Published in final edited form as:

J Control Release. 2011 August 10; 153(3): 233–239. doi:10.1016/j.jconrel.2011.04.004.

Preferential cellular uptake of amphiphilic macromolecule–lipid complexes with enhanced stability and biocompatibility

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Abstract

Amphiphilic macromolecules (AM) were electrostatically complexed with a 1:1 ratio of 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) to form AM–lipid complexes with drug delivery applications. The complexes exist as AM-coated liposomes and their drug delivery properties can be tuned by altering the AM–lipid weight ratio. The complexation and tuning are achieved in a simple, efficient, and scalable manner. The gradual increase in lipid ratios concurrently increased the zeta potential of the complexes, which directly correlates to increased cell uptake of the complexes *in vitro* with preferential uptake noted in BT-20 carcinoma cells versus normal fibroblasts. Increasing AM content increased complex steric stability in the presence of serum proteins and reduced the inherent cytotoxicity towards fibroblasts *in vitro*. AM–lipid complexes solubilized paclitaxel and showed drug-mediated, dose-dependent cytotoxicity towards target BT-20 cells *in vitro*. AM–lipid complexes make good candidates as drug delivery systems due to their tunable zeta potential, steric stability, inherently low cytotoxicity, and ability to load and deliver insoluble chemotherapeutic agents. Significantly, their preferential uptake in a carcinoma cell line over normal cells *in vitro* demonstrates a unique, passive targeting approach to delivery anti-cancer therapeutics.

Keywords

Liposomes; Amphiphilic macromolecules; Preferential cell uptake; Drug delivery; Paclitaxel

1. Introduction

Cationic lipids alone or in combination with neutral lipids have been used to form liposomes as delivery vehicles for therapeutic agents [1,2]. Specifically, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (Fig. 1, top) and N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) (Fig. 1 middle) form heterogeneous liposomes [1,3]. When used in therapeutic delivery vehicles, DOPE is considered a “helper” lipid due to its ability to destabilize endosomal membranes, facilitating the release of the drug delivery system into the cytoplasm [4]. DOTAP is utilized because it imparts an overall positive charge to the drug delivery system. While cationic liposomes have been extensively studied, their positive charge correlates with cytotoxicity [5–7]. Also, under physiological conditions, cationic liposomes exhibit poor stability due to their interaction with serum proteins inducing liposome flocculation, fusion, and aggregation [8].

Many research groups have combined amphiphilic block copolymers with liposomes to form heterogeneous complexes for drug delivery applications. Liposome structural stability [9,10] and steric stability [11–13] was increased with the addition of di-block or tri-block copolymers. This increase occurred both when the polymer participated in the bilayer formation and when the polymer was absorbed onto the pre-formed liposome [13]. Similarly, the addition of amphiphiles to liposomes formed complexes with increased cellular uptake versus the vesicles alone [14]. However, these reported systems did not manipulate the polymer–lipid ratio to optimize drug delivery properties such as stability and cytotoxicity. Also, the rates of cellular uptake in normal and carcinoma cell lines were not quantitative and directly compared.

In this work, we evaluate the impact of polymer–lipid ratios on stability and cytotoxicity, and directly compare cellular uptake in cancer and normal cells. The polymers are amphiphilic macromolecules (AMs) that self-assemble at low critical micelle concentrations to form micelles [15–22]. AMs are designed to be non-cytotoxic, non-immunogenic, biodegradable, and allow for the inclusion of biological functionalities for targeted drug delivery. Generally, these AMs consist of a mucic acid-modified hydrophobic domain and a methoxy-terminated poly(ethylene glycol) (PEG) hydrophilic domain (Fig. 1, bottom). The four hydroxyl groups of mucic acid are derivatized with twelve-carbon aliphatic chains, resulting in a multi-branched hydrophobic region efficient in self-assembly and capable of binding bioactive molecules [22]. The AMs are similar to conventional polymeric micelles, existing as thermodynamic aggregates with inherent thermodynamic instability [23]. However, unlike conventional polymeric micelles, they have high solution stability [21] and form significantly smaller micelles [22].

Amphiphilic macromolecule addition to the surface of cationic liposomes enables the PEG component of the AM to impart steric stability to the complexes. The gradual addition of negatively charged amphiphilic macromolecules will also gradually decrease the charge-mediated cytotoxicity associated with cationic liposomes. Varying the ratio of amphiphilic macromolecules to lipids mitigate the AM–lipid complex charge to optimize cellular uptake.

The goals of this study were to evaluate the ability of AM–lipid complexes to load and preferentially deliver water-insoluble drugs *in vitro*. Varying concentrations of AMs were added to preformed 1:1 DOPE:DOTAP liposomes to form complexes that were assessed for size, charge, serum stability, cell viability and uptake, and drug loading capability.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were obtained from Avanti Polar Lipid (Alabaster, AL). Poly(ethylene glycol) 5 kDa was purchased from Polysciences, Inc. (Warrington, PA) and dried by azeotropic distillation from toluene before use. Functionalized PEG, H₂N–PEG–FITC, was purchased from Laysan Bio, Inc. (Arab, AL) and used as received. Paclitaxel, Cremophor EL, and absolute ethanol were obtained from Sigma-Aldrich (St. Louis, MO). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer was obtained from MP Biomedicals, LLC (Aurora, OH). Primary human normal dermal human fibroblasts (NDHF) and human intraductal carcinoma cells (BT-20) were obtained from American Type Culture Collection (Manassas, VA). Non-functionalized AMs were prepared as previously described [22]. All other solvents and reagents, unless otherwise stated, were purchased from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich (St. Louis, MO) and used as received.

2.2. Polymer characterization

Proton nuclear magnetic resonance (^1H -NMR) spectra of the products were obtained using a Varian 400 MHz or 500 MHz spectrophotometer. Samples were dissolved in chloroform- d , with minimal dimethyl sulfoxide- d_6 if necessary, and with tetramethylsilane as an internal reference. Molecular weights (M_w) were determined using gel permeation chromatography (GPC) with respect to poly(ethylene glycol) standards (Sigma-Aldrich) on a Waters Styragel® HR 3 THF column (7.8×300 mm). The Waters LC system (Milford, MA) was equipped with a 2414 refractive index detector, a 1515 isocratic HPLC pump, and 717plus autosampler. An IBM ThinkCentre computer with Waters Breeze Version 3.30 software installed was used for collection and processing of data. Samples were prepared at a concentration of 10 mg/mL in tetrahydrofuran, filtered using 0.45 μm pore size nylon or polytetrafluoroethylene syringe filters (Fisher Scientific; Pittsburg, PA) and placed in sample vials to be injected into the system. Melting points were determined by differential scanning calorimetry on a TA DSC Q200. TA Universal Analysis 2000 software was used for data collection on a Dell Dimension 3000 computer. Samples (3–5 mg) were heated under dry nitrogen gas. Data were collected at heating and cooling rates of 10 $^{\circ}\text{C}/\text{min}$ with a two-cycle minimum.

2.3. Synthesis and characterization of AM-FITC

The AM-FITC synthesis is outlined in Fig. 2 and prepared as previously described [25]. In brief, the carboxylic acids of AM were activated with excess thionyl chloride at 90 $^{\circ}\text{C}$ overnight. Residual thionyl chloride was removed by rotary evaporation and the resulting yellow oil used immediately without further purification. The activated compound (0.33 g, 0.34 mmol) was dissolved in anhydrous CH_2Cl_2 (5 mL) under argon. Anhydrous pyridine (0.080 mL, 0.99 mmol) was then added. Subsequently, H_2N -PEG-FITC (0.35 g, 0.070 mmol) dissolved in anhydrous CH_2Cl_2 (5 mL) and anhydrous pyridine (0.080 mL, 0.99 mmol) was added to the reaction flask drop wise *via* syringe pump at a rate of 1.5 mL/h. The reaction was stirred under argon gas and light restrictive conditions for 24 h at room temperature. The solution was then acidified with 0.05 N HCl and the organic phase washed with 50:50 brine: H_2O (2×), dried over MgSO_4 , and concentrated to a yellow oil. Minimal CH_2Cl_2 was then added and product precipitated by the addition of 10-fold chilled diethyl ether. The solid was then collected by centrifugation and supernatant removed by decanting. Solid was dried under ambient atmosphere and light restrictive conditions for 12 h and under high vacuum for 12 h. Yield: 0.32 g, 77%. ^1H -NMR (CDCl_3): δ 7.08 (m, 3H, ArH), 6.88 (d, 1H, ArH), 6.75 (m, 3H, ArH), 6.60 (m, 4H, ArH), 3.62 (m, ~0.50 kH, CH_2O), 2.66 (m, 4H, CH_2), 2.33 (m, 4H, CH_2), 1.62 (m, 8H, CH_2), 1.26 (m, 58H, CH_2), 0.88 (t, 12H, CH_3) (see Supplemental Fig. A in supplemental material for NMR spectrum). T_m = 48 $^{\circ}\text{C}$. GPC: M_w : 6.3 kDa (see Supplemental Fig. B in supplemental material for GPC curve). PDI: 1.1.

2.4. Preparation of AM-lipid complexes

AM-lipid complexes were prepared as weight-to-weight mixtures of AM and total weight of DOPE:DOTAP (1:1) lipids. The AM-lipid weight ratios assayed were 0:1, 1:10, 1:5, 1:2, 1:1, 2:1, 5:1, 10:1 and 1:0.

Liposomes were produced by co-dispensing aliquots of lipid in chloroform into glass vials and solvent removed by rotary evaporation. The remaining film was dried overnight under vacuum at room temperature. The films were hydrated with 10 mM HEPES buffer and incubated on a rotary shaker overnight at room temperature. Materials were then processed through eight freeze-thaw cycles by immersion in a dry ice-methanol bath followed by immersion in a water bath at 60 $^{\circ}\text{C}$. Materials were then passed 27 times through a 100 nm filter using a mini-extruder (Avanti Polar Lipids). Separately, dried AM powder stock was hydrated in 10 mM HEPES. Concentrated aliquots of pre-formed SUV were added to

concentrated aliquots of AM in 10 mM HEPES and brought to desired volume. Samples were statically incubated for at least 1 h at 25 °C. Processed materials were stored at 4 °C until use. All samples were used within 4 to 7 days of production.

2.5. Paclitaxel in cremophor:ethanol formulation

Paclitaxel in methanol was dispensed in glass vials and the solvent removed by evaporation. To the vial was added a 1:1 mixture of Cremophor EL and absolute ethanol. The final concentration of paclitaxel in cremophor:ethanol (Cr:Eth) was 6 mg/mL. The reagent was further diluted in buffer as required.

2.6. Complex size and zeta potential by DLS

AM–lipid complexes were assayed for size and zeta potential by dynamic light scattering (DLS) using a NanoZS90 Instrument (Malvern Instruments, Malvern, UK). Three 1-mL samples were evaluated at a minimum of 1 mg/mL total AM–lipid complex in 10 mM HEPES at room temperature. Each sample was run three separate times with ten measurements per run. Values reported represent the average of the three samples.

2.7. AM–lipid complex stability in serum

100 μ L fetal bovine serum (FBS) was added to 900 μ L solutions of AM–lipid complexes in 10 mM HEPES to create a 10% FBS final concentration. The samples were then incubated on a rotary shaker at 37 °C for 7 days. AM–lipid complex size was evaluated by DLS at days 0, 4 and 7. Three 1-mL samples were evaluated. Each sample was run three times with ten measurements per run. Values reported represent the average of the three samples.

2.8. In vitro cell expansion

Normal dermal human fibroblasts (NDHF) were expanded in Dulbecco's Modified Eagle's Media (DMEM) (Gibco, Grand Island, NY) supplemented with 10% FBS (HyClone, Logan, UT) and 1% penicillin/streptomycin (P/S) (Gibco, Grand Island, NY). Human intraductal carcinoma BT-20 cells were expanded in Eagle's Minimum Essential Medium (ATCC, Manassas, VA) and supplemented with 10% FBS and 1% P/S. Prior to assays, NDHF and BT-20 cells were plated at 5.0×10^3 cells/cm² in T flasks (Corning, Corning, NY) and expanded to approximately 2.5×10^4 cells/cm². The cells were harvested with 1–3 mL of trypsin-EDTA (Gibco, Grand Island, NY) and counted using a Guava PCA Instrument (Guava Technologies, Hayward CA) as per manufacturer's instructions.

2.9. Complex cellular uptake monitored with AM-FITC

For these studies only, AM-FITC was used in place of AM. Harvested NDHF and BT-20 cells were seeded into 96 well plates (Corning, Corning, NY) at 2×10^4 cells/well (0.2 mL media per well) in DMEM supplemented with 10% FBS and 1% P/S and incubated overnight at 37 °C, in a humidified 5% CO₂ incubator (Thermo Electron Corp, Marietta, OH). The media was then removed by aspiration and replaced with 0.2 mL AM-FITC–lipid complex containing OptiMEM (Gibco, Grand Island, NY) supplemented with 1% FBS and 1% P/S ($n=4$ per complex concentration). Untreated control wells received vehicle control media. After 24 h, cells were observed by fluorescent microscopy. The cells were then harvested by trypsinization and quantitatively analyzed using a Guava EasyCyte Plus Instrument (Guava Technologies, Hayward CA) on the green fluorescence channel.

2.9.1. AM–lipid complex in vitro cytotoxicity—Harvested NDHF cells were seeded into 96 well plates (Corning, Corning, NY) at 5×10^3 cells per well (200 μ L media per well) in DMEM supplemented with 10% FBS and 1% P/S and incubated overnight at 37 °C, in a humidified 5% CO₂ incubator (Thermo Electron Corp, Marietta, OH). The media was

removed by aspiration and replaced with 200 μ L AM-lipid complex containing DMEM supplemented with 2% FBS and 1% P/S ($n=5$ per complex concentration). Untreated control wells received vehicle control media. After 24 h, the cells were counted. Briefly, cells were harvested by trypsinization (75 μ L trypsin-EDTA followed with 75 μ L complete media to neutralize trypsin) and 50 μ L of staining solution (48:1:1 media, DMSO, Guava ViaCount Flex reagent (Guava Technologies, Hayward, CA)) was added to each well. Cells were counted using a Guava EasyCyte Plus Instrument (Guava Technologies, Hayward CA) instrument with an original volume of 0.2 mL and a dilution factor of one.

2.9.2. Paclitaxel encapsulation—The material:drug weight ratio for all studies was defined as 30:1 [26]. Paclitaxel in chloroform was added with lipids prior to solvent evaporation. Immediately prior to freeze-thaw, free paclitaxel was removed by filtration through a 0.22 μ m PTFE syringe filter (Nalgene, Rochester, NY). The samples were then processed using the methods described earlier. Concentrated pre-formed paclitaxel-loaded SUV was added to concentrated pre-formed micelles in 10 mM HEPES and brought to volume. Samples were statically incubated for at least 1 h at 25 $^{\circ}$ C. Paclitaxel encapsulation efficiency was evaluated by diluting an aliquot of paclitaxel loaded AM-lipid complexes 1:10 in methanol. The diluted sample was then assayed by UV/Vis spectroscopy. Scans from wavelength 200–400 nm were performed using material only samples as reference standards. The absorption at 230 nm was recorded and used to calculate drug concentration using a previously generated calibration curve ($R^2=99.0\%$).

2.9.3. Paclitaxel loaded AM-lipid complex in vitro cytotoxicity—Harvested BT-20 cells were seeded into 96 well plates (Corning, Corning, NY) at 2×10^4 cells per well (200 μ L media per well) in EMEM supplemented with 10% FBS and 1% P/S and incubated overnight at 37 $^{\circ}$ C, in a humidified 5% CO₂ incubator (Thermo Electron Corp, Marietta, OH). The media was removed by aspiration and replaced with 200 μ L paclitaxel loaded or unloaded AM-lipid complex-containing media ($n=4$ per complex concentration). Untreated control wells received vehicle control media. At 72 h, cells were counted. Briefly, cells were harvested by trypsinization (75 μ L trypsin-EDTA followed with 75 μ L complete media to neutralize trypsin) and 50 μ L of staining solution (48:1:1 media, DMSO, Guava ViaCount Flex reagent) was added to each well. Cells were counted using a Guava EasyCyte Plus instrument with an original volume of 0.2 mL and a dilution factor of one. Percent viable cells were calculated as number of viable treated cells versus number of viable untreated control cells.

2.9.4. Data calculations and representation—All data were reported as the mean plus or minus the standard deviation. All calculations were performed using Microsoft Excel for Windows software.

3. Results and discussion

3.1. AM-lipid complex formation and stability

AMs were combined with a 1:1 ratio of DOPE:DOTAP liposomes to form tunable AM-lipid complexes. DOPE:DOTAP has previously shown to be effective in the delivery of nucleic acids [3]. However, this formulation of cationic and neutral lipids has not been extensively evaluated for the solubilization and delivery of insoluble anti-cancer drugs. A range of complexes were prepared by varying the weight ratio of AM to lipids, ranging from predominately AM complexes to predominately lipid complexes. To maximize the observed effect of varying the weight ratio of the AM-lipids, weight ratios ranges spanning one log order from predominately one component were used. Because the complexation process

occurs relatively quickly, at room temperature and in aqueous media, variable complex structures are achieved in a simple, efficient, and scalable manner.

AM–lipid complex size was assayed by DLS. Fig. 3 shows that the AM alone values (~20 nm) were consistent with previous data [22] and the liposome alone values (~100 nm) were consistent with the 100 nm filter used in the extrusion process. DLS histograms for all samples produced showed a single peak curve with low standard deviation (data not shown) indicating a distinct, single structure. Upon addition to the liposome suspension, the negatively charged AM micelles are electrostatically attracted to the positively charged cationic liposomes. When micelle solutions above their CMC are diluted into suspensions of liposomes, the micelle monomers (acting as surfactants) will partition into the lipid bi-layer membrane forming a mixed lipid–surfactant bilayers [27]. This partitioning leads to an increase in the mean vesicle size [28]. Although, the observed increase in complex size with increasing AM concentration infers an AM-coated liposome, a bilayer structure could not be confirmed by TEM (Supplemental Fig. C).

Fig. 4 shows the effect of serum protein on AM–lipid complex size over time. AM–lipid aggregate formation was immediate and visually observed upon serum addition to the complexes with low AM concentrations. These aggregates were not observed in complexes with high AM concentrations. The gradual addition of AM to the liposome protected the structures from serum-induced aggregation over time. Non-serum treated AM–lipid complexes also showed no significant increase in size over the same time period (data not shown). Overall, AM–lipid complexes demonstrated excellent stability in serum for one week.

3.2. Influence of AM on liposomal biocompatibility and cellular uptake

The positive charge of cationic liposomes typically correlates with cytotoxicity [5–7] mediated by the generation of reactive oxygen intermediates, toxic oxidative bursts [7], and a disruption of cellular and sub-cellular membrane functions [6]. To evaluate cytotoxicity, AM–lipid complexes were cultured with NDHFs for 24 h. As shown in Fig. 5, the viability of DOPE:DOTAP-treated fibroblasts was less than 50% of the viable untreated control cells after 24 h of culture. Increasing concentrations of AM in the complexes showed increased cell viability. Some AM–lipid complex treated cells showed a viability that was above 100% versus the untreated controls. Micelle-induced increases in proliferation previously occurred with AM [15] as well as other micelle forming block co-polymers materials [29].

The attraction and binding of drug delivery vehicles to target cell surfaces are mediated largely by nonspecific ionic interactions [4,30], where increased cytotoxicity towards carcinoma cells correlates to increases in surface charge [4]. Therefore, the correlation of AM–lipid complex charge and cellular uptake was carefully evaluated. Higher AM concentrations in AM–lipid complexes decrease the zeta potential in a highly controlled fashion (Fig. 6). To investigate the role of zeta potential in cellular uptake, a fluorescently tagged AM (i.e., AM-FITC) was included in the AM–lipid complexes. BT-20 and NDHFs were both treated with the AM-FITC–lipid complexes and quantitatively analyzed for fluorescence. Fluorescent microscopy revealed the peri-nuclear localization of the AM-FITC within the cytoplasm (data not shown). For both cell types, increased AM–lipid complex uptake correlated with increased lipid concentrations, as noted by increased mean fluorescence (Fig. 7). At any given AM–lipid complex concentration, cell uptake was greater in the BT-20 carcinoma cells than the normal fibroblast cells. While the mechanism of cell uptake is unknown, others have reported endocytosis as the major mechanism for the uptake of cationic nanoparticles [30]. Further studies will need to be conducted to determine the exact mechanism of AM–lipid complex cell uptake.

Previous investigators used flow cytometry assays to quantitatively demonstrate the uptake of drug delivery systems into target carcinoma cells *in vitro* [31,32]. Other investigators used active targeting motifs to mediate preferential uptake of a drug delivery system in heterogeneous *in vitro* carcinoma cultures [33]. Our study directly and quantitatively compared the uptake of the AM–lipid complex in carcinoma and normal cell lines. The results infer a preferential uptake of AM–lipid complexes in the carcinoma cell line tested and indicate a unique passive cell targeting system. Studies with additional carcinoma and normal cell types are required to further investigate the mechanism of action.

3.3. AM–lipid complex drug loading and delivery

Lastly, AM–lipid complexes were evaluated as delivery systems for the anticancer drug paclitaxel. Paclitaxel is highly hydrophobic and poorly soluble in water [34,35]. Clinically, paclitaxel is formulated in a 1:1 mixture with Cremophor® EL and absolute ethanol as Taxol™. However, serious side effects are associated with Taxol™ including hypersensitivity, neurotoxicity, and nephrotoxicity; these side effects are mostly attributed to the Cremophor® EL component [36] and mediate Taxol's maximum tolerated dose. The development of delivery systems for paclitaxel remains an active field of study [37,38] with many systems utilizing nanoparticles composed of mixed materials to enhance the loading and delivery of paclitaxel [39–43].

The encapsulation efficiency and loading of paclitaxel into DOPE: DOTAP liposomes averaged 93.7% (S.D. 4.5%) and 6.2% (S.D. 0.3%), respectively. The high encapsulation efficiency demonstrates the ability of DOPE:DOTAP to effectively solubilize insoluble therapeutics, such as paclitaxel. At all AM–lipid ratios, the addition of AM to the paclitaxel-loaded liposomes did not significantly affect the encapsulation efficiency of the drug within the complex as compared to the paclitaxel-loaded liposome alone (*t*-test, $p > 0.05$ for all ratios). The encapsulation efficiencies for all AM–lipid ratios ranged from 88.3 to 95.2% with analysis of variance showing no significant difference between the encapsulation efficiencies of all AM–lipid ratios (one-way ANOVA, $p = 0.75$) (see Supplemental Fig. D). AM addition to paclitaxel-loaded liposomes at all ratios significantly lowers the loading efficiency as compared to the paclitaxel-loaded liposome alone (*t*-test, $p < 0.01$ for all ratios). Drug loading ranged from 2.8 to 0.28% with increasing AM content correlating with decreased loading (see Supplemental Fig. E). These results are consistent and expected—adding AM to the lipids increases the overall mass of drug carrier whereas the drug mass remains constant.

The human intraductal carcinoma cell line BT-20 was used as a representative breast cancer target cell for *in vitro* cytotoxicity studies. As representative data, Fig. 8 shows the dose-dependent effect of paclitaxel-loaded AM–lipid complexes at a 1:1 AM–lipid weight ratio on BT-20 cell viability as compared to paclitaxel-loaded liposomes alone (i.e., no AM present) and paclitaxel in a cremophor:ethanol (Cr: Eth) formulation similar to Taxol™. 1:1 AM–lipid complexes represent the minimal amount of AM required to eliminate liposome cytotoxicity and instability in serum proteins while maintaining a high level of cellular uptake in BT-20 cells. The PTX concentration range evaluated is similar to paclitaxel's maximum plasma concentration during the clinical administration of Taxol™ [44]. At PTX concentrations of 10^{-3} mM to 5×10^{-5} mM, the cytotoxicity of PTX-loaded AM–lipid complexes (Fig. 8, top) was significantly higher than corresponding material alone samples (Fig. 8, bottom), inferring the cytotoxicity observed is mediated by the paclitaxel (*t*-test, $p < 0.01$). The cytotoxicity of the drug-loaded AM–lipid was not significantly different from a Taxol™-like formulation at all concentrations (*t*-test, $p > 0.5$) except for the 5×10^{-5} mM PTX sample, which was significantly lower (*t*-test, $p < 0.05$). Drug-loaded liposomes at all drug concentrations were also not significantly different from drug loaded AM–lipid (*t*-test, $p > 0.5$) except for 5×10^{-5} mM PTX (*t*-test, $p < 0.01$). This result infers that AM addition did

not interfere with the efficacy of the drug-loaded liposome. All other AM–lipid complex weight ratios, when loaded with PTX, showed a similar dose-dependent and drug-mediated cytotoxicity with samples from 10^{-3} to 10^{-5} mM PTX having significantly higher cytotoxicity than corresponding material alone as controls (*t*-test, $p < 0.05$ for AM–lipid ratios and all concentrations) (data not shown). To date, the mechanisms of paclitaxel release is unknown; the components of AM–lipid complexes may mediate or enhance the intracellular release of paclitaxel. The role of AM–lipid complex components, including the possible role of DOPE in endosomal disruption, should be further investigated to further optimize AM–lipid complexes for improved intracellular drug release. Additionally, the stability of PTX loaded AM–lipid complexes and the PTX release profile from AM–lipid complexes has been completed and will be the topic of a future manuscript.

4. Conclusions

AMs combined with DOPE:DOTAP create AM-coated liposome complexes with a range of properties desirable for drug delivery applications. The AM–lipid complexation is achieved in a simple, efficient, and scalable manner. With the AM–lipid complexes, we demonstrated that the incorporation of AM into DOPE:DOTAP liposomes i) does not significantly influence the liposome size, ii) correlates to enhanced cellular uptake, iii) increases uptake in carcinoma cells relative to normal cells, iv) improves serum stability and v) enables drug loading. Further, paclitaxel-loaded AM–lipid complexes exhibit cytotoxicity *in vitro* towards target carcinoma cell lines in a dose-dependent manner. AM–lipid complexes show promise for delivery of anticancer drugs, as their preferential uptake in a carcinoma cell line demonstrates a passive targeting mechanism that is highly effective *in vitro*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Bryan Langowski, Ph.D. and Sarah Hehir, Ph.D. for their critical review for the article and Anna Gosiewska, Ph.D. at Advanced Technologies and Regenerative Medicine, LLC (Johnson & Johnson, Inc.) for access to instruments and facilities.

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Fig. 1. (Top) 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) [24], (middle) 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) [24], (bottom) mucic acid-based AM [21].

Fig. 2.
Synthesis and structure of AM alone and conversion into fluorescently labeled AM-FITC.

Fig. 3. AM-lipid complex in 10 mM HEPES buffer as assayed for size by DLS within 2–4 h after production. Similar results are seen for complexes assayed up to 7 days after production. Lipid content for all samples was fixed at 5 mg/mL.

Fig. 4. AM-lipid complex sizes in 10% FBS for up to 7 days at 37 °C as assayed by DLS. Lipid content for all samples was fixed at 1 mg/mL.

Fig. 5. Percent of viable NDHF treated for 24 h with AM-lipid complexes vs. untreated cells. All lipid concentrations were fixed at 0.1 mg/mL. CE—co-evaporation, PA—post-addition.

Fig. 6. AM-lipid complex zeta potential in 10 mM HEPES as assayed by DLS. Lipid content for all samples was fixed at 5 mg/mL.

Fig. 7.

Mean fluorescence of BT-20 and NDHF treated for 24 h with FITC-labeled AM-lipid complexes as measured by Guava 96.

Fig. 8.

Top—percent of viable BT-20 treated for 72 h with paclitaxel loaded 1:1 AM-lipids vs. untreated cells. Bottom—percent of viable BT-20 treated for 72 h with vehicle alone controls vs. untreated cells.