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pH-Responsive Artemisinin Dimer in Lipid Nanoparticles Are Effective Against Human Breast Cancer in a Xenograft Model

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Abstract

Artemisinin (ART), a well-known antimalaria drug, also exhibits anticancer activities. We previously reported a group of novel dimeric artemisinin piperazine conjugates (ADPs) possessing pH-dependent aqueous solubility and a proof-of-concept lipid nanoparticle formulation based on natural egg phosphatidylcholine (EPC). EPC may induce allergic reactions in individuals sensitive to egg products. Therefore, the goal of this report is to develop ADP-synthetic lipid particles suitable for *in vivo* evaluation. We found that ADP binds to 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) with greater than 90% efficiency and forms drug-lipid particles ($d \sim 80$ nm). Cryo-electron microscopy of the ADP drug-lipid particles revealed unilamellar vesicle-like structures. Detailed characterization studies show insertion of the ADP lead compound, ADP109, into the DPPC membrane and the presence of an aqueous core. Over 50% of the ADP109 was released in 48 hours at pH4 compared with less than 20% at neutral. ADP109-lipid particles exhibited high potency against human breast cancer, but was tolerated well by nontumorigenic cells. In MDA-MB-231 mouse xenograft model, lipid-bound ADP109 particles were more effective than paclitaxel in controlling tumor growth. Cellular uptake studies showed endocytosis of the nanoparticles and release of core-trapped marker throughout the cytosol at 37°C. These results demonstrate, for the first time, the *in vivo* feasibility of lipid-bound ART dimer for cancer chemotherapy.

Keywords

liposome; cancer chemotherapy; nanoparticle; cancer; glass transition; pH responsive delivery system; lipid-drug interaction; membrane integrity

INTRODUCTION

Artemisia annua L., an annual plant first documented in the Wushi'er Bingfang of ancient China in 200 BC, has long been used to treat fever and malaria-associated symptoms with a proven safety profile.^{1,2} The plant's active agent, artemisinin (ART), was identified to be effective against malaria in the early 1970s.^{3,4} Since the late 1980s, numerous studies have

suggested antiproliferative, antiangiogenic, and anti-inflammatory properties of ART-derived compounds.^{5–7} Among the ART derivatives, dimeric ART linked at the C10 position is more potent than their monomeric analogues against various types of human cancer cell lines.^{8,9} ART dimer succinate, for example, is over 100-fold more potent than its monomer analogue, Artesunate, against a panel of human breast cancer cell lines (mean Log concentration required to inhibit maximal growth by 50% of -7.01^{10} and -4.7^{11} respectively). Another major advantage of ART-based chemotherapeutics is their safety. Lai and Singh¹² reported that rats could be dosed with 8 mg/kg per day of ART mixed in food for 40 weeks without any adverse effects. Although a number of ART dimer derivatives are active in rodent xenograft models,^{13–16} limited solubility and bioavailability pose challenges for this class of hydrophobic compounds for clinical development, as organic cosolvents such as dimethylsulfoxide (DMSO) are not suitable for use in parenteral dosage forms. Association of ART to nanoparticles or microparticles has been shown to improve *in vitro* solubility and *in vivo* pharmacokinetic profiles.^{17–20}

Liposomes and lipid nanoparticles, now commonly employed in clinical applications, have been proven to alter the pharmacokinetics and tissue distribution of active compounds.²¹ For example, Doxil[®], a liposome formulation of the chemotherapeutic compound doxorubicin, showed reduced cardiotoxicity and uncompromised efficacy, thus widening the therapeutic window compared with the free drug.^{21–24} An ideal drug delivery system should stably retain the drug molecules under conditions where prolonged circulation or accumulation is desired and release the loaded drug in response to a stimulus. Responsiveness to environmental pH is one feasible intracellular trigger for drug release, such as in late-stage endosome ($\sim$ pH 5) or lysosome (as low as $\sim$ pH 4)²⁵ of the cell.^{26–28} Although aqueous-soluble drugs can be successfully released, hydrophobic compounds incorporated in the bilayer have demonstrated little evidence suggesting comparable release from particles.²⁹

Previously, we reported a proof-of-concept study where the pH-responsive ART dimer, artemisinin dimer piper-azine conjugate (ADP109) (Scheme 1), was bound to egg phosphatidylcholine (EPC) liposome and the drug–lipid nanoparticles demonstrated equally high potency as the free drug in cell culture.³⁰ Although EPC is useful in initial *in vitro* studies, it has a number of limitations for clinical use. Being a mixture of phospholipids extracted from natural sources, EPC may have variation in fatty acyl chain compositions among batches, increasing the risk of poor reproducibility. In addition, patients who are allergic to egg products may be at risk of an immune response to the EPC. Thus, despite the gaining clinical use of liposomal drug therapeutics,³¹ few contain natural EPC lipids.³²

The goal of this study, thus, is to characterize the ADP109–lipid interactions and formulate nanoparticles using lipids with well-defined, saturated fatty acid chains for preclinical development. The ADP109–lipid particles in this study are based on a more robust synthetic lipid with saturated fatty acid chains [1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)] and a polyethylene glycol shell. The pH-responsive loading and release mechanisms are examined and the drug–lipid interactions are delineated to understand the effect of incorporating bulky hydrophobic compounds on membrane molecular packing. Antibreast cancer activity, evaluated both in cell culture and xenograft model, indicates enhanced selectivity and potency of the pH-responsive ART dimer liposome nanoparticles.

MATERIALS AND METHODS

Materials

Lipids DPPC and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] sodium salt (mPEG₂₀₀₀-DPPE) were purchased from Genzyme (Cambridge, Massachusetts) in powder form. Chloroform (hazardous volatile solvent, handle in ventilated hood) was dried with molecular sieves for at least 24 h before use. Calcein was purchased from TCI America (Portland, Oregon), and 1,6-dipheyl-1,3,5-hexatriene (DPH) was bought from Life Technologies (Formerly Invitrogen, Carlsbad, California). Thiazolyl blue tetrazolium bromide, (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (MTT), powder was purchased from Sigma–Aldrich (St. Louis, Missouri). Commercially prepared 1× phosphate-buffered saline (PBS) solution was obtained from Thermo Scientific (Waltham, Massachusetts). Glow-discharged Quantifoil R-2/2 grid (300 mesh) for electron microscopy (EM) imaging was purchased from Electron Microscopy Sciences (Hatfield, Pennsylvania). Mice experiments were conducted in the facility of Washington Biotechnology Inc. (Baltimore, Maryland). Animal protocol was reviewed and approved by the company's IACUC, approval number 12-002.

Preparation and Size Characterization of DPPC-Based Liposome (NP209) of Artemisinin Dimer Pyridylmethylpiperazine (ADP109)

The general procedure followed that of the previously published protocol.³³ ADP109 was dissolved in chloroform to make a 50-mg/mL stock solution. The DPPC and mPEG₂₀₀₀-DPPE were dissolved in chloroform at the concentration of 100 mg/mL. Aliquots of ADP109, DPPC, and mPEG₂₀₀₀-DPPE stock solutions were added to a screw-capped glass test tube. Organic solvent was removed under a gentle stream of nitrogen followed by further drying *in vacuo* overnight to form a thin film on the inner wall. The film was rehydrated with 1× PBS at approximately 47°C for 1–3 h, depending on concentration, to give a liposome suspension at desired lipid concentration (20–200 mM). The mixture was then sonicated at 45°C for 5 min three times to give a translucent suspension without observable particles and then gradually cooled down to well below the lipid phase transition temperature (4°C), to afford the desired nanometer-sized liposomes. NP209 was diluted to 2 mM for sizing measurements on a Zetasizer Nano ZS (Malvern Instrument, Worcestershire, UK) with argon laser ($\lambda = 633.0$ nm) at room temperature. Sizing data were reported as the average ($\pm$ SD) of five measurements with 13 runs per measurement.

Cryo-EM Characterization of NP209

To prepare NP209 nanoparticle samples for cryo-EM imaging, 4 μ L of 1 mM liposome suspension were applied on a glow-discharged Quantifoil R-2/2 grid (300 mesh; Electron Microscopy Sciences). The sample was blotted by a piece of filter paper to remove excess solution, and then plunged into liquid ethane, which was cooled by liquid nitrogen. Cryo-EM samples were stored in liquid nitrogen till observation. Cryo-EM images were recorded on an Eagle 4k×4k camera (FEI Co., Hillsboro, Oregon) using a Tecnai G2 F20 microscope (FEI) at 200 kV with a 20- μ m objective aperture at -180°C . The electron dose for each exposure was around 2000 e^{-}/nm^2 . Cryo-EM images were taken at -4 μ m defocus, and the effective pixel size was 0.44 nm. The size of nanoparticles was analyzed using ImageJ.³⁴

Effects of ADP109 on Membrane Phase Transition Behavior

NP209 was diluted to 1 mM in 1× PBS and warmed to 60°C for 5 min. Stock solution of DPH, was prepared at 2 mM in tetrahydrofuran. 0.05% volume of the DPH stock was mixed with the liposome suspensions, vortexed briefly, incubated at 60°C for 15 min in the dark, then cooled down to room temperature (21°C–24°C) for fluorescence polarization measurements on a Hitachi F4500 fluorescence spectrophotometer (Tokyo, Japan). A circulating water bath (Fischer Scientific model 800; Pittsburgh, Pennsylvania) maintained stable temperature during measurements. A digital thermo probe (Fischer Scientific) was used to monitor the exact temperature within the cuvette and ensure stability of temperature while the liposome sample was heated. Fluorescence measurements were taken as an average of 10 s of continuous reading. Triplicate measurements in each direction of emission polarization: 0° ($I_{\parallel}$) and 90° ($I_{\perp}$) with respect to 0° excitation, were measured with liposomes in 1× PBS. Curve fitting was based on average and SD calculated from triplicate readings. The fluorescence anisotropy was calculated using Eq. 1 below:

$$\text{Fluorescence anisotropy} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

ADP109 Incorporation into Liposomes and pH-Responsive Release Efficiencies

For loading efficiency studies, liposome suspensions in original concentrations were placed into dialysis tubing (MWCO 6000–8000 Da; Spectrum Labs, Rancho Dominguez, California) and dialyzed against at least 1000× volume of 1× PBS buffer overnight at room temperature. Aliquots × (30 µL) of both dialyzed and undialyzed samples were collected in Eppendorf tubes, crashed with 300 µL of cold acetonitrile, vortexed for 30 s, and centrifuged at 18,000 *g* for 5 min. The supernatant of the centrifuged samples was collected for UV absorbance measurement from 200 to 400 nm or DU 640 spectrophotometer (Beckman Coulter, Indianapolis, Indiana).

The loading efficiency was calculated according to the following equation (Eq. 2) below:

$$\text{Loading efficiency (\%)} = \frac{A_{263}(\text{D})}{A_{263}(\text{UD})} \times 100\%$$

where $A_{263}(\text{D})$ is the absorbance at 263 nm of the dialyzed sample, and $A_{263}(\text{UD})$ is that of the undialyzed sample.

For the release efficiency studies, aliquots of 300 µL of 20 mM dialysis-purified liposome suspensions were dialyzed against 500 mL of pH 7.4 PBS, pH 6 citrate, or pH 4 citrate buffers at ambient temperature. Aliquots (30 µL) were removed from each dialysis at times $t = 0.5, 2, 6, 24$, and 48 h for UV studies with the same workup procedure as that of the loading efficiency studies. Values were plotted as the average ($\pm$ SD) of three independent experiments.

The release efficiency was calculated according to Eq. 3 below:

$$\text{Release efficiency (\%)} = 100\% - \left(\frac{A_{263}(\text{pH})}{A_{263}(\text{D})} \times 100\% \right)$$

where $A_{263}(\text{pH})$ is the absorbance at 263 nm of the sample dialyzed at indicated pH, and $A_{263}(\text{D})$ is that of the initial dialysis-purified sample.

Encapsulation of Calcein in NP209 Nanoparticles

Thin film of lipids with or without ADP109 was generated as described above in the preparation of NP209 lipid–drug particles. Aqueous calcein stock was prepared at 200 mM in double-deionized and 0.2 μm filtered water with pH adjusted to 7.8. The dried thin film was rehydrated with 50 mM calcein (diluted from stock with 2 $\times$ PBS) for 1 h at 47°C and sonicated three times at 45°C for 5 min each to yield a translucent suspension of nanoparticles at 20 mM lipid concentration. The suspension was gradually cooled to 25°C over duration of at least 2 h, allowing membranes to anneal as described above. The calcein-trapped liposomes were then separated from the free dye on a Sephadex G50 gel column with 2 $\times$ PBS of matching osmolality, and stored at 4°C until use.

pH-Dependent Release of Encapsulated Aqueous Marker Calcein from NP209 Nanoparticles

In 96-well plates, calcein-trapped NP209 was diluted four times with 2 $\times$ PBS at indicated pH for pH-dependent fluorescence regeneration at 37°C. Positive control was generated by dilution with 2 $\times$ PBS in the presence of 0.15% sodium deoxycholic acid (DOC) for complete calcein release from aqueous core. All buffers used were tested for matching osmolality to prevent particle burst as a result of osmotic pressure. The fluorescence of calcein was measured using a Victor²D fluorometer (Perkin Elmer, Waltham, Massachusetts) equipped with excitation and emission filter wavelengths of 495 and 515 nm, respectively. The quenching efficiency was calculated according to Eq. 4 below:

$$\text{Percent quenching (\%)} = \left(1 - \frac{F(\text{NP})}{F(\text{DOC})} \right) \times 100$$

where $F(\text{NP})$ is the fluorescence intensity of NP209 and $F(\text{DOC})$ is that of NP209 in 0.15% DOC.

The pH-dependent fluorescence regeneration was calculated according to Eq. 5 below:

$$\text{Fluorescence regeneration (\%)} = \frac{F_{\text{NP}}(\text{pH})}{F_{\text{NP}}(\text{DOC})} \times \frac{F_{\text{Cal}}(\text{DOC})}{F_{\text{Cal}}(\text{pH})} \times 100$$

where $F_{\text{NP}}(\text{pH})$ is the fluorescence intensity of NP209 at various pH, $F_{\text{NP}}(\text{DOC})$ is that of NP209 in 0.15% DOC, $F_{\text{Cal}}(\text{pH})$ is the fluorescence intensity of free calcein at various pH, and $F_{\text{Cal}}(\text{DOC})$ is that of free calcein in 0.15% DOC.

A calcein fluorescence-concentration standard curve was generated to define self-quenching threshold, and linear range was defined below the self-quenching concentration to estimate calcein concentrations trapped inside particles and to ensure that data points collected fell within the linear range. Each fluorescence regeneration data point was performed as triplicates and values were reported as the average $\pm$ SD.

Effects of Lipid Association on ADP109 Potency Against Human Breast Cancer Cells

In 96-well plates were seeded approximately 5000 cells/well of BT474 or MDA-MB-231 cells. The cells were incubated for 20–30 hours until fully adhered. The incubation condition was a humid chamber at 37°C with 5% CO₂ supply, and the culture medium was Dulbecco's modified Eagle's medium (high glucose, with L-glutamine) containing 10% fetal bovine serum (complete medium). For MCF10A, 15,000 cells/well were seeded. MCF10A culture medium and assay medium were prepared according to literature.³⁵ Serial dilutions of ADP109 stock solution at 20 mM in DMSO were made to eight appropriate concentrations ranging from 1 to 100nM, in complete medium for cancerous cell lines or assay medium for MCF10A, with 1% DMSO. Two-hundred microliter of the compound-containing medium was added to each well after removal of the seeding medium. Sterile dialysis-purified NP209 was diluted in complete medium or assay medium assuming 100% loading. Three wells were run in parallel for any given compound and concentration in one experiment. The cells were incubated with the drugs for 48 h at 37°C before the medium was replaced with 90 μ L of fresh complete medium plus 10 μ L of MTT solution at 5 mg/mL and incubated further for 4 h. At the end of incubation time, exhausted medium was gently removed. The purple formazan crystals (MTT) were dissolved in 50 μ L of DMSO, and incubated for 10 min before the absorbance at 570 nm was read on Microplate Reader model 680 (Bio-Rad, Berkeley, California). Concentration required to inhibit cell proliferation by 50% versus control (IC₅₀) values were defined as drug concentration that resulted in 50% viability of cells compared with medium control (with or without 1% DMSO), and three independent experiments were performed to calculate the reported average IC₅₀.

Effects of NP209 Liposomes on Human Breast Cancer Mice Xenograft Model

Animal experiments were carried out by Washington Biotechnology, Inc. under IACUC approved protocol. Maximum tolerated dose (MTD) study was carried out on healthy 4–6-week-old female Swiss-Webster mice. Dose range study of single injections, chronic dosing, and intermittent dosing studies were carried out with five mice in each group with subcutaneous injections. Body weight and clinical adverse reactions (piloerection, signs of distress) were recorded to determine the dose regimen for the efficacy study.

In the single dose efficacy study, 30 female athymic nude mice (Harlan Sprague–Dawley, Inc. Frederick, Maryland), 5–6-week old, were implanted with 1×10^6 MDA-MB-231 cells (passage 24) in 20% Matrigel (BD Biosciences, San Jose, California) subcutaneously in the right flank, and left to develop solid tumor for 10 days (therapy day –10 to day 0). Animals were grouped into three groups: blank NP209 vehicle, NP209, and paclitaxel control. Starting from day 0, mice were injected with 40 mg/kg drug of NP209 or lipid equivalence of blank NP209 subcutaneously every other day for 2 weeks, or 10 mg/kg paclitaxel (Hospira, Lake Forest, Illinois) intravenously every day until day 4. All mice were weighed

every Monday, Wednesday, and Friday, and tumor sizes were estimated by calculating tumor volume according to Eq. 6 below.

$$\text{Tumor volume} = \text{length} \times \text{width}^2 \times \frac{1}{2}$$

Statistical comparison was evaluated by two-tail Student's *t*-test. Data were presented as mean [$\pm$ SEM (standard error of the mean)] of seven animals in each group. Extra animals were accounted for in each group in consideration of outliers and anomalies.

Cellular Uptake Study of NP209

For cellular uptake studies, sterile calcein-trapped NP209 liposomes were prepared and purified as described above in 1 $\times$ PBS. Osmolality of the nanoparticle suspension was tested on a vapor pressure osmometer (model 5520; Wescor Vapor[®], Princeton, New Jersey) to ensure biocompatibility. 1.5×10^5 cells per well of BT474 cells were seeded into 12-well plates. The cells were incubated for 24 hours at 37°C in a humidified atmosphere containing 5% CO₂ until fully adhered. On the day of imaging experiment, complete medium was replaced by 0.5 mL of blank PBS (Blank), 1 mM calcein-trapping NP209, or 0.4 μ M free calcein in PBS. Two time points for NP209 up-take were studied in different wells with varying start time so that the end of incubation time coincided. At the end of the incubation period, cells were washed three times with 1 mL PBS each, and imaged live with 0.5 mL PBS in each well. All images were taken on a Carl Zeiss Axiovert 200M inverted microscope equipped with XBO 75 light source and an AxioCam MRm Camera, using a FITC/GFP filter cube (EX/BP 485/20) and Plan Neofluar 40X/0.75 D objective (Göttingen, Germany). Image analysis was performed with AxioVision V4.8 software. Image settings, exposure levels, brightness, and contrast were held constant for all samples incubated under the same temperature for qualitative comparison.

RESULTS AND DISCUSSIONS

The feasibility of substituting egg derived phosphatidylcholine with a well-defined synthetic lipid, DPPC, was validated by preparing ADP109-bound liposome composed of 95% DPPC and 5% polyethylene glycol-2000 (PEG₂₀₀₀) at the same drug-to-lipid (D-L) ratio as that of the EPC-liposomes for comparison of drug association efficiency and particle size (Table 1). PEG₂₀₀₀ was included at 5% (mole) to provide higher degree of hydration intended for *in vivo* stealth (reduced nonspecific uptake by phagocytes) effect.^{36–38} Our data in Table 1 demonstrate that replacement of EPC with DPPC has no impact on percent drug loading or particle diameter. Therefore, all studies were performed with DPPC-based composition below.

ADP109 Liposome Nanoparticle Characterization and Optimization

ADP109-bound lipid nanoparticles, NP209, were prepared by thin-film-rehydration method and particle size was reduced by bath sonication.³³ Particles with varied D-L ratio were prepared to study the loading capacity of bulky hydrophobic ART dimers in liposomes and drug-lipid binding interactions. ART dimer loading was optimum at D-L ratio of 1:10.

Insoluble ADP109 precipitated when the D–L ratio was 1:3 or 1:5. When the D–L ratio decreased to 1:7, no precipitates were observed but the efficiency of drug incorporation was only $50 \pm 3\%$. These data suggest that there exists an upper limit to the amount of ADP109 that is able to bind to the lipid membrane, either interacting with the surface of or incorporated into the hydrophobic membrane. Thus, NP209 was prepared at D–L ratio of 1:10 for further characterizations to most efficiently load the drug into the lipid particles that are under 100 nm in diameter.

Physical properties such as size, drug loading efficiency, and aqueous core presence were characterized for the ART dimer (ADP109)–lipid nanoparticle. At 1:10 ratio of ADP109–total lipid, NP209 sized at 76 ± 10 nm in diameter with polydispersity index of 0.205 ± 0.015 . The drug loading efficiency was $90 \pm 6\%$ at lipid concentrations between 20 and 200 mM, with a maximum concentration of 15.6 mg of ADP109 dissolved per milliliter with 200 mM lipid in PBS (Table 1). The existence of an aqueous core within NP209 was confirmed by trapping an aqueous soluble fluorescent marker, calcein. High concentrations of calcein result in self-quenching phenomenon. When 50 mM of calcein is stably encapsulated in the aqueous core of liposome particles, fluorescence is reduced because of self-quenching; when the core is unstable or the liposome is lysed, calcein escapes from the aqueous core and the concentration is diluted, resulting in regeneration of fluorescence. In NP209, calcein showed $98.5(\pm 0.5)\%$ quenching, indicating a stable aqueous core capable of trapping small water-soluble marker at a ADP109–lipid ratio of 1:10.

Morphological characterization by cryo-EM showed spherical nanoparticles with an average particle size at 55.9 nm ($n = 468$). Comparison between 0° - and 45° -tilted imaging proved that the ADP109-bound liposome particles are three-dimensional spheres. The NP209 cryo-EM images are reflective of literature published unilamellar vesicles³⁹ (Fig. 1), suggesting that these NP209 nanoparticles exist as liposome-like structures with a single bilayer enclosing an aqueous core. Membrane inserted ADP109 at 10 mol % does not seem to significantly impact the formation of these DPPC-based unilamellar vesicles.

Effect of ADP109 Binding on Lipid Membrane Phase Transition Behavior

Although the above study denoted that ADP109 was associated with the lipid membrane, it is not yet clear how the ART dimer interacts with the membrane. Drug incorporation in the lipids alters membrane behavior, whereas encapsulation within the aqueous core may have minimal impact on membrane lipid organization. To study this lipid–drug interaction, a fluorescent polarization probe, DPH,^{40,41} was used to determine the effect of ADP109 incorporation on the packing order of lipids. The rotational freedom of the inserted DPH corresponds to the viscosity of the membrane environment. Thus gel state, or high viscosity, membranes yield high fluorescence anisotropy values, and depolarization are observed as membranes become more disordered during and after phase transition.^{42,43} Depolarization of DPH as particle membranes melt can be modeled to estimate the gel–liquid phase transition temperature (T_m). As shown in Figure 2, when ADP109 was incorporated into the lipid membranes at 1:10 D–L ratio, fluorescence anisotropy of DPH in NP209 at gel state ($\sim 23^\circ\text{C}$) was lower than that of control liposome without drug (blank NP209), suggesting that ADP109 presence resulted in less-ordered packing of lipid molecules even when the

particles were in solid phase (Fig. 2a). The presence of ADP109 lowered the T_m of the membrane system in NP209 compared with Blank NP209 by 3°C (Fig. 2b). Together, these data suggest that ADP109 molecules insert into the lipid membrane with sufficient depth, such that the interactions impacted both the phase transition temperature and membrane packing or “order” in both gel and fluid phase.

pH-Responsive Release of Lipid-Bound ADP109 and Aqueous Core Entrapped Calcein

ADP109 possesses a pyridine-methyl-piperazine tail (Scheme 1) to allow pH-dependent aqueous solubility, as previously reported.³⁰ To evaluate pH-responsive drug release, NP209 was exposed to pH 7.4, 6.0, and 4.0 to measure the ART dimer release over time. NP209 released ADP109 more efficiently at pH 4 than physiological neutral. At pH 4, over 50% of the hydrophobic drug escaped from the liposomes by 48 h, whereas only 20% release was recorded at pH 7.4 in the same time period (Fig. 3).

The protonation of ADP109 incorporated in the liposome membrane resulted in perturbation of membrane and destabilization of the particle aqueous compartment, as measured by an increase in fluorescence generated by released calcein. The encapsulated calcein release profile, albeit also dependent on pH of the environment, was different from that of ADP109. The time course of fluorescence regeneration was recorded over 48 h at indicated pH and 37°C (Fig. 4). While drug-free blank NP209 showed little fluorescence regeneration regardless of pH, NP209 released calcein in a clear pH-dependent manner (Fig. 4). It should be noted that NP209 regenerated 30% of calcein fluorescence after 24 h even at pH 7.4 when the blank control regenerated less than 1% under the same conditions. This was likely because of the T_m of NP209 (37.4°C) being close to the incubation temperature (37.0°C), resulting in the most leaky state of the membrane during incubation.^{44,45} Improvements in drug retention and particle stability under physiological conditions are being investigated and will be reported separately.

Effects of Liposome Association on ADP109 *In Vitro* Cytotoxicity Against Human Breast Cancer Cells

We assessed the potency of NP209 on two human breast cancer cell lines with different degrees of drug resistance. BT474 is a HER2⁺ cell line that has been shown to be susceptible toward the ART pharmacophore; MDA-MB-231 is a triple-negative breast cancer (TNBC) cell line (lacking estrogen receptor, progesterone receptor and HER2) that is more resistant toward ART derivatives.⁴⁶ In addition, to evaluate selectivity and safety, a nontumorigenic human breast epithelial cell line, MCF10A, was also used in the cytotoxicity assay. We found that lipid nanoparticle-bound ADP109 exhibited similar potency to that of free ADP109 (Table 2), with IC₅₀ values in the low nanomolar range for BT474 and single digit micromolar range for MDA-MB-231. The NP209 nanoparticles showed similar anticancer potency as free drug in 1% DMSO against BT474 cells, and a threefold reduction in the IC₅₀ value ($p = 0.03$) on MDA-MB-231 cells.

Although the exact mechanism leading to the antitumor effects of ADP109 or NP209 nanoparticles is not clear, our previous study with NP109 showed down regulation of proteins such as survivin and cyclin D1.³⁰ Survivin has been reported to correlate strongly

with antiapoptotic ability of cancer cells⁴⁷ and cyclin D1 with human cancer incidence.⁴⁸ The immortal nontumorigenic human breast epithelial cell line, MCF10A, was less susceptible to both the free compound and its liposome formulations. In both NP109³⁰ and NP209 assays, more than 50% of MCF10A cells were still viable after 48 h of incubation at 10 μ M ADP109 concentration. We have yet to test concentrations higher than 10 μ M on MCF10A cells. Under our experimental conditions, NP209 was about 100 times more selective against BT474 cells than MCF10A cells, and threefold more potent against MDA-MB231 cells. Although MCF10A is often used as a nontumorigenic control, it in fact differs from real healthy cells found *in vivo* as it has been induced to proliferate in culture. Thus, the selectivity recorded for ADP109 and its lipid particles may represent underestimated values.

Effect of NP209 on Suppressing Tumor Growth in MDA-MB-231 Mice Xenograft

Prior to the efficacy study, the *in vivo* safety of NP209 was carried out to define a MTD. We found that a maximum dose of 40 mg/kg every 2 days (q2d) could be tolerated for at least 3 weeks without significant body weight loss in healthy mice. Therefore, a dose of 40 mg/kg q2d subcutaneous injection was chosen for the initial proof-of-concept efficacy study.

Despite lower potency reported for ADP109 compared with other breast cancer cells tested, MDA-MB-231 was chosen for the *in vivo* study. The use of TNBC cells, MDA-MB-231, in immune-compromised mice as xenograft models provide a more challenging model which requires a treatment that addresses both drug resistance and drug-sensitive scenarios. Currently, there are only a few treatment choices for TNBC patients. Standard cytotoxic agents such as taxanes, anthracyclines, and platinum-based agents, are used either as single or combination therapies to control tumor growth, but with limited efficacy.⁴⁹ Thus, to explore the potency of our pH-responsive ADP109-bound lipid nanoparticle on TNBC *in vivo*, female athymic nude mice were implanted with MDA-MB-231 cells in the mammary pad and allowed 10 days for tumor development until solid volume reached approximately 100 mm³. The mice were then randomly assigned into three groups: NP209, blank NP209, or paclitaxel (positive control) at typically recommended dose and frequency. The NP209 test group received a 40-mg ADP109/kg dose (q2d for 14d) in lipid nanoparticle formulation, whereas the vehicle control group received equivalent dose of blank NP209, both given subcutaneously on the back of the animal. As shown in Figure 5, although tumor size in all groups increased, the rate of tumor growth was suppressed significantly in mice receiving NP209 compared with that of the lipid control ($p < 0.05$) on day 14. Furthermore, NP209 outperformed paclitaxel, a standard chemotherapy for breast cancer,⁴⁹ given under standard dose regimen for this TNBC xenograft mouse model. The apparent low efficacy of paclitaxel found in both our study and the literature^{50,51} is consistent with the fact that MDA-MB-231 cells are relatively insensitive to paclitaxel-induced mitotic arrest that leads to apoptosis.⁵⁰ Although the mechanism of action of ART dimers' antiproliferative effect is still a subject of active investigation, our results showed that MDA-MB-231 cells were susceptible to the ART dimers both *in vitro* and *in vivo*. This initial xenograft efficacy study provided us with the first insight to support our continued effort of ART dimer nanoparticle development.

Our finding on the effective growth suppression but not regression of the TNBC tumor by pH-responsive ART dimer liposomes is consistent with literature consensus that the ART pharmacophore is cytostatic but not cytotoxic at the concentrations used in these animal studies.⁵² This suggests that ART-dimer-based compounds may best be administered as adjuvant therapy to manage the disease progression. We showed that aqueous marker, calcein, can be effectively coreleased with the membrane-incorporated ADP109. Therefore, one can design a water-soluble cytotoxin encapsulated within the core of these ADP109-bound lipid nanoparticles to simultaneously deliver multiple drugs to cancer cells to achieve a maximal potency against hard-to-treat cancers such as TNBC. Dose-dependent pharmacology and toxicology as well as pharmacokinetic and efficacy studies would be needed to confirm the potential of

ADP109-bound lipid nanoparticles as a mono or combination therapeutic candidate. Such studies are beyond the scope of this report and are part of our on-going effort to optimize the lipid-drug nanoparticle's properties for enhanced stability, safety, and efficacy. The pH-responsive ART dimer nanoparticle xenograft study reported here is the first *in vivo* experiment to deliver these hydrophobic dimer derivatives in aqueous solutions, to the best of our knowledge. We hope that the better understanding of drug-lipid interactions and promising potency shown in this report will enable further development of ART-based nanoparticle therapeutics.

Cellular Uptake of Calcein-Encapsulating NP209 Shows Energy-Dependent Uptake Mechanism

To shed light on uptake mechanisms of NP209 by human breast cancer cells, BT474 cells were incubated with 1 mM NP209 for 10 or 120 min at 37°C, physiological temperature or 4°C, where no energy-dependent uptake was feasible.⁵³ Figure 6 shows representative fluorescence microscopy images of cells incubated with blank PBS for 120 min, 1 mM NP209 for 10 or 120 min and free calcein at a concentration equivalent to that released by 1 mM NP209 in 120 min. Intact calcein-trapped NP209 particles show up as punctated fluorescence, whereas when particle membrane disintegrates and calcein is released, diffused fluorescence is observed throughout the cell. Only at 37°C were we able to see clear diffused calcein fluorescence inside the cytoplasm of the cell, suggesting release of the aqueous core-trapped content into intracellular compartments and cytosol of the cells. Longer incubation time afforded higher fluorescence intensity, correlating to greater amount of uptake and cargo release.

At 4°C, however, where all energy-dependent uptake pathways are inhibited, we no longer were able to observe diffused fluorescence inside the cell membranes. The rigidity of cell and drug-bound lipid membranes at lower temperature also likely resulted in less fusion of NP209 to cell membranes, further decreasing amount of delivery to the cells. In case of 1 mM NP209 incubated for 120 min at 4°C, few of the faintly fluorescent NP209 could be observed attached to cell surface under microscope. These cellular uptake results confirm our *in vitro* and *in vivo* data that the lipid-associated ADP109 are being delivered into the cytoplasm and other organelles within breast cancer cells, thus leading to observed suppression of tumor cell growth.

CONCLUSIONS

We have successfully developed and characterized a pH-responsive ART dimer in PEG-coated lipid formulation, NP209, with synthetic lipids of saturated fatty acid chain. The lipid composition was able to incorporate ADP109 with near-complete efficiency and showed acid-induced drug released. Binding and insertion of ADP109 into lipid membrane was confirmed by DPH depolarization studies, which showed a 3°C reduction in phase transition temperature because of drug incorporation. The NP209 formulation was potent and selective against both HER2+ and TNBC cell lines. Initial MDA-MB-231 mice xenograft studies validated the feasibility of ADP109–lipid nanoparticle formulations for *in vivo* delivery of ART dimer derivatives as chemotherapeutic agents, inhibiting growth of the triple negative tumor cells that usually respond poorly to current drug therapies. The ability of the NP209 formulation to suppress drug-resistant MDA-MB-231 cell growth beyond that achieved with paclitaxel in mice xenograft provide a step toward finding a therapeutic modality effective against TNBC.

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Abbreviations used

ART	artemisinin
ADPs	artemisinin dimer piperazine conjugates
DMSO	dimethylsulfoxide
DOC	deoxycholic acid
DPH	1,6-diphenyl-1,3,5-hexatriene
DPPC	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphocholine
EPC	egg phosphatidylcholine
IC₅₀	concentration required to inhibit cell proliferation by 50% versus control
mPEG₂₀₀₀-DPPE	1,2-dipalmitoyl- <i>sn</i> -glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] sodium salt
MTD	maximum tolerated dose
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)
PBS	phosphate-buffered saline
SEM	standard error of the mean
T_m	gel–liquid phase transition temperature

TNBC

triple-negative breast cancer

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Scheme 1.

Chemical structure of ART and ADP109.

Figure 1.

(a) Representative cryo-EM images of NP209 liposomes viewed as untilted (left, 0°) and tilted (right, -45°). Scale bar = 100 nm. (b) Enlarged images showing individual particles selected from untilted view.

Figure 2.

Effects of ADP109 on phase transition behavior of lipids containing 95% DPPC and 5% mPEG₂₀₀₀-DPPE, determined by DPH fluorescence anisotropy as a function of temperature.

(a) Fluorescence anisotropy of DPH incorporated in lipid membranes of control blank NP209 (○) or drug-bound NP209 (●) with change in temperature. (b) Gel-fluid phase transition temperatures calculated from fluorescence polarization studies. Values plotted and reported as average ($\pm$ SD) of triplicate measurements.

Figure 3. pH-dependent ADP109 release from NP209 (20 mM lipid) dialyzed under ambient temperature at pH 7.4, 6, and 4. Values plotted as average ($\pm$ SD) of three independent experiments.

Figure 4. pH-dependent calcein fluorescence regeneration profiles of (a) NP209 and (b) the drug-free blank NP209. Values plotted as average with SD as error bars of triplicate readings, after adjustment of pH effect on calcein fluorescence.

Figure 5.

Average tumor volume measured of NP209 (●) test group and blank NP209 (—) control group treated with 40 mg/kg drug every other day subcutaneous injection of nanoparticles from day 0 to day 15, and intravenous injection of paclitaxel (△) once per day from day 0 to day 4 at 10 mg/kg. Day 0 is defined as 10 days after tumor implant. Values plotted as average with error bars as SEM of seven animals in each group. Significant difference was defined by the *t*-test where $p < 0.05$: # $p < 0.1$, * $p < 0.05$ between NP209 and blank NP209. Paclitaxel group did not show significant difference compared with control or test groups.

Figure 6.

Representative fluorescence microscopy images of BT474 cells incubated at 37°C (left two panels) and 4°C (right two panels) with blank PBS, NP209, or free calcein concentration equivalent to that released by NP209 in 120 min at 37°C. Energy-dependent uptake of intact particles and release of aqueous core-trapped calcein are observed at both 10 and 120 min. Images taken with 40× magnification and scale bar = 10 μm.

Table 1

Comparison of Size and Incorporation Efficiency of NP109 and NP209

	Lipid Composition	Drug–Lipid Ratio	Average Size (d, nm)	Percent Drug Incorporation
NP109 ³⁰	EPC	1:10	70(±20)	91(±9)%
NP209	95% DPPC–5% DPPE-mPEG ₂₀₀₀	1:10	76(±10)	90(±6)%

Average size and percent drug incorporation values reported as average (±SD) of three independent experiments.

Table 2Calculated IC₅₀ Values of ADP109, NP109, and NP209 on BT474, MDA-MB-231, and MCF10A Cell Lines

	IC ₅₀ (μM ± SD)		
	BT474	MDA-MB-231	MCF10A
ADP109	0.07 ± 0.01	10 (± 3)	>10 ^a
NP109	0.08 ± 0.01 ³⁰	7(± 2) ³⁰	>10 ^a
NP209	0.105 ± 0.003	3 (± 1) ^b	>10 ^a

Values reported as average (±SD) of three independent experiments, with each experiment performed in triplicates.

^aMore than 50% cell living after 48-h incubation with 10 μM.^bSignificantly different from IC₅₀ of free ADP109 (*p* = 0.03).