Abstract

Malignant melanoma is a difficult cancer to treat due to the rapid development of resistance to drugs targeting single proteins. One response to this observation is to identify single pharmacologic agents that, due to a unique mechanism of action, simultaneously target multiple key pathways involved in melanoma development. Leelamine has been identified as functioning in this manner but has poor bioavailability in animals and causes lethality when administered intravenously. Therefore, a nanoliposomal-based delivery system has been developed, called Nanolipolee-007, which stably loads 60% of the compound. The nanoparticle was as effective at killing melanoma cells as leelamine dissolved in DMSO and was more effective at killing cultured melanoma compared with normal cells. Mechanistically, Nanolipolee-007 inhibited PI3K/Akt, STAT3, and MAPK signaling mediated through inhibition of cholesterol transport. Nanolipolee-007 inhibited the growth of preexisting xenografted melanoma tumors by an average of 64% by decreasing cellular proliferation, reducing tumor vascularization, and increasing cellular apoptosis, with negligible toxicity. Thus, a unique clinically viable nanoparticle-based drug has been developed containing leelamine for the treatment of melanoma that acts by inhibiting the activity of major signaling pathways regulating the development of this disease. Mol Cancer Ther; 13(10); 1–13. ©2014 AACR.

Introduction

Malignant melanoma is one of the most difficult cancers to treat due to the development of recurrent resistant disease (1). Although encouraging recent studies showed promising results with Zelboraf, a selective V600E-B-Raf kinase inhibitor, development of drug resistance remains a problem (2). To address this concern, one approach has been to develop liposomes containing novel agents specifically targeting multiple key pathways or signaling cascades, leading to recurrent resistance disease development (3).

Loading of drugs into nanoparticles to circumvent bioavailability, toxicity, or lethality can be used to overcome limitations of standard drug delivery systems, which involve nonspecific targeting and biodistribution with low solubility at a modest therapeutic index (4, 5). Nanoparticles can be designed with optimal size and surface characteristics to increase the circulation time and biodistribution (6). One advantage of nanoparticles is the enhanced permeability and retention (EPR) effect that enables nanoparticles to accumulate in tumors at much higher concentrations than in normal tissue (6). The EPR effect occurs because the vasculature of tumors is poorly developed and leaky, enabling nanoparticles and macromolecules to preferentially accumulate and concentrate in tumors (7). Natural or synthetic polymers or lipids can be used as drug delivery vectors (7). Lipid-based delivery vehicles have established track records, and have suitable biological properties, including biocompatibility, biodegradability, and the ability to accommodate both hydrophilic and hydrophobic drugs (8).

Liposomes primarily consist of phospholipid amphiphiles that self assemble to form bilayer membranes. Liposomes have been extensively studied as drug-delivery systems and are used as biocompatible carriers of drugs, enzymes, peptides, vaccines, imaging agents, and/or genetic material (9). Depending on the properties of the drugs, they can be loaded into the aqueous or lipid compartments of liposomes and can be effective for delivering the therapeutic cargo into tumors in...
animals (9). In addition, liposomes can be used as models for artificial cells that are able to mimic cell membrane trafficking and studying drug movement (10). Currently, nanoliposomal-based therapeutics are used clinically and many are at various stages of clinical development (3, 11).

Recently, a cell-based screen of a natural product library was undertaken to identify a pharmacologic agent that can decrease melanoma development by targeting the PI3K, STAT3, and MAPK signaling cascade, which is detailed in the article by Gowda and colleagues (12). Leelamine-mediated inhibition of these cascades was attributed to the inhibition of receptor-mediated endocytosis of receptor tyrosine kinases (RTK) causing aberrant accumulation of these proteins in the perinuclear region of cells, which is detailed in the article by Kuzu and colleagues (13). Using a combination of protein arrays and systems biology followed by validation studies, leelamine was found to inhibit the PI3K/Akt, STAT3, and MAPK pathways by disrupting cancer cell cholesterol transport. These are key driver pathways in melanoma cells constitutively activated in 50% to 70% of melanomas, functioning to reduce cellular apoptosis, increase proliferation and aid the invasive processes, promoting melanoma progression (14–16).

Leelamine dissolved in DMSO and administered by intraperitoneal injection, inhibited the growth of prexistening xenografted melanoma tumors by 60% without significant toxicity. However, due to poor bioavailability, toxicity and lethality of this agent when administered intravenously, a stable PEGylated nanoliposomal delivery system has been developed called Nanolipolee-007. The nanoparticle has an average size of 80 nm and is stable in saline for 1 year at 4°C. Nanolipolee-007 was 5.69-fold more effective at killing melanoma than normal cells, decreased cellular proliferation, and triggered apoptosis through a G₀/G₁ block, resulting in fewer cells in the S-phase. Intravenously administered Nanolipolee-007 had negligible toxicity and retarded existing xenograft melanoma tumor growth by up to 60% without affecting animal weight or organ function by decreasing melanoma cell proliferation, increasing apoptosis, and decreasing vascular development. Therefore, Nanolipolee-007 is a potentially clinically viable intravenous nanoparticle formulation that can be used to treat melanoma in mice and has potential for use in humans.

Materials and Methods

Cell lines and culture conditions

Normal human primary melanocytes FOM103 and wild-type B-Raf containing SbCl2 (provided by Dr. Herlyn between 2003 and 2005; Wistar Institute, Philadelphia, PA) were cultured as described (14). Human fibroblast FF2441 cells were provided by Dr. Craig Myers lab between 2005 and 2006, Penn State College of Medicine, Hershey, PA. Mutant type B-Raf melanoma cell lines UACC 903 were provided from Mark Nelson between 1995 and 1999, University of Arizona, Tucson, AZ and 1205 Lu provided by Dr. Herlyn between 2005 and 2006, Wistar Institute, Philadelphia, PA. Wild type B-Raf melanoma cell line containing CB161.C19 was provided by Dr. Danny Welch (2003), University of Kansas, Kansas City, KS and MelJuSo provided by Dr. Judith Johnson (between 1995 and 1999), Institute for Immunology, Germany. Cell lines were maintained in a 37°C humidified 5% CO₂ atmosphere incubator and periodically monitored for genotypic characteristics, phenotypic behavior, and tumorigenic potential to confirm the cell line identity (14).

Intravenous administration of free leelamine and liposomal leelamine

Four- to six-week-old female Athymic-Foxn1nu nude mice (Harlan) were injected intravenously with 30 mg/kg body weight leelamine dissolved in DMSO or Nanolipolee-007. Vehicle control animals were treated with DMSO or empty liposome. Animal mortality or hemolysis and coagulation was recorded after 1 hour (n = 3).

Generation of Nanolipolee-007

Leelamine hydrochloride (Tocris Biosciences) was encapsulated into a nanoliposome (called Nanolipolee-007) prepared by combining egg L-α-Phosphatidylcholine (ePC) and 1,2-Dipalmitoyl-sn-Glycero-3-Phosphoethanolamine-N-[Methoxy(Polyethylene glycol)-2000] ammonium salt (DPPE-PEG-2000) in chloroform at 80:20 mol% and a final lipid concentration of 25 mg/mL in buffer solution (Avanti Polar Lipids Inc). The same lipid formulation was found to load Abietic acid (Sigma Chemical Co.) and an empty control nanoliposome was also made. 7.5 mg of leelamine hydrochloride and/or abietic acid (in methanol) was added to 1.0 mL of nanoliposome solution, resulting in a 22.6 mmol/L concentration. Mixture was then dried under nitrogen gas and resuspended in 0.9% saline at 60°C. Following rehydration, the mixture was sonicated at 60°C for 30 minutes followed by extrusion at 60°C through a 100-nm polycarbonate membrane using Avanti Mini Extruder (Avanti Polar Lipids Inc). The particle size and charge characteristics were determined using a Malvern Zetasizer (Malvern Instruments).

Characterization of Nanolipolee-007

Loading efficacy. Amount of leelamine encapsulated into the nanoliposome was measured by calculating the amount of tritium-labeled leelamine incorporated into the nanoparticle. Tritium-labeled leelamine was synthesized by bromination, followed by replacing the bromine atom with tritium with a specific activity of 25 Ci/mmol (American Radio Chemicals Inc; http://www.arc-inc.com/index.php). Dialysis and size exclusion chromatography were used to calculate tritium-labeled leelamine loading efficacy (17, 18). One milliliter of Nanolipolee-007 made with tritium-labeled leelamine was placed into a 1 × 5 cm long dialysis membrane bag (molecular weight cutoff: 25
kDa; Spectra Por). The dialysis bag was suspended in 1 L of 0.9% saline with constant stirring (300 rpm) for 12 hours and the amount of tritium-labeled leelamine remaining in the dialysis bag was measured by liquid scintillation counter (LS-6500- Beckman Coulter). Sepharose CL-4B (wet bead size, 45–165 μm; Sigma Chemical Co.) was loaded into a column (0.6 × 60 cm) equilibrated in 0.9% saline, pH 7.0. Next, 200 μL of 1 × 10^6 cpm units was loaded onto the column and eluted with 0.9% saline at room temperature. Eluted fractions were collected at a flow rate of 10 mL/hours and the amount of tritium in each fraction was measured. Peak fractions were pooled and the amount of tritium-labeled leelamine encapsulated in Nanolipolee-007 was measured. The percentage incorporation of leelamine was determined using the following formula: amount of radioactivity in peak-I (liposomal encapsulated)/amount of radioactivity in peak-I + amount of radioactivity in peak-II (free leelamine) × 100.

**Stability of Nanolipolee-007.** Nanolipolee-007 stored in sterile saline at 4°C was measured at 1, 3, 6, 9, and 12 months by comparing size, charge, and efficacy for killing UACC 903 melanoma cells. During this interval, the particle size and charge characteristics were determined by Malvern Zetasizer Nano, (Malvern Instruments). Efficiency of Nanolipolee-007 for killing UACC 903 was measured using the MTS assay (Promega). Briefly, 5 × 10^3 UACC 903 cells per well in 100 μL of media were plated and grown in a 96-well plate for 48 hours and treated with 0.62 to 100 μmol/L of control nanoliposome alone and Nanolipolee-007 for 24 hours. IC_{50} values for each treatment agent in μmol/L for respective cell lines were calculated from two independent experiments using GraphPad Prism version 4.01 (GraphPad Software).

**In vitro drug release kinetics.** The in vitro release of leelamine from the nanoparticle was measured at room temperature by dialysis through a molecular weight cutoff 25-kDa membrane (Spectra Por). Briefly, 1 mL of tritium-labeled Nanolipolee-007 was placed into a dialysis membrane and suspended in 1 L of 0.9% saline (or 10 mmol/L GSH medium) with constant stirring at 300 rpm for 12 hours. Free unbound leelamine was separated, and the dialysis bag containing the encapsulated tritiated leelamine was immersed in 500 mL of releasing medium containing 0.9% saline (or 10 mmol/L reduced glutathione). A 1-mL sample from the releasing medium was taken at 1, 2, 4, 8, 12, 24, 36, and 48 hours, and radioactivity associated with the tritiated leelamine measured using a liquid scintillation counter (LS-6500- Beckman Coulter).

**Hemolytic activity.** Hemolytic activity assay was as described by Nie and colleagues (19). In brief, fresh human blood was drawn and placed into an EDTA test tube. Erythrocytes were separated from plasma by centrifugation at 1,500 rpm for 10 minutes at 4°C using PBS. Human mouse erythrocytes pellet was diluted with PBS to a give a 5% v/v solution and 50 μL added into microcentrifuge tubes and treated with leelamine in DMSO (100 μmol/L), nanolipolee-007 (100 μmol/L), DMSO, or empty liposome or 1% Triton X-100 (positive control). Samples were incubated at 37°C for 60 minutes and then centrifuged at 12,000 rpm for 10 minutes. Next, supernatants were transferred to a 96-well plate and absorption measured at 540 nm. Amount of hemoglobin released in the presence of 1% Triton X-100 was set as 100% lysis and % hemolysis was calculated as: (absorbance of the samples at 540 nm/absorbance of the positive control) × 100.

**Pharmacokinetics.** Leelamine contained in Nanolipolee-007 was extracted from serum as reported previously (20). Swiss Webster (n = 5) mice were intravenously injected with 30 mg/kg body weight of Nanolipolee-007, animals were euthanized, and blood was drawn by cardiac puncture at various time periods. Samples were kept at room temperature for 30 minutes followed by serum separation by centrifugation for 5 minutes at 5,000 rpm. Next, 20 μL of the collected serum was added to 80 μL of acetonitrile along with 5 μL of propanolol (RS)-1-(1-methylethylamino)-3-(1-naphthoxy)propan-2-ol, an internal standard (transition of m/z 259.9–116.0). Solution was vortexed for 30 seconds and then centrifuged at 10,000 rpm for 10 minutes. Supernatant was transferred to the autosampler vials and subject to LC/MS using LC/MS-MS 2010 EV system (Shimadzu).

**Cell viability, proliferation, apoptosis, and cell-cycle analysis.** Viability and IC_{50} of normal human melanocytes, fibroblast, and melanoma cells (UACC 903 and 1205 Lu) following treatment was measured by MTS assay (Promega; ref. 21). Briefly, 5 × 10^3 melanoma or fibroblast (FF2441) or 20 × 10^3 melanocytes (FOM103) cells per well in 100 μL of media were plated and grown in a well of a 96-well plate for 48 or 72 hours and treated with 0.62 to 100 μmol/L of empty nanoliposome, abietic acid control nanoliposome, or Nanolipolee-007 for 24 hours.

**Cellular proliferation and apoptosis rates.** A total of 5 × 10^3 UACC 903 or 1205 Lu melanoma cells were seeded in 96-well plates, followed by treatment with 0.62 to 100 μmol/L of empty control or abietic acid nanoliposomes or Nanolipolee-007 for 24 hours. Percentage proliferating or apoptotic cells were quantified by a colorimetric cell proliferation ELISA BrdU Kit (Roche Applied Sciences) or fluorimetric Apo-ONE Homogenous caspase-3/7 assay kit as previously reported (Promega; ref. 21).

**Cell-cycle analysis.** Cells in each phase of the cell cycle were calculated by growing UACC 903 or 1205 Lu melanoma cells in 100-mm culture dishes followed by treatment with empty control nanoliposome or Nanolipolee-007 (2–3 μmol/L) for 24 hours. Total floating and adherent cells were collected following trypsinization and stained with a 1-mL propidium iodide solution containing 100 μg/mL propidium iodide; (Sigma), 20 μg/mL Ribonuclease A (Roche diagnostics) and 3 μg/mL Triton X-100 dissolved in 0.1% (W/V) sodium citrate for 30 minutes at 4°C. Stained cells were analyzed using the FACScan analyzer (Becton Dickinson), and data were processed.
utilizing ModFit LT software (Verity Software House; ref. 21).

Western blot analysis

Cell lysates treated with empty control nanoliposome or Nanolipolee-007 (3–5 μmol/L) for 3 to 24 hours were harvested in RIPA lysis buffer containing protease and phosphatase inhibitors (Pierce Biotechnology; ref. 21). Blots were probed with antibodies according to each supplier’s recommendations: antibodies to total Akt, phospho-Akt (Ser473), phospho-PRAS40 (Thr246), total Bad, pBad (Ser112), total Erk1/2, phospho-Erk1/2 (Thr202/Tyr204), total CDK2, phospho-CDK2 (Thr160), phospho-Rb (Ser807), total Stat, phospho-Stat3 (Tyr705), caspase-3, and cleaved PARP from Cell Signaling Technology; total PRAS40 from Invitrogen; cyclin D1, α-enolase, and secondary antibodies conjugated with horseradish peroxidase from Santa Cruz Biotechnology.

Tumorigenicity assessments

Tumor kinetics were measured by subcutaneous injection of 1.5 × 10⁶ UACC 903 or 1205 Lu cells in 0.2 mL of DMEM supplemented with 10% FBS. Cells were injected above both left and right rib cages of 3- to 4-week-old female Athymic-Foxn1nu nude mice (Harlan). Six days later, when a fully vascularized 50 to 75 mm³ tumor had formed, mice were randomly divided into vehicle control or experimental groups (5 mice/group; 2 tumors/mouse) and treated intravenously everyday for 3 to 4 weeks with 30-mg/kg body weight of Nanolipolee-007 or control abietic acid or empty nanoliposome. Body weight in grams and dimensions of developing tumors in mm³ were measured on alternate days (21).

Size and time match tumors for analysis of tumorigenic processes regulating tumor development

Mechanism by which Nanolipolee-007 delayed tumor growth was determined by comparing size and time matched xenografted melanoma tumors treated with Nanolipolee-007 or empty control nanoliposome. UACC 903 cells (2.5 × 10⁶) were injected subcutaneously into nude mice, generating tumors of the same size developing at parallel time points. Six days later, mice were treated intravenously with empty nanoliposome or Nanolipolee-007 (30 mg/kg body weight) daily up to day 15. Tumors were harvested at 11, 13, and 15 days for comparison of rates of cellular proliferation, apoptosis, and vessel density by IHC (22, 23).

Toxicity assessments

Four to six weeks old female Athymic-Foxn1nu nude mice (Harlan) were treated intravenously either empty or abietic acid control nanoliposomes or Nanolipolee-007 (n = 5). Animals were weighed daily to ascertain toxicity leading to changes in body weight. At the end of treatment, blood was collected from each of the euthanized animals in a serum separator tube with lithium heparin (BD Microtainer) following cardiac puncture and analyzed for blood makers of major organ function indicative of toxicity (24). A portion of liver, heart, kidney, pancreas, and spleen tissue from each animal was formalin fixed and paraffin embedded to examine changes in cell morphology and tissue organization following hematoxylin and eosin (H&E) staining (24).

Statistical analysis

Statistical analysis was performed using Prism 4.0 GraphPad Software. One-way or two-way ANOVA was used for group wise comparisons (24). Results represent at least three to four independent experiments and are shown as averages ± SEM. The number of asterisks in the figures indicates the level of statistical significance as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Results

Development of Nanolipolee-007 and its efficacy for killing melanoma cells

Natural Product Library (NPL-480) consisting of 480 compounds derived from plants, animal, bacteria, and fungal sources was used to identify the agents effective at killing melanoma cells, which is detailed in the manuscript by Gowda and colleagues (12). Leelamine was found to be the most potent of the agents identified. Leelamine simultaneously inhibits the PI3K/Akt, STAT3, and MAPK cascades to inhibit melanoma development by disrupting cholesterol transport and endosomal trafficking, which are detailed in the article by Kuzu and colleagues (13). Leelamine and a structurally similar inactive control compound called abietic acid are shown in Fig. 1A. The IC₅₀ of leelamine dissolved in DMSO for killing UACC 903 or 1205 Lu melanoma cells are 1.78 and 2.49 μmol/L, respectively (Fig. 1A). In contrast, control abietic acid had no effect on cell viability at concentrations >100 μmol/L. Therefore, abietic acid that shares structural similarity with leelamine was used as a control (Fig. 1A).

Use of leelamine in animals or humans is limited by its poor bioavailability and insolubility in saline (Supplementary Fig. S1). It had previously been dissolved in DMSO for use in animals, which is not useful for clinical applications requiring intravenous administration (25, 26). Intravenously administered DMSO is lethal to mice necessitating the development of a clinically viable formulation (27). Toxicity limiting the potential clinical utility of free leelamine in DMSO, but not liposomal leelamine, was corroborated in animal studies (Supplementary Table S1). Free leelamine in DMSO at 30 mg/kg body weight led to death of all animals within 1 hour of treatment, whereas liposomal leelamine at this concentration exhibited no mortality. Because of the toxicity associated with leelamine, a clinical viable delivery system was developed. A novel PEGylated neutral leelamine loaded liposomal system (80:20 mol% of ePC: DPPE PEG-2000) called Nanolipolee-007 was developed (Fig. 1B), which was based on the size, zeta potential, efficacy,
Targeting Melanoma with Nanolipolee-007

Figure 1. Development of Nanolipolee-007 and comparison of its killing efficacy on normal as well as on melanoma cells. A, to identify a novel drug targeting multiple key pathways in melanoma, a cell-based screen was undertaken of a natural product library, identifying leelamine (A, left). Abietic acid, structurally similar to leelamine, had no effect on melanoma cell viability (A, right). B, schematic of Nanolipolee-007. C, size and charge of Nanolipolee-007 was 70 to 80 nm in size with a neutral surface charge in saline. The particle size and charge characteristics were established using a Malvern Zetasizer. D, efficacy of Nanolipolee-007 for killing normal and melanoma cells. Nanolipolee-007 was 5.69-fold more effective at killing metastatic melanoma than normal cells, suggesting potential cancer therapeutic utility at concentrations <2.26 μmol/L. Data represent an average of at least three independent experiments.

Nanolipolee-007 stably loaded leelamine

Parameters to examine the stability of Nanolipolee-007 such as size, charge, and melanoma cell killing efficacy were analyzed for multiple batches (28). Loading of leelamine into the nanoliposomal formulation was assessed using tritium-labeled drug during the manufacture of Nanolipolee-007, followed by dialysis to remove free compound. Tritium-labeled leelamine was synthesized by bromination, followed by replacing the bromine atom with tritium with a specific activity of 25 Ci/mmol and purity of the compound was determined by HPLC (Supplementary Fig. S2A). Biological efficacy of tritiated leelamine for killing melanoma cells was compared with leelamine by MTS assay (Supplementary Fig. S2B). Size exclusion chromatography was used to measure removal of loosely bound leelamine from that contained more tightly in the nanoliposome. Leelamine loading after dialysis showed that 64.1% of the drug was incorporated into the nanoliposomal formulation (Fig. 2A). Column chromatography of samples before and after dialysis showed the disappearance of the loosely bound leelamine after dialysis (Fig. 2B). Loading efficiency was also measured by UV-visible spectrophotometry following
centrifugation using 10-kDa Centricon filters to remove free drugs from the nanoparticle. The loading efficiency of leelamine was found to be 70.6% (Supplementary Fig. S3). Stability of Nanolipolee-007 stored in sterile saline at 4°C was measured at 1, 3, 6, 9, and 12 months, and size, charge as well as efficacy for killing UACC 903 melanoma cells were compared (Fig. 2C). During this period, the nanoliposomes retained similar size and charge distributions as well as efficacy for killing UACC 903 melanoma cells with an IC_{50} ranging from 1.96 to 2.60 μmol/L (Fig. 2C). No aggregation or precipitation of the nanoparticles occurred during this period.

**Nanolipolee-007 decreased the hemolytic activity of leelamine**

Liposomal formulations of a hydrophobic drug can overcome solubility and hemolysis occurring with the free drug (29). A hemolytic assay was performed to examine whether Nanolipolee-007 caused the same level of red blood cell lysis occurring with leelamine. Leelamine in DMSO induced 15.81% hemolysis, which is significant compared with 3.29% occurring with Nanolipolee-007 (Fig. 2D).

**Leelamine released from Nanolipolee-007 was present in the serum of animals**

Leelamine release from Nanolipolee-007 was measured in vitro by dialysis in 0.9% saline for 24 hours, which showed that approximately 91.1% of leelamine was released. Release initially occurred slowly for the first four hours and reached a maximum level 12 hours later (Fig. 2E). Leelamine release was also examined with 10 mmol/L glutathione, leading to similar results, suggesting that unencapsulated drug is not observed at the concentrations released from the individual nanoliposomes (Supplementary Fig. S4). Next, presence of leelamine in the serum of mice was measured following intravenous injection of 30 mg/kg body weight of Nanolipolee-007 and serum analyzed for the presence of the drug over a 24-hour period by LC/MS-MS. Leelamine contained in Nanolipolee-007 was present in the serum of mice for >10 hours after intravenous administration (Fig. 2F).

**Nanolipolee-007 inhibited melanoma tumor development with negligible major organ-related toxicity**

The study by Gowda and colleagues (12) showed that intraperitoneal injection of leelamine dissolved in DMSO at 7.5 mg/kg body weight retarded existing xenograft melanoma tumor growth by up to 60%. To determine whether intravenously administered Nanolipolee-007 would function in a similar manner, mice were injected subcutaneously with 1.5 million UACC 903 or 1205 Lu melanoma cells and tumors let develop for 6 days at which time fully vascularized tumors had formed. Animals were then treated by daily intravenous injections of 30 mg/kg body weight of Nanolipolee-007 and compared with controls. Nanolipolee-007 decreased tumor volume by approximately 55% for UACC 903 (Fig. 3A) and 1205 Lu (Fig. 3B) cells compared with controls. Nanolipolee-007 treated animals did not show any significant changes in body weight, suggesting negligible toxicity (Fig. 3A and B; inset). No noticeable changes in the serum parameters indicative of vital organ toxicity were observed (Fig. 3C), but an increase in cholesterol and triglyceride levels was seen following nanoliposomal treatment after 24 days. This is expected following daily administration of lipid-based nanoparticles. However, analysis of the H&E-stained liver from vehicle or Nanolipolee-007–treated mice showed no changes in the morphology or histologic architecture of the organ (Fig. 3D). Furthermore, no changes were detected in histologic sections from heart, lung, kidney, or spleen (Fig. 3D). These data demonstrated that Nanolipolee-007 effectively inhibited xenografted melanoma tumor development without significant organ-related toxicity other than increased lipid levels that could be controlled pharmacologically or by administering the agent on alternate days.

**In vivo mechanistic study of Nanolipolee-007 in size and time matched xenograft tumors**

To investigate the mechanism by which Nanolipolee-007 delayed tumor growth, the rates of cell proliferation, apoptosis, and tumor angiogenesis occurring in time and size-matched xenograft tumors treated with Nanolipolee-007 were compared with empty control nanoliposome-treated animals (22, 23). Size and time matched tumors at days 11, 13, and 15 were compared to identify statistically quantifiable differences in cell proliferation, apoptosis, or vascular development affected by Nanolipolee-007 treatment. At day 11, a statistically significant 60% reduction in proliferating cells was observed as well as an increased number of cells undergoing apoptosis compared with control-treated animals (Fig. 4A and B). Furthermore, differences in vascular development were also detected in all tumors compared with vehicle controls. Thus, intravenously developed Nanolipolee-007 at 30 mg/kg inhibited melanoma tumor development by decreasing proliferation, triggering apoptosis, and decreasing vascular development (Fig. 4A–C).

**Nanolipolee-007 decreased cellular proliferation, triggered apoptosis, and arrested melanoma cells in the G_0-G_1 phase of the cell cycle**

To further unravel the mechanisms leading to cell growth inhibition after treatment of mice with Nanolipolee-007, the rates of cellular proliferation, apoptosis, and the percentage of cells in the various phases of the cell cycle were measured. Increasing concentrations of Nanolipolee-007 from 0.62 to 10 μmol/L decreased the cellular proliferative potential as measured by bromodeoxyuridine (BrdUrd) incorporation (Fig. 5A) and increased cellular apoptosis measured by caspase-3/7 activity (Fig. 5B). Cell-cycle analysis of propidium iodide–stained UACC
Figure 2. Physicochemical characterization of Nanolipolee-007. A, leelamine loading into Nanolipolee-007. Loading was measured using tritium-labeled leelamine when making Nanolipolee-007, showing 64.1% loading. Data represent an average of at least three independent experiments. B, characterization of loosely and tightly bound leelamine in Nanolipolee-007. Both dialysis and size exclusion chromatography-based approaches were used to measure drug loading efficiency. The amount of tritium-labeled leelamine remaining in Nanolipolee-007 was measured. Data represent an average of at least three independent experiments. C, long-term stability of Nanolipolee-007. Nanolipolee-007 was stable in saline stored at 4°C for a year; maintaining its activity for killing UACC 903 cells with an IC₅₀ of 1.96 to 2.60 μmol/L. D, hemolytic activity of Nanolipolee-007 compared with leelamine. (Continued on the following page.)
903 and 1205 Lu cells following 24 hours of Nanolipolee-007 treatment showed increases in the sub-G0/G1 and G0–G1 cell populations, with a corresponding decrease in the S-phase population (Fig. 5C). Thus, Nanolipolee-007 reduced melanoma cell survival by decreasing proliferation and triggering apoptosis mediated through a G0–G1 block, resulting in fewer cells in the S-phase population of the cell cycle.

Nanolipolee-007 inhibited the activity of three driver pathways promoting melanoma development

Pathways targeted by leelamine in melanoma cells were previously identified and detailed in the articles by Gowda and colleagues (12) as well as Kuzu and colleagues (13), using a Kinexus antibody microarray and Ingenuity Pathway Analysis (IPA) followed by validation using Western blotting. Nanolipolee-007 treatment did not alter the histologic structure of liver, kidney, lung, spleen, or heart. *, P < 0.05; **, P < 0.01; ***. P < 0.001.
Figure 4. Mechanistic basis for tumor inhibition mediated by Nanolipolee-007. Formalin-fixed paraffin-embedded size- and time-matched tumors sections were subjected to Ki67 staining for cell proliferation (A), TUNEL staining for apoptosis (B), CD31 staining for tumor angiogenesis (C), and compared with empty nanoliposome-treated animals. From day 11, a statistically significant decrease in proliferating tumor cells, increase in number of cells undergoing apoptosis, and reduction in vascular development were identified compared with vehicle-treated animals (A–C). **, \( P < 0.01; \) ***, \( P < 0.001 \).
decreased the activity of the PI3K/Akt, STAT3, and MAPK pathways, which are major signaling cascades promoting melanoma development (14–16). Decreased signaling through both UACC 903 and 1205 Lu melanoma cells following treatment with 3 to 6 μmol/L of Nanolipolee-007 for 3 to 24 hours is shown for the PI3K/Akt (Fig. 6A) and STAT3 (Fig. 6B) pathways. Nanolipolee-007 significantly inhibited Akt phosphorylation without affecting total Akt protein levels in a dose- and time-dependent manner (Fig. 6A). In addition, Nanolipolee-007 inhibited the phosphorylation of other downstream signaling components of the cascade regulating cell survival. It also decreased expression of cyclin D1 and increased cleaved caspase-3 and PARP protein levels at later time points (Fig. 6A). STAT3 is also constitutively activated in melanomas and contributes to tumor cell growth, proliferation, metastasis, and angiogenesis in various cancers (30, 31). Nanolipolee-007 inhibited phosphorylation of STAT3 without affecting total STAT3 protein levels (Fig. 6B). Nanolipolee-007 also had a minor effect on the MAPK pathway (Fig. 6C), which was likely due to its unique mechanism of action and is detailed in the article by Kuzu and colleagues (13).

Discussion

Melanoma is the most deadly type of skin cancer diagnosed in the United States (32). Incidence and mortality rates continue to increase annually and it remains one of the most invasive as well as drug-resistant cancer types (33). Although Zelboraf has been approved by the FDA for the treatment of malignant melanomas harboring a V600E-B-Raf mutation, the development of drug resistance is raising concerns about the long-term utility of Zelboraf as a single-agent therapy for malignant melanoma (34). Moreover, Zelboraf is effective only in approximately 50% of patients with melanoma having mutant V600E-B-Raf, leaving the other 50% in need of other treatment modalities (34). Therefore, agents targeting multiple driver pathways in patients with melanoma that could lead to lower rates of resistance development are needed, as well as drugs for treating those patients lacking the V600E-B-Raf mutation (35).
Natural products have played an important role in the development of new anticancer drugs and currently constitute more than 60% of cancer therapeutics in the clinic (36, 37). Leelamine was identified from a natural product library screen, to inhibit melanoma cells growth but intravenous administration was limited by hemolysis and animal death. Liposomal formulation of drugs such as leelamine can be used to moderate these concerns (29). Leelamine in DMSO induced 15.81% hemolysis, which is considered high, compared with 3.29% observed with Nanolipolee-007. This difference might be due to the rigidity of the liposome and electrostatic repulsion of anionic RBCs (29). Rigid molecules such as DPPE PEG-2000 are less prone to attach to the RBC membrane compared with flexible molecules (29).

Nanotechnology can be used to improve solubility, pharmacokinetics, and reduce side effects associated with various drugs (38, 39). Among different nanoparticles, liposomes are well-studied colloidal particles delivering drugs to tumors and increasing the solubility of amphiphilic agents (40, 41). Liposomes less than 100 nm can enter tumors due to the leaky vasculature, which does not occur in the normal vasculature due to the EPR effect (4). Nanolipolee-007 fulfills all these criteria having an average size of 70 to 80 nm and a neutral charge. The most common surface modification of nanoparticles is PEGylation, in which polyethylene glycol is covalently linked through lipids to the surface of the liposome (42). PEGylated liposomes tend to be stable, have enhanced circulation time, avoid clearance by the reticulo-endothelial system, and have minimal toxicity (43). In this report, leelamine was loaded into a PEGylated neutral liposomal formulation that was stable at 4°C for a year and had an increased circulating half-life, promoting accumulation at the tumor site.

In clinical studies, liposomes have improved the pharmacokinetics and bio-distribution properties of therapeutic agents as well as an ability to reduce toxicity by accumulation in tumors due to the EPR effect (44). Currently, there are 12 liposome-based drugs approved for...
clinical use and others are in various stages of clinical development (3, 5, 11). For example, PEGylated liposomal formulations of doxorubicin such as Doxil and Lipo- dox are approved for intravenous application with minor dose-limiting toxicity compared with doxorubicin (45, 46). Nab-paclitaxel, a nanoparticle formulation of pacli- taxel, has also demonstrated higher therapeutic efficacy against breast cancer than paclitaxel and several other nab-based chemotherapeutics are currently under clinical evaluation (47, 48).

Mechanism of leelamine-mediated cell death has been investigated and reported in the manuscript by Kuzu and colleagues (13). Briefly, leelamine is a lysosomotropic compound accumulating inside acidic cell compartments such as lysosomes and endosomes. Accumulation leads to disruption of intracellular cholesterol homeostasis and interferes with autophagic flux as well as receptor-mediated endocytosis. Inhibition of receptor-mediated endocytosis shuts down RTK signaling and inhibits the activa- tion of downstream PI3K/Akt, STAT3, and MAPK signaling cascades. B-Raf mutation is not able to trigger melanoma development alone and requires cooperation with other cellular alterations in RTK signaling such as the Akt pathway (49) and leelamine has the potential to target these pathways as well.

Nanolipolee-007 inhibited the phosphorylation of Akt without affecting total Akt protein levels in a dose- and time-dependent manner. This, in turn, decreased down- stream levels of active PRAS40 and BAD proteins. In addition, Nanolipolee-007 decreased expression of prolif- eration marker cyclin D1 as well as increasing levels of apoptosis markers cleaved caspase-3 and FARP proteins (14). Nanolipolee-007 also inhibited Stat3 signaling in melanomas. Targeted inhibition of Stat3 is known to retard melanoma development and other studies also demonstrated that targeting Stat3 in conjunction with Akt synergistically inhibits melanomas (16, 50).

In conclusion, Nanolipolee-007 retained the tumor inhibitory activity of leelamine dissolved in DMSO and improved the solubility of the drug with negligible tox- icity in mice, suggesting its potential as a therapeutic agent for the treatment of melanoma or other cancers in which the PI3/Akt kinase, and STAT3, and to a lesser extent MAPK pathways are deregulated.

Disclosure of Potential Conflicts of Interest
The Pennsylvania State University has patented this discovery and licensed it to Melanovus Oncology, Inc. for commercialization. Dr. Robert- son has a financial interest (equity ownership) and business interests (Chief Scientific Officer and member of the Board of Directors) in Mela- novus Oncology, Inc. The Pennsylvania State University also has equity and royalty interests in Melanovus, Oncology, Inc. These interests and positions have been reviewed and managed by the University in accord- ance with its Conflict of Interest policies.

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Acknowledgments
The authors thank Katie Huber for technical assistance.

Grant Support
This work was supported by NIH grants R01 CA-136667-02, R01 CA-113863-02, R01 CA-127892-01A (to G.P. Robertson), The Foreman Foun- dation for Melanoma Research (to G.P. Robertson), and the H.G. Barsu- manian, M.D. Memorial Fund (to A. Sharma).

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Received April 24, 2014; revised May 7, 2014; accepted July 21, 2014; published OnlineFirst July 31, 2014.

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Molecular Cancer Therapeutics

Nanolipolee-007, a Novel Nanoparticle-Based Drug Containing Leelamine for the Treatment of Melanoma

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Mol Cancer Ther  Published OnlineFirst July 31, 2014.

Updated version  Access the most recent version of this article at: doi:10.1158/1535-7163.MCT-14-0357

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