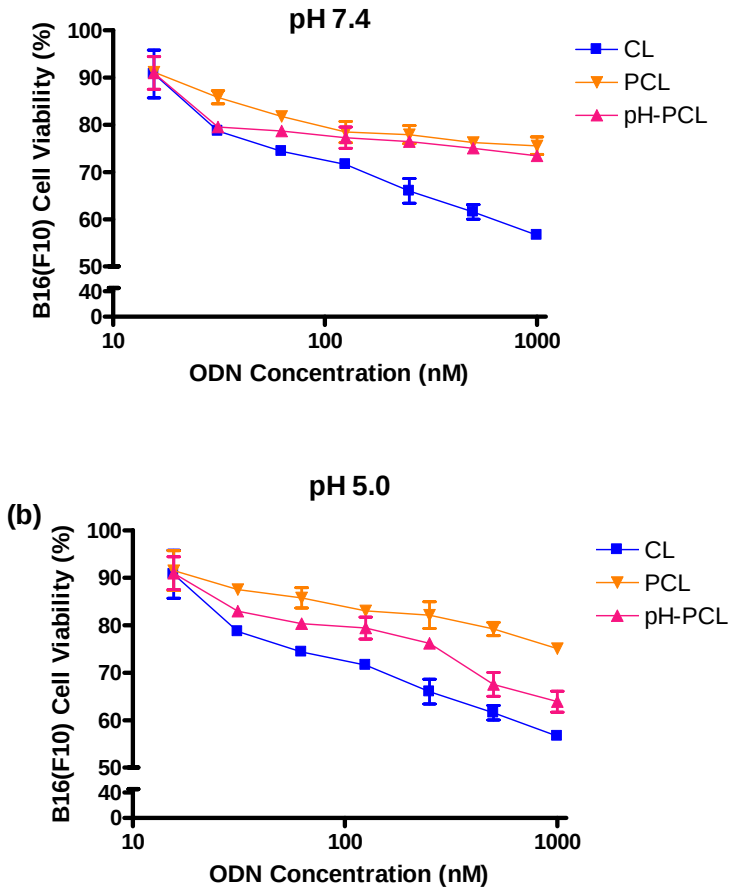


Figure 20. Percentage B16(F10) cell viability (mean ± SEM, with n=3) after treatment with different multifunctional liposomes pre-incubated for 3 hours at 37^0C in (a) pH 7.4 or (b) pH 5.0 buffers.

More importantly, however, was the fact that pH-PCL had differential activity when pre-incubated in neutral or lowered pHs. The importance of such differential

activity is that pH-PCL particles can have a prolonged blood residence time due to the presence of PEG and can also be less toxic to healthy tissues due to the shielding of the positive charges of the liposomes. However, when they reach tumor sites through the EPR effect, the lowered pH of the tumor tissue can promote cleavage of the PEG chains and the liposomes, now unshielded, can interact with and be taken up by the cancer cells.

5) DISCUSSION

5.1. Formulation development of CL, containing D-(KLAKLAK)$_2$/G3139 complexes.

The multifunctional liposomes developed in this study were based on cationic liposomes prepared from the initial complexation between two oppositely charged macromolecules. The positively charged pro-apoptotic peptide D-(KLAKLAK)$_2$ was mixed with an excess of negatively charged antisense oligodeoxynucleotide (ODN) G3139 to produce negatively charged complexes. These complexes were incorporated inside cationic liposomes, during lipid film hydration and extrusion, through interaction between the negative complexes and the positively charged phospholipids in excess. This procedure was a modification of a previously described study, where PEI/ODN polyplexes were encapsulated in liposomes [83], and analogous methods can be found, such as the one which generated "pre-condensed stable plasmid lipid particles" (pSPLP) [84] or a "multifunctional envelope-type nano device"(MEND) [85].

Although the methodology employed was not completely novel, it had some distinct features. While cationic liposomes have provided an efficient tool for the incorporation and the intracellular delivery of nucleic acid molecules, such as G3139, it is unusual to use these carriers for the delivery of positively charged peptides, such as D-(KLAKLAK)$_2$, because of charge repulsion. This was possible because of the initial complexation between the peptide and the antisense, which allowed encapsulation of both macromolecules. Other possible methods to encapsulate D-(KLAKLAK)$_2$ in liposomes are the use of a pH gradient or negatively charged lipids. In the pH gradient method [86],

the use of a low pH buffer to prepare neutral liposomes and later adjust the external pH to higher values with a buffer containing D-$(KLAKLAK)_2$ could result in a high peptide loading efficiency. However, the liposome formulation would need an extra component to help them interact with, and be internalized by the cells, such as a cell penetrating peptide [87]. Attempts to use negatively charged liposomes to encapsulate D-$(KLAKLAK)_2$ were made, but the particles obtained did not show cytotoxicity *in vitro*.

In the latter two methods, the liposomal formulations would not have phospholipids such as DOTAP and DOPE that can better interact with cells and help them escape endosomes, respectively [10, 11, 56, 57]. Although these phospholipids can be toxic to cells, there was no major toxicity to B16(F10) and MCF-7 cells at the concentrations used (up to 54μM) in this study. Also, it was shown that these cationic liposomes were taken up by B16(F10) cells, indicated by the association of green fluorescence from the labeled D-$(KLAKLAK)_2$ with cells. Since there was no specific targeting moiety on the surface of the liposomes that could bind and deliver the cargo preferentially to one cell than the other, it is likely that the CL formulations, containing the macromolecules, were also taken up by MCF-7 cells. Thus, the therapeutic outcome of these multifunctional liposomes was due to D-$(KLAKLAK)_2$ and G3139 macromolecules delivered to the cells.

5.2. *In vitro* and *in vivo* activities of D-$(KLAKLAK)_2$/G3139 complexes in CL.

The D-$(KLAKLAK)_2$ and G3139 macromolecules had distinct cytotoxic effects that were dependent on the cell line used. After 24 hours, there was no reduction in MCF-

7 cell viability with G3139 alone or when complexed with D-$(KLAKLAK)_2$ in CLs. By contrast, viability was reduced by up to 40% in B16(F10) cells when these macromolecules were complexed in the CL formulation. Because of this difference in viability, all experiments were done with B16(F10) melanoma cells. These results do not necessarily mean that these macromolecules have no effect on MCF-7 cells. Similar to a previous study [7], these cells may require a second dose of the formulations at the same concentrations and a longer exposure time for G3139 to down-regulate bcl-2 protein and cause MCF-7 cells to lose viability.

While the ODN concentration range applied once to MCF-7 cells did not cause a decrease in viability, in B16(F10) over the same concentration range, the highest concentrations also resulted in cell death mediated by the non-specific sequence G3622, the reverse sequence of G3139. It seems that the G3139 concentration range necessary to obtain down-regulation of bcl-2 protein is cell-dependent. In fact, G3139 has been used at 1-4μM for K562 leukemia cells [24, 25], 0.5-2 μM for KB oral carcinoma [74], and 25-200nM for 518A2 human melanoma cells [26, 66]. Since these 518A2 cells are related to B16(F10) murine melanoma, the ODN concentration range was decreased, and cells were incubated with up to 400nM of ODNs.

In the 6-400nM ODN concentration range, it was possible to separate the cytotoxic effects promoted by G3139 until 200-250nM from toxicity promoted by the non-specific sequence (G3622) from 400nM. After 48 hours incubation, the same 40% reduction in B16(F10) viability was achieved. There was no need to use a concentration of 500nM for 24 hours which had also elicited cell death with the non-specific sequence. This 40% reduction in viability was obtained with 200nM of G3139 alone or with 100nM

of G3139 complexed with 150nM of D-$(KLAKLAK)_2$ in CLs. Also, after 48 hours incubation, the dose necessary to kill 50% of B16(F10) cells (LC_{50}) was achieved when 300nM of D-$(KLAKLAK)_2$ was complexed with 200nM of G3139 in CL. This quantity of D-$(KLAKLAK)_2$ was 0.1% of the concentration needed to kill 50% of cancer eukaryotic cells in a monolayer when the peptide was used alone in solution [6]. **This result clearly demonstrates that a combination of the peptide with G3139 in an efficient delivery system, such as a cationic liposome, can drastically reduce the amount of drugs needed for anti-cancer therapy.**

Later, it was found that the anti-cancer activity of the multifunctional liposomes occurred through programmed cell death (apoptosis). The D-$(KLAKLAK)_2$/G3139 complexes elicited higher caspase activity than G3139 alone at 125 and 250nM. Then, analyses of proteins were performed to study the mechanism by which D-$(KLAKLAK)_2$/G3139 complexes promoted increased caspase activity. Bcl-2 protein levels were examined through Western blotting to check for the down-regulation of this protein by G3139. However, after 24 hours incubation with B16(F10) cells, 200nM of G3139 alone or complexed with D-$(KLAKLAK)_2$ in CLs did not decrease bcl-2 protein levels.

In these studies, it was not possible to incubate B16(F10) cells for 48 hours and then measure bcl-2 protein levels, as done by other research groups [24, 74, 75], because of the pronounced cell viability reduction, after those 48 hours of incubation, by CL formulations containing D-$(KLAKLAK)_2$/G3139 complexes at 200nM ODN concentration. Because of the difference in viable cells among the formulations, the total protein loading would not be the same for Western blotting. Even if there were bcl-2

down-regulation at 48 hours, it does not explain a 40-50% reduction in B16(F10) viability during this time span if G3139 should first promote down-regulation of the protein and then cause cells to lose their viability or to make them more susceptible to chemotherapeutic agents. Bcl-2 mRNA levels could have been checked through real time-polymerase chain reaction (RT-PCR) to detect knock-down of the mRNA by G3139. However, even with a decrease in bcl-2 mRNA levels, it may be necessary some time to detect decrease in bcl-2 protein levels because bcl-2 protein has a half-life of approximately 10 hours [88]. Therefore, there was another mechanism (a non-antisense effect) which reduced viability of the B16(F10) cells, through apoptosis, that occurred before the antisense G3139 down regulated bcl-2 protein levels.

Non-antisense effects of G3139 have been discovered in the 518A2 human melanoma cells [37]. Although bcl-2 protein levels were reduced after G3139 treatment, it occurred after cytochrome c was released from the mitochondria to the cytoplasm which then promoted the apoptosis. Furthermore, it was found that cytochrome c was released after the mitochondrial VDAC channel was locked in an open conformation by G3139 in the same cells [38, 39]. Because 518A2 and B16(F10) are closely related cells, it suggests cytochrome c release may be involved in the early cytotoxicity of G3139 in the murine melanoma cells. The detection of cytochrome c levels in the cytoplasm of B16(F10) cells is currently under investigation.

In addition, the use of D-$(KLAKLAK)_2$/G3139 complexes in CL may have contributed to a shift in the pharmacological action of these multifunctional liposomes towards the release of cytochrome c because both macromolecules work by interacting with mitochondria and promote the release of the cytochrome c protein. Their

combination is useful because D-$(KLAKLAK)_2$ works by disrupting the mitochondrial membrane potential with consequent mitochondrial swelling [6, 46] and G3139 keeps the VDAC channel opened [38, 39] so that both mechanisms could contribute to an enhanced release of cytochrome c.

The antisense (down-regulation of bcl-2 protein) and non-antisense (release of cytochrome c) effects of G3139 seem to be cell-dependent. While the non-antisense effects were mainly found in melanoma cells [37-40], G3139 works well as an antisense molecule in other cells [7, 20, 24, 25, 74, 75]. It is not understood yet why this ODN has different mechanisms of action depending on the cell line used. The non-antisense effect, also called off-target effect, is not restricted to G3139. An antisense molecule targeting the MDR1 mRNA, which translates to p-glycoprotein, knocked down not only the targeted mRNA but also affected 37 other genes/mRNAs tested [89]. Moreover, off-target effects are not exclusive to antisense oligonucleotide molecules. Some small interfering RNAs (siRNAs) also presented off-target effects [90, 91].

The anti-cancer therapy of the multifunctional liposomes was also tested *in vivo*, through intratumoral injections in B16(F10) tumor-bearing mice. Significant tumor growth inhibition compared to the control group was obtained only with CL formulations containing D-$(KLAKLAK)_2$/G3139 complexes. The anti-cancer effect depended on apoptosis, as in the *in vitro* studies. Although intratumoral injections can deliver the highest amount of formulations to an easily accessible tumor tissue and can have some clinical applications [92-94], most tumors are internal or widespread. The DOPE/DOTAP CL formulations initially developed could not be administered intravenously because they would be quickly bound to opsonins and eliminated by organs of the RES, before

reaching the tumor tissue. Therefore, it was necessary to further develop this cationic liposomal formulation to escape this rapid capture and to accumulate in tumor sites of all kinds, once injected by the intravenous route.

5.3. Surface modification of CLs containing D-$(KLAKLAK)_2$/G3139 complexes.

After developing the first multifunctional liposome formulation, containing D-$(KLAKLAK)_2$/G3139 complexes encapsulated in CL, studies moved towards the improvement of this CL formulation. This improvement consisted of adding PEG chains to the surface of the liposomes. With this surface modification, it was expected that the new multifunctional liposome formulation, PEG-Cationic Liposome (PCL), should escape rapid capture by organs of the RES and have prolonged blood residence time [9, 13]. This was an important step because the first multifunctional liposomes, CLs, if administered intravenously, could not only be rapidly sequestered by the RES but also could interact with and be taken up by non-cancer cells, rendering them very toxic to healthy tissues. Various concentrations of PEG-DSPE, as well as PEG-DSPE molecules with different molecular weights of PEG were tried to determine, *in vitro*, what concentration and size of PEG could better shield the positive charges of the PCL formulation. It was observed that 1% mol of PEG2000 or PEG5000 significantly reduced interactions of PCL with B16(F10) cells, compared to CL formulation, through less reduction in cell viability. This shielding is estimated to considerably reduce the interaction of the PCL formulation with non-cancer cells and with opsonins, reducing

their interaction with healthy tissues and enabling them to be long-circulating particles, respectively.

The PCL particles developed should circulate in the blood until they reach tumor tissues. There, the gaps among cancer endothelial cells should allow the extravasation of PCL particles to the tumor tissue. Since this tumor tissue does not have a proper lymphatic system that can efficiently drain the particles, PCL should be retained in the tumor, a phenomenon known as the EPR effect [12, 59]. However, as the *in vitro* results indicated, PCL should have minimal activity against the cancer cells because the positive charges, which initiate the interaction with cancer cell membranes, are shielded by PEG. PEG would have to be removed in the tumor tissue to restore the toxic activity of the liposomes loaded with the macromolecules against the cancer cells.

Thus, the third multifunctional liposomal formulation was developed with PEG molecules that could be removed once inside a tumor. Advantage was taken of a physiological feature of tumor tissues, a lowered pH environment, to elaborate pH-cleavable PEG-lipids. These molecules were synthesized through the formation of hydrazone bonds which can be hydrolyzed at a relatively acidic pH [67-69].

After the pH cleavable PEG-Hz-PE molecules were obtained and their hydrolyses were demonstrated in a low pH buffer *in vitro*, a pH-sensitive PCL (pH-PCL) formulation was prepared. The hydrolysis of PEG-Hz-PE was also confirmed in pH-PCL after the mean size and zeta-potential of these particles changed, which was due to the cleavage of PEG in an acid buffer. Although encapsulation efficiency of the peptide was reduced in PCL and pH-PCL compared to plain CL, the amount of the loaded macromolecules may be increased if PEG molecules, both non-cleavable and cleavable, are added as loose

micelles after the liposomes are formed [80, 82]. This post-insertion procedure avoids PEG chain insertion oriented towards the core of the liposomes and prevents the repulsion of the negatively charged complexes. However, it may also generate particles of many different sizes, increasing the overall size distribution of the liposomes.

When tested with B16(F10) cells, pH-PCL was the only multifunctional liposomal formulation which had differential activity when pre-incubated at neutral or acidic pHs. At neutral pH, PEG-Hz-PE molecules of pH-PCL shielded the positive charges of these liposomes, similar to shielding of PCL formulation by non-cleavable PEG2000-DSPE molecules. The shielding promoted a low cytotoxic activity in both formulations and should allow both liposomal particles to have a prolonged blood residence time. At an acidic pH, to simulate the lowered pH environment of a tumor tissue, cytotoxic activity of PCL was still low due to shielding by PEG2000-DSPE. However, the lowered pH promoted hydrolysis of PEG-Hz-PE and the unshielded positive charges of pH-PCL were able to interact with cells and the cytotoxic activity of the liposomes with the macromolecules was restored, becoming closely related to CL cytotoxicity.

Potential future *in vitro* studies for these multifunctional liposomes are differential uptake to evaluate the incorporation of the particles by the cells and the differential apoptotic activity among the three formulations when pre-incubated at neutral or lowered pHs. Finally, *in vivo* studies should be performed to compare tumor growth inhibition, as well as biodistribution, among the multifunctional liposomes when they are injected intravenously.

6) SUMMARY AND CONCLUSION

- Cationic liposomes (CL) loaded with D-$(KLAKLAK)_2$ and G3139 were obtained after the initial complexation between the two macromolecules and charge alternation among the macromolecules and phospholipids.
- *In vitro*, D-$(KLAKLAK)_2$ and G3139 complexes in CL demonstrated increased cancer cell cytotoxicity and enhanced apoptotic activity against B16(F10) melanoma cells compared to G3139 alone in CLs.
- *In vivo*, D-$(KLAKLAK)_2$/G3139 complexes in CLs significantly inhibited tumor growth, through induction of apoptosis.
- The mechanism involved in CL (D-$(KLAKLAK)_2$/G3139) enhanced apoptotic activity and anti-cancer effect against B16(F10) melanoma cells was independent of bcl-2 down-regulation.
- PEG2000-DSPE molecules at 1% mol concentration at the surface of PEG-Cationic Liposomes (PCLs) can reduce their cytotoxicity towards B16(F10) cells.
- pH-sensitive PEG-CL (pH-PCL) with cleavable PEG-Hz-PE molecules had differential cytotoxic activity towards B16(F10) melanoma cells when pre-incubated at neutral pH (similar to PCLs) or when at a lowered pH (similar to CLs).

The multifunctional liposomes developed in this study (CL, PCL and pH-PCL) are novel since these drug carriers encapsulated and delivered two therapeutic macromolecules, D-$(KLAKLAK)_2$ and G3139, in a single formulation. The clinical

advantages of such a formulation include the enhanced anti-cancer therapy promoted by the combination of both macromolecules and suggest ways to decrease the number of separate medications that a cancer patient may have to take. Such a combination may also lead to an increase in treatment compliance.

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