Leelamine Mediates Cancer Cell Death through Inhibition of Intracellular Cholesterol Transport

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Abstract

Leelamine is a promising compound for the treatment of cancer; however, the molecular mechanisms leading to leelamine-mediated cell death have not been identified. This report shows that leelamine is a weakly basic amine with lysosomotropic properties, leading to its accumulation inside acidic organelles such as lysosomes. This accumulation leads to homeostatic imbalance in the lysosomal endosomal cell compartments that disrupts autophagic flux and intracellular cholesterol trafficking as well as receptor-mediated endocytosis. Electron micrographs of leelamine-treated cancer cells displayed accumulation of autophagosomes, membrane whorls, and lipofuscin-like structures, indicating disruption of lysosomal cell compartments. Early in the process, leelamine-mediated killing was a caspase-independent event triggered by cholesterol accumulation, as depletion of cholesterol using β-cyclodextrin treatment attenuated the cell death and restored the subcellular structures identified by electron microscopy. Protein microarray-based analyses of the intracellular signaling cascades showed alterations in RTK–AKT/STAT/MAPK signaling cascades, which was subsequently confirmed by Western blotting. Inhibition of Akt, Erk, and Stat signaling, together with abnormal deregulation of receptor tyrosine kinases, was caused by the inhibition of receptor-mediated endocytosis. This study is the first report demonstrating that leelamine is a lysosomotropic, intracellular cholesterol transport inhibitor with potential chemotherapeutic properties leading to inhibition of autophagic flux and induction of cholesterol accumulation in lysosomal/endosomal cell compartments. Importantly, the findings of this study show the potential of leelamine to disrupt cholesterol homeostasis for treatment of advanced-stage cancers. Mol Cancer Ther; 13(7): 1690–703. ©2014 AACR.

Introduction

Melanoma is the deadliest and most metastatic form of skin cancer (1). If it is detected early, surgery still is the most feasible option to cure the disease. However, if it metastasizes to other organs, less than 36% of the patients survive for longer than 1 year (1). Targeted therapies such as Zelboraf and dabrafenib, two U.S. Food and Drug Administration (FDA)–approved mutant B-RAF inhibitors, can be used for the short-term management of melanoma. However, an aggressive drug-resistant disease usually develops, limiting survival benefit to only a few months (2). Cancer cells were able to bypass these targeted therapies by compensating with alternative activation of the mitogen-activated protein kinase (MAPK) pathway, several by activating upstream receptor tyrosine kinases (RTK; ref. 3). Therefore, targeting these alternative escape routes through a combinatorial drug treatment approach or development of a drug that suppresses multiple disease-driving pathways is indispensable for a successful treatment of melanoma or prevention of recurrent resistant disease.

Natural compounds are important sources of new drug development and represent a significant portion of the FDA-approved cancer therapeutics portfolio (4). Through a natural compound library screen, leelamine, a tricyclic diterpene molecule that was extracted from the bark of pine trees, is identified and reported in the article by Gowda and colleagues (in the current issue of this journal) as a potential drug effective against advanced-stage melanoma. This agent has been shown to induce death of advanced-stage melanoma cell lines 3- to 5-fold more effectively than normal cells and has led to a 60% decrease in tumor burden compared with control vehicle-treated animals. In these studies, leelamine inhibited AKT and STAT3 signaling in xenografted tumors. No obvious systemic toxicity of the compound was detected as assessed by body weights of animals or by examination of the various blood parameters that are indicative of organ distress. However, the molecular mechanisms underlying the therapeutic efficacy of leelamine were unknown;
therefore, in this report, the mechanism of action of leelamine for induction of cell death in cancer cells has been identified.

Before this study, leelamine was reported to be a poor agonist of cannabinoid receptors (CBR) and a weak inhibitor of pyruvate dehydrogenase kinases (PDK; 5, 6). However, data presented here suggest that leelamine-mediated melanoma cell death does not involve modulation of any of these reported targets, but was rather initiated by the lysosomotropic property of the compound, which triggers accumulation of compound inside the acidic cell compartments. This accumulation led to the disruption of cholesterol homeostasis and intracellular vesicle transport systems as well as inhibition of autophagic flux. As a consequence, caspase-independent cell death was induced. Western blot analyses revealed inhibition of RTK, AKT, ERK, and STAT3 signaling cascades, which are reported to be important for the survival of melanoma cells. Lee-lamine-mediated inhibition of these cascades was attributed to the inhibition of receptor-mediated endocytosis of RTKs causing aberrant accumulation of these proteins in the perinuclear region of cells.

Materials and Methods

Cell lines, culture conditions, and plasmids
Metastatic melanoma cell line UACC 903 was provided by Dr. Mark Nelson (University of Arizona, Tucson, AZ) between 1995 and 1999, 1205 Lu cell line was provided by Dr. Herlyn (Wistar Institute, Philadelphia, PA) between 2003 and 2005, human fibroblast cell line FF2441 was provided by Dr. Craig Myers (Penn State College of Medicine, Hershey, PA) in 2003 and 2005, and these cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Invitrogen) supplemented with 1% GlutaMAX (Invitrogen) and 10% FBS (Hyclone) in a 37°C humidified 5% CO2 atmosphere incubator. Melanoma cell lines were periodically monitored for genotypic characteristics, phenotypic behavior, and tumorigenic potential, and DNA fragmentation assays were performed. However, in the case of β-cyclodextrin pretreatment, β-cyclodextrin was washed away after 60 minutes of treatment and subsequently the cells were treated either with dimethyl sulfoxide (DMSO) or leelamine. Twenty-four hours after treatment, MTS assay was performed.

Caspase dependence, mitochondrial membrane potential, and DNA fragmentation assays
Caspase dependence of cell death was measured by growing cells in a 96-well plate and preincubating with pan-caspase inhibitor z-VAD-fmk (20 μmol/L) for 1 hour before drug treatments. TRAIL (50 ng/mL) treatment was used as a positive control for induction of caspase-dependent cell death. Twenty-four hours after treatment, cell viability was measured by MTS assay. Mitochondrial membrane potential (ΔΨm) was measured via the TMRE Mitochondrial Membrane Potential Assay Kit (Abcam) according to the manufacturer’s protocol. For the DNA fragmentation assay, total DNA was collected using the DNeasy (Qiagen) Kit according to the instructions and loaded into 1% agarose gels for electrophoresis.

Electron microscopy analyses
UACC 903 cells growing at 70% to 80% confluency on 35-mm Permanox Petri dishes (Electron Microscopy Sciences) were treated with leelamine (3 μmol/L) alone or in combination with B-cyclodextrin (1 mmol/L) for 3 hours, washed with PBS, and then fixed with fixative (0.5% glutaraldehyde/4% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.3) for 1 hour. The cells were washed in 0.1 mol/L sodium cacodylate and post-fixed overnight in buffered 1% osmium tetroxide and 1.5% potassium ferrocyanide. After post-fixation, cells were rinsed with buffer, dehydrated in a graded series of ethanol, and embedded in EMbed812 (Electron Microscopy Sciences). After sectioning, samples were stained with 2% aqueous uranyl acetate and lead citrate followed by analysis with JEOL JEM1400 Digital Capture transmission electron microscope (TEM).

Kinexus and RTK protein arrays
UACC 903 cells treated with leelamine (3 μmol/L for 3, 6, 12, or 24 hours) were collected with Kinexus lysis buffer according to the Kinexus protocol and shipped to Kinexus for analyses with Kinexus Antibody Microarray Chip 1.3 (812 antibodies). The array data were normalized through Z-score transformation, and Z-ratios between treated samples and corresponding controls were calculated as described elsewhere (9). The data were analyzed through Ingenuity Pathway Analyses (IPA; version 17199142) software based on the alterations with a Z-ratio > 1.5 or Z-ratio < −1.5 with the software’s default settings. The Human Phospho-Receptor Tyrosine Kinase Array Kit was obtained from R&D Systems, and experiments were performed according to the manufacturer’s protocols. The blot images were quantified using Quantity One 1-D Software (Bio-Rad Laboratories).
Figure 1. Lysosomotropic property of leelamine inhibits autophagic flux. A, light microscopic images show vacuolization of melanoma cells after leelamine treatment. B, transmission electron micrographs show DMSO-treated control cells (a), leelamine-treated cells displaying formation of lipofuscin-like material (b), web-like membrane whorls (c), and increased number of autophagosomes (d). C, Western blot analyses showing LC3B and P62 protein levels as a marker of autophagic flux; Erk-2 served as a loading control. (Continued on the following page.)
Leelamine Inhibits Intracellular Cholesterol Transport

Cholesterol localization, quantitation, and thin layer chromatography analyses

Localization of intracellular cholesterol was detected through Cayman’s Cholesterol Cell-Based Detection Assay Kit (Cayman Chemical) according to the manufacturer’s protocol. Lipid extraction from cell cultures was achieved through the Bligh and Dyer method (10). The lipid extract was dissolved in chloroform:methanol (2:1) and stored at −20°C. Thin layer chromatography (TLC) analyses were undertaken according to a published approach (11). High-performance TLC (HPTLC) Silica gel 60 plates (Merck) were developed with iodine vapor. The bands were analyzed with ImageJ, an image analysis software (v1.44; NIH, Bethesda, MD). Retention factors were calculated as 0.35 for cholesterol, 0.1 for phosphatidylcholine, and 0.17 for phosphatidylethanolamine. These values were consistent with the values observed in the aforementioned published study (11).

Evaluation of endocytosis

Endocytic capacity of the cells was measured through evaluation of receptor-mediated endocytosis of Alexa Fluor 488–conjugated transferrin protein (Molecular Probes). Briefly, cells were seeded into chamber slides and treated with leelamine for 2 hours. Next, transferrin protein was added at a final concentration of 5 μg/mL and incubated for 30 minutes. Cells were then washed with PBS, trypsinized, and collected for flow cytometry analysis or fixed on a slide with 4% paraformaldehyde for fluorescence microscopy analysis.

Analysis of drug uptake using 3H-labeled leelamine

To analyze the kinetics of leelamine uptake, 3H-labeled leelamine was used (specific activity of 25 Ci/mmol; American Radio Chemicals Inc.). UACC 903 cells (70%–85% confluent in a 150-mm plate) were treated with 25 mL of DMEM containing 3 μmol/L leelamine and 5 μL of 20 μmol/L treated leelamine (1 million CPM/μL). At various time points, 20 μL of media from the plate was collected and radioactivity associated with the tritiated leelamine was measured using the LS-6500-Beckman Coulter liquid scintillation counter.

Analysis of lysosomotropism

UACC 903 cells were plated into 6-well plate and grown to 75% to 90% confluency. Cells were treated with 1 μmol/L LysoTracker Red DND-99 (Life Technologies) for 15 minutes. Cells were subsequently treated with leelamine or chloroquine for 45 minutes and collected for flow cytometry analysis.

Western blot analysis

Melanoma cells (1–1.5 × 10⁶) were plated in 100-mm culture dishes and grown to 75% to 90% confluency. After treatments, at indicated time points, cells were harvested in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (Pierce Bio-technology). Proteins were quantitated using the BCA assay from Pierce. Thirty micrograms of protein per lane was loaded onto a NuPage gel from Life Technologies and electrophoresed according to the manufacturer’s instructions. Proteins were transferred to polyvinylidene difluoride (PVDF) membrane, and blots were probed with antibodies according to the supplier’s recommendations (for detailed antibody information, see Supplementary Table S2). Immunoblots were developed using the enhanced chemiluminescence (ECL) detection system (Thermo Fisher Scientific).

siRNA transfections

siRNA (1 pmol/well) was transfected into UACC 903 cells that were seeded into 96-well plates using Lipofectamine RNAiMAX (Life Technologies) reagent according to the manufacturer’s protocols. Forty-eight hours after transfection, effect on cell viability was measured by MTS assays. Mutant BRAF siRNA and scrambled siRNA were used as positive and negative controls, respectively. Viability of cells was plotted against scrambled siRNA-transfected cells (siRNA sequences are provided in Supplementary Table S3).

Statistical analysis

The statistical analysis was performed using the unpaired Student t test. A P < 0.05 was considered statistically significant. * indicates P < 0.05.

Results

Leelamine inhibits autophagic flux in melanoma cells

To dissect the mechanism by which leelamine kills cancer cells, UACC 903 melanoma cells treated with leelamine were examined by light and electron microscopy. Light microscopy showed rapid and widespread vacuolization of the cells (Fig. 1A), followed by membrane blebbing, cell shrinkage, and cell rounding. Compared with control DMSO-treated cells (Fig. 1B, box a), TEM showed accumulation of lipofuscin-like material (Fig. 1B, box b; undegraded lysosomal waste), formation of web-like membrane whorls (Fig. 1B, box c), and increased number of autophagosomes (Fig. 1B, box d).

TEM analysis suggested that leelamine treatment led to autophagosome accumulation; therefore, the effect of leelamine on autophagic flux was next evaluated through

(Continued.) BafA1 treatment was used as a positive control for inhibition of autophagic flux. Bottom, confocal microscopy of GFP-tagged LC3B accumulation in leelamine- or BafA1-treated UACC 903 cells. D, light microscopic images of melanoma cells following leelamine treatment either alone or in combination with V-ATPase inhibitors Conc-A and BafA1. E, kinetics of 1H-labeled leelamine uptake. F, viability of cells exposed to conditioned media that is collected from leelamine-treated melanoma cells at different time points. G, histogram showing lysosomotropic property of leelamine, assessed by its competition with LysoTracker Red DND-99 dye, which was similar to chloroquine, a well-known lysosomotropic compound.
Western blotting. Treatment with Bafilomycin A1 (BafA1), a specific inhibitor of vacuolar type H\(^+\)-ATPase that blocks autophagic flux, induced accumulation of LC3B (an autophagosome marker) and p62/SQSTM1 (an autophagic flux marker) proteins, indicating the inhibition of autophagosomal degradation (Fig. 1C; ref. 12). Likewise, leelamine treatment induced the accumulation of both proteins in a dose-dependent manner, suggesting inhibition of autophagic flux. Accumulation of LC3B protein was detectable via fluorescence microscopy of UACC 903 cells expressing GFP-tagged LC3B protein (Fig. 1C). Leelamine treatment also dose-dependently increased the intensity ratio of LC3B-II to LC3B-I, further indicating enhanced autophagic activity (Fig. 1C).

**Leelamine possesses lysosomotropic property leading to accumulation inside the acidic cell compartments**

Because observations such as vacuolization of cells, accumulation of lipofuscin-like material, formation of web-like membrane whorls, and inhibition of autophagic flux are commonly attributed to lysosomal storage diseases and can be mimicked by some lysosomotropic compounds, the lysosomotropic potential of leelamine was next investigated (13, 14). As a weakly basic primary amine, leelamine has a \( pK_a \) of 9.9 (calculated by ACD Labs Precepta software v14.0), and it was therefore predicted to be a lysosomotropic compound. Treatment with vacuolar H\(^+\)-ATPase inhibitors can suppress the activity of lysosomotropic compounds by inhibiting the acidification of cell compartments (15). Pretreatment of UACC 903 melanoma cells with two vacuolar H\(^+\)-ATPase inhibitors that target different subunits of the H\(^+\)-ATPase complex, BafA1 and Concanaamycin A (Conc-A), suppressed leelamine-mediated cell vacuolization (Fig. 1D), suggesting that leelamine is a lysosomotropic compound.

Lysosomotropic compounds can be rapidly taken up by cells due to trapping inside the acidic organelles such as lysosomes and late endosomes (16, 17). To measure the kinetics of leelamine uptake, UACC 903 cells were treated with tritiated leelamine, and every 10 minutes, media samples were collected to quantify levels of tritiated compound remaining in the media. Sixty percent of the tritiated leelamine was internalized into cells in less than 30 minutes after treatment, which supports the lysosomotropic property of the compound (Fig. 1E). This was further confirmed by testing the efficacy of collected samples to decrease cell viability. UACC 903 cells were treated with 3 \( \mu \)mol/L leelamine in a 100 plate, and every 10 minutes, 300 \( \mu \)L of media was collected from the plate to treat UACC 903 cells that were plated in a 96-well plate (100 \( \mu \)L 3 wells/sample). Twenty-four hours later, cell viability was assessed by MTS assay. Consistent with the uptake kinetics of leelamine, samples collected 30 minutes after preincubation with cells did not induce cell death in UACC 903 cells (Fig. 1F).

To further validate the lysosomotropic property of leelamine, a modified LysoTracker Red DND-99 compen-
Figure 2. Lysosomotropic property of leelamine induced caspase-independent cell death leading to disruption of Δψm. A, viability of melanoma cells treated with leelamine in the absence or presence of V-ATPase inhibitors BafA1 or Conc-A. B, caspase dependence of leelamine-mediated cell death measured through treatment of melanoma cells with leelamine in the absence or presence of pan-caspase inhibitor, z-VAD-fmk. C, DNA laddering assay showing absence of DNA fragmentation following leelamine treatment. Stauroporine was used as a positive control for apoptosis-mediated DNA fragmentation. D, histogram showing Δψm following leelamine or FCCP (positive control) treatment. E, viability of wild-type or BAX-knockout HCT116 cells after 24 hours of treatment with increasing concentrations of leelamine. F, viability of wild-type or atg5-knockout MEF cells after 24 hours of treatment with increasing concentrations of leelamine. Images showing that leelamine did not cause vacuolization of ATG5-knockout MEF cells. (*, P < 0.05, t test.)
Leealmine caused lysosomal membrane permeabilization, lysosomal peptidase inhibitors, ALLN (calpain/cathepsin-inhibitor 1), ALLM (calpain/cathepsin-inhibitor 2), leupeptin (cysteine, serine, and threonine peptidase inhibitor), Pepstatin-A (aspartic proteinase inhibitor), and AEBSP (irreversible serine protease inhibitor), were used. However, none of these inhibitors were able to alter leelamine-mediated cell death, suggesting that lysosomal membrane permeabilization is not involved in leelamine-mediated cell death (Supplementary Fig. S2B).

Because disruption of mitochondrial function plays a key role in the execution of several cell death programs, mitochondrial membrane potential (Δψm) of UACC 903 cells was measured after leelamine treatment (22). In the positive control, 20 μmol/L of FCCP, a very potent uncoupler of oxidative phosphorylation, significantly hindered Δψm of treated cells. Leelamine treatment also decreased the Δψm of treated melanoma cells in a time- and dose-dependent manner (Fig. 2D). Of note, Δψm was found to be diminished in more than 70% of the cells when melanoma cells were treated with 3 μmol/L leelamine for 24 hours, suggesting that leelamine triggers significant perturbations in mitochondrial stability.

Bcl-2-associated X protein (BAX) and BH3 interacting-domain death agonist (BID) are two well-studied apoptosis regulators that induce the opening of the mitochondrial barrier of oxidative phosphorylation, significantly hindered Δψm of treated cells. Leelamine treatment also decreased the Δψm of treated melanoma cells in a time- and dose-dependent manner (Fig. 2D). Of note, Δψm was found to be diminished in more than 70% of the cells when melanoma cells were treated with 3 μmol/L leelamine for 24 hours, suggesting that leelamine triggers significant perturbations in mitochondrial stability.

Phenotypes induced by some lysosomotropic compounds (e.g., U18666A and imipramine) resemble those occurring with Niemann–Pick type C (NPC) disease (26, 27). NPC is a well-studied lysosomal storage disease, which leads to neurodegeneration and cell death through late endosomal/lysosomal (LE/L) accumulation of unesterified cholesterol due to loss-of-function mutations in NPC proteins (28). These compounds also lead to the accumulation of endosomal cholesterol upon treatment (26, 27). To investigate whether leelamine triggered a phenotype similar to NPC, cholesterol localization following leelamine treatment was analyzed via Filipin-III staining. Under steady-state conditions, UACC 903 cells stained weakly for Filipin at the periphery of the nucleus

**Activity of leelamine was not mediated by PDKs or CBRs**

Pyrubate dehydrogenase kinases (PDK) and cannabinoi

**Leealmine Inhibits Intracellular Cholesterol Transport**

Leelamine Inhibits Intracellular Cholesterol Transport

Figure 3. Leelamine-mediated cell death depends on intracellular cholesterol accumulation caused by inhibition of cellular endocytosis. A, fluorescence microscopy of cholesterol localization following leelamine or U18666A treatment. B, HPTLC showing cholesterol accumulation in melanoma cells with increasing leelamine concentrations. The numbers under the cholesterol band show the quantitation of the cholesterol band with respect to the DMSO treatment; PC, phosphatidylcholine; PE, phosphatidylethanolamine. C, viability of melanoma cells following leelamine treatment alone or in combination with co- or pretreatment with β-cyclodextrin. D, transmission electron micrographs of leelamine- and β-cyclodextrin–cotreated UACC 903 cells. E, fluorescence microscopic images showing endocytosis of Alexa Fluor–conjugated transferrin protein following DMSO control or leelamine treatment in the absence or presence of β-cyclodextrin–mediated cholesterol depletion. F, flow cytometry–based analyses of Alexa Fluor–conjugated transferrin protein endocytosis following leelamine treatment. DAPI, 4',6-diamidino-2-phenylindole. (*, P < 0.05, t test.)
Leelamine inhibited cellular endocytosis

Endosomes are major sorting compartments within cells functioning not only for the uptake of extracellular material, but also for the maintenance of cell signaling through recycling of membrane receptors (30, 31). Because endosomal accumulation of cholesterol has the potential to disrupt the endocytic system, the integrity of the system was assessed by measuring endocytic uptake of Alexa Fluor–conjugated transferrin protein (32, 33). Fluorescence microscopy analysis showed robust suppression of endocytosis following leelamine treatment for both UACC 903 and 1205 Lu melanoma cells (Fig. 3E). Depletion of cholesterol through β-cyclodextrin cotreatment prevented endocytosis (Fig. 3D). It is important to note that the inhibition of cholesterol transport but not the cholesterol synthesis led to cell death, as statins were not able to induce cell death in these cell lines (Supplementary Fig. S4).

Leelamine inhibited signaling pathways driving melanoma cell survival

Because leelamine disrupted cellular endocytosis, inhibition of this process was predicted to disrupt key signaling pathways important for melanoma survival. Therefore, signaling pathways altered following leelamine treatment were assessed. Since cycloheximide treatment suggested that protein synthesis was not involved in the activity of leelamine, the primary effect of the compound was predicted to occur at the posttranslational level. Therefore, to assess these changes, high-throughput antibody microarray analysis was undertaken using Kinexus Antibody Microarray Chip 1.3 (Kinexus Bioinformatics Corporation). This analysis simultaneously assessed the expression and phosphorylation status of various cell signaling proteins. Cell lysates were collected at various time points from 3 to 24 hours following leelamine or control treatment and analyzed using the Kinexus arrays. Data were normalized through Z-score transformation and significant alterations were identified by calculation of Z-ratios between treated samples and corresponding controls (Table 1; see Supplementary Table S4 for array results). Results suggested alterations in the members of the RTK–AKT signaling pathway [e.g., insulin—like growth factor 1 receptor (IGF1R), IRS1, ALK, EPHA1, ERBB2, GSK3, and FOXO3 (FKHR1.1); Fig. 4A]. Analyzes of the data through IPA software suggested that the insulin and phosphoinositide 3-kinase (PI3K)–AKT pathways were the most prominent pathways that were altered following leelamine treatment (Supplementary Fig. S5).

Involvement of key proteins that were downstream of RTK signaling was subsequently validated by Western blotting. Significant suppression of the active AKT (pAKT) and STAT3 proteins (pSTAT3) was identified (Fig. 4B). Suppression of several other signaling proteins [e.g., MAPK3/MAPK1 (Erk1/2), AKTS1 (PRA540), CREB, and RPS6KB1 (p70S6K)] in these pathways has been validated by Gowda and colleagues in the current issue of this journal. Phosphorylation of EIF4EBP1 (4EBP1), an important regulator of cap-dependent protein translation, was significantly decreased by leelamine, suggesting that the AKT/mTOR branch of the cascade was also inhibited following leelamine treatment (34). Most importantly, BafA1 cotreatment reversed the effect of leelamine on these signaling cascades, suggesting that these alterations were triggered by the lysosomotropic properties of leelamine (Fig. 4B).

Leelamine disrupted RTK signaling via interference with intracellular vesicular transport systems, which was reversible by cholesterol depletion

Because protein microarray analysis suggested alterations in RTK signaling, the activities of 42 different RTKs were analyzed using a protein array that is specific to RTKs (Fig. 4C). Alterations in tyrosine phosphorylation of several RTKs, such as decreases in ERBB4 and platelet—derived growth factor receptor (PDGFR) receptors, as well
Cellular vesicular transport system (Fig. 4E).

accumulation, further supporting inhibition of the intra-

in INSR/IGF1R-AKT signaling, displayed perinuclear

Moreover, IGF1R also displayed slight accumulation that was more

time-dependent accumulation of HGFR precursor protein

identified increases in HGFR and IGF1R phosphorylation

receptors (Fig. 4D). The precursor form of

receptor (HGFR) receptors were observed. However,

Western blot analyses displayed a dose- and

significant decrease in mature forms of both HGFR

as an increase in IGF1R and hepatocyte growth factor

receptor (HGFR) receptors were observed. However,

identified increases in HGFR and IGF1R phosphorylation

were associated with intracellular accumulation of these

receptors. Western blot analyses displayed a dose- and

time-dependent accumulation of HGFR precursor protein

with significant decrease in mature forms of both HGFR

and IGF1R receptors (Fig. 4D). The precursor form of

IGF1R also displayed slight accumulation that was more

significant after 12 hours of leelamine treatment. Accu-
mulation of these precursor proteins were possibly related
to the disruption of the endocytic system (33). Moreover,
immunofluorescence staining of various RTKs (IGF1R,
PGF1R, and TRK receptors) and IRS1, an adaptor protein in
INSR/IGF1R-AKT signaling, displayed perinuclear
accumulation, further supporting inhibition of the intra-
cellular vesicular transport system (Fig. 4E).

To demonstrate that signaling alterations were induced
by disrupted cholesterol homeostasis, β-cyclodextrin
cotreatment was used to deplete cholesterol, and effects
on inhibited signaling pathways were examined by
Western blotting (Fig. 4F). β-cyclodextrin cotreatment
restored phosphorylation of AKT and STAT3 proteins,
suppressed accumulation of IGF1R and HGFR precu-
sors, inhibited upregulation of CDKN1B (p27) protein,
reinstated CCND1 (cyclin D1) to control levels, and
decreased PARP cleavage (Fig. 4F). Thus, these observa-
tions suggested that leelamine-mediated signaling altera-
tions were initiated by disruption of cholesterol homeo-

Discussion

In this study, leelamine has been identified as a
lysosomotropic compound that disrupts intracellular

Table 1. Alterations in protein expression or activity following leelamine treatment

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NOTE: Values in bold indicate Z-Ratio > 1.5 or Z-Ratio < –1.5. % CFC: percentage change from control treatment.

*Antibody spots that were not reliable due to technical issues.

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cholesterol homeostasis to induce cell death more selectively in melanoma cells compared to normal cells. Cholesterol is an essential component of cell membranes and occupies vital roles in intracellular transport and signaling systems (36, 37). Its homeostasis is strictly regulated as proper functioning of several organelles, such as golgi, endoplasmic reticulum, and mitochondria, relies on cholesterol abundance in the membranes of these organelles (38, 39). Late endosomes and lysosomes have an important role in maintaining this homeostasis. Cholesterol that is derived from the membranes of the endocytotic vesicles and cholesteryl esters that are derived from the imported low-density lipoprotein (LDL) molecules or from the autophagic flux, converge on the lysosomal cell compartments where cholesteryl esters are hydrolyzed to free cholesterol molecules (39–41). Excess free cholesterol should be either esterified in the endoplasmic reticulum or removed from the cell through the efflux pathway (39).

NPC1 and NPC2 proteins function together to export free cholesterol from the LE/L cell compartments, and loss-of-function mutations in these genes give rise to the accumulation of free cholesterol in the LE/L compartments (42). Because lysosomes are a convergent point for the endocytic and autophagic pathways, cholesterol accumulation potentially shuts down both of these pathways.

Inhibition of autophagic flux is potentially detrimental to cells due to insufficient disposal of toxic protein aggregates and inadequate recycling of unnecessary cellular components to maintain intracellular homeostasis (17). Recent studies link autophagy to cholesterol homeostasis (41). Elrick and colleagues identified autophagy as an important source of accumulated cholesterol in NPC disease (43). In their study, ATG5-null-MEF cells accumulated less cholesterol in the LE/L compartments upon U18666A treatment. In agreement with this observation, ATG5-null-MEF cells were more resistant to leelamine-mediated cell death compared with wild-type counterparts. These observations suggested autophagy as an important source for accumulated cholesterol in leelamine-treated cells. Moreover, endosomal cholesterol accumulation not only inhibits autophagic flux but can also induce autophagy itself (44). Leelamine treatment dose-dependently increased...

Figure 4. Leelamine mediated alterations in signaling pathways. A, schematic summary of signaling alterations in melanoma cells occurring following leelamine treatment based on Kinexus antibody array analysis. B, Western blot (WB) analysis of pAKT (S472), total AKT, 4EBP1(T70), pSTAT3(Y705), STAT3, and ERK-2 proteins function together to export free cholesterol from the LE/L cell compartments, and loss-of-function mutations in these genes give rise to the accumulation of free cholesterol in the LE/L compartments (42). Because lysosomes are a convergent point for the endocytic and autophagic pathways, cholesterol accumulation potentially shuts down both of these pathways.

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Figure 5. Schematic summary of cellular alterations mediated by leelamine in melanoma cells.
LC3BII to LC3BII ratio, as well as decreased the phosphorylation of 4EBP1, suggesting the sustained inhibition of mTOR signaling and induction of autophagy. Thus, the autophagic process creates a vicious cycle between cholesterol accumulation and autophagy induction in which endosomal cholesterol accumulation triggers autophagy, and autophagy subsequently induces further endosomal cholesterol accumulation, which is summarized in Fig. 5.

In contrast to autophagy, inhibition of endocytosis disrupts intracellular signaling processes, as receptor-mediated signaling depends on endocytosis and endocytic recycling of internalized receptors to the cell membrane (45). RTKs are an important family of membrane receptors that are regulated through receptor-mediated endocytosis. Upon ligand binding and activation, they are internalized through endocytosis and transported to the late endosomes where they are either recycled back to the membrane or directed to the lysosomes for degradation (45). This process is important for downregulation of initiated signal transduction and also is required for transduction of various signals from the cell periphery to the nucleus (46). RTKs play vital roles in the progression of several cancers including melanoma (47). Hyperactivation of several RTKs, such as PDGFR, ERBB4, AXL, and IGFR, contribute to mutant BRAF inhibitor resistance (3). They induce PI3K–AKT, Stat, and mitogen—activated protein kinase (MAPK) signaling cascades in response to extracellular factors. Leelamine-mediated disruption of RTK signaling led to the inhibition of these three signaling cascades. However, inhibition of MAPK signaling was not very prominent in contrast to AKT3 and STAT3 pathway shutdown, as the constitutive activation of the MAPK cascade is triggered by mutant V600E BRAF protein and does not require RTK activity (48). Silencing of AKT activity significantly suppresses melanoma tumor growth (49). Cell lines with over-activated AKT signaling show increased sensitivity to the inhibition of the PI3K–AKT signaling pathway (50). Because leelamine inhibits AKT signaling, it is effective for killing cells in which the PI3K pathway is activated. RTKs also mediate induction of Stat signaling, which is reported to be essential for the transforming activity of the various RTKs such as IGFR (51). Under steady-state growth conditions, activity of STAT proteins is transient and tightly regulated by various signaling pathways (52). However, STATs are constitutively activated and promote tumor development in several malignancies, including melanoma (53). Niu and colleagues reported constitutive activation of STAT3 in more than 80% of melanoma cell lines in which hyperactivated STAT3 inhibits apoptotic pathways through induction of BCL2L1 (BCL-XL) expression (54). Our studies showed that leelamine significantly hinders STAT3 activity and decreases BCL2L1 protein levels as observed in Kinexus array analysis and in subsequent validation studies.

In summary, this study identifies leelamine as a lysosomotropic agent that triggers cell death through cholesterol accumulation in LE/L cell compartments. The accumulated cholesterol inhibits autophagic flux, disrupts receptor-mediated endocytosis, and subsequently inhibits signaling pathways that are key to melanoma development. These findings not only suggest significant potential of leelamine for the treatment of melanoma, but also identify a new approach for induction of melanoma cell death and possibly that of other cancer types.

Disclosure of Potential Conflicts of Interest
Penn State has patent protected this discovery, which has subsequently been licensed to Melanovus Oncology. Melanovus Oncology is partly owned by Penn State University and Gavin P. Robertson, who is also CSO of the company. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: O.F. Kuzu, R. Gowda, A. Sharma, G.P. Robertson Development of methodology: O.F. Kuzu, R. Gowda, A. Sharma Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): O.F. Kuzu, A. Sharma Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): O.F. Kuzu Writing, review, and/or revision of the manuscript: O.F. Kuzu, A. Sharma, G.P. Robertson Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Gowda, G.P. Robertson Study supervision: R. Gowda, A. Sharma, G.P. Robertson

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References


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