Targeting Multiple Key Signaling Pathways in Melanoma using Leelamine

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Running title: Targeting melanomas using leelamine.

Key words: Melanoma, natural products, leelamine, tumor development, PI3K, MAPK, STAT3.

Conflict 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.

Grant support: NIH grants R01 CA-136667-02, RO1 CA-1138634-02, RO1 CA-127892-01A (GP, Robertson), The Foreman Foundation for Melanoma Research (GP Robertson) and H.G. Barsumian, M.D. Memorial Fund (A Sharma).
ABSTRACT

Melanoma is a highly drug resistant cancer with resistance developing to agents targeting single proteins. To circumvent this problem, a new class of agent inhibiting multiple key pathways important in this disease is being developed to reduce the likelihood of developing resistant disease. The PI3 kinase (PI3K), MAP kinase (MAPK) and STAT3 pathways are constitutively activated in 50-70% of melanomas promoting disease development. To identify a drug simultaneously targeting the PI3K, MAPK and STAT3 cascades, a natural product library was screened to identify leelamine as a potential inhibitor. Leelamine was 4.5-fold more effective at inhibiting cultured melanoma cell survival than normal cells, with average IC$_{50}$ values of 2 and 9.3 µmol/L, respectively. It inhibited cellular proliferation at a concentration of 2.5 µmol/L by 40-80% and longer exposure increased apoptosis 600% through a mechanism detailed in the article in the current issue of this journal by Kuzu OF et al. Leelamine inhibited the growth of preexisting xenografted melanoma tumors by an average of 60% by targeting the PI3K, MAPK and STAT3 pathways without affecting animal body weight or blood markers of major organ function. The mechanism of action of leelamine is mediated by disruption of cholesterol transport, causing decreased cellular proliferation and, consequently leading to increased tumor cell apoptosis as well as decreased tumor vascularization. Thus, a unique agent and novel mechanism of action has been identified for the treatment of melanoma that acts by inhibiting the activity of three major signaling pathways regulating the development of this disease.
INTRODUCTION

Melanoma remains a highly drug resistant tumor type (1). Resistance develops relatively quickly to drugs targeting single proteins as occurs with agents such as Zelboraf targeting mutant V600E-B-Raf, which is present in approximately 50% of sporadic melanomas (2). To combat the development of resistance, one approach has been to identify a new class of agent inhibiting multiple key pathways important in melanoma (3, 4).

Agents simultaneously inhibiting several key pathways aiding melanoma development would be a first in class type of new drug for treating melanoma. An agent of this type would be predicted to more effectively reduce the likelihood of recurrent resistant disease, which is occurring with targeted agents such as Zelboraf (4). However, agents of this type do not currently exist. The PI3 kinase (PI3K), MAP kinase (MAPK) and STAT3 pathways play major roles in melanoma (5-8). These signaling pathways are constitutively activated in up to 70% of melanomas, functioning to reduce cellular apoptosis, increase proliferation and aid the invasive processes promoting melanoma progression (5-11). Currently, no approach has been identified to simultaneously target these pathways to treat melanoma.

Natural products can be a source of effective cancer drugs and several are being used for treating a wide variety of cancer types (12-14). Over 60% of anti-cancer agents are derived from plants, animals, marine sources or microorganisms (14, 15). Examples are taxanes, vinca alkaloids, and camptothecins, used as chemotherapeutic agents to treat breast, lung, ovarian, bladder, head and neck, cervical and skin cancers (16, 17). Leelamine (also called as Dehydroabietylamine) is derived from the bark of pine trees (18). Relatively little is known regarding its mechanism of action other than it is weakly binding to the cannabinoid receptor CB1 but not stimulating G-protein activity (19) and possible modulation of pyruvate dehydrogenase kinase activity (20).

To identify a drug that might simultaneously target the PI3K/Akt, MAPK and STAT3 cascades, a natural product library (NPL-480) consisting of 480 compounds derived from plants, animals, bacterial and fungal sources were screened to identify those that would inhibit melanoma cell survival by targeting key pathways needed for melanoma cell survival while not modulating others. Leelamine
was 4.5-fold more effective at inhibiting cultured melanoma cell survival than normal cells. It inhibited cellular proliferation and increased apoptosis by targeting the PI3K/Akt, MAPK and STAT3 pathways. Leelamine inhibited the growth of preexisting xenografted melanoma tumors by an average of 60% without affecting animal body weight or blood markers of major organ function. The mechanism of action of leelamine occurred through simultaneous inhibition of pAkt, pErk and pStat3 activity in these pathways through a unique mechanism detailed in the article in the current issue of this journal by Kuzu OF et al. Collectively, these discoveries provide novel insights into the therapeutic implications of using leelamine for the treatment of melanoma by simultaneously inhibiting multiple key driver signaling pathways promoting this disease.
MATERIALS AND METHODS

**Cell line and culture conditions.** Human primary melanocyte FOM103 provided by Dr. Herlyn (between 2003-2005), Wistar Institute, Philadelphia, PA, human fibroblast FF2441 and human foreskin keratinocyte provided by Dr. Craig Myers (in 2005), Penn State College of Medicine, Hershey, PA cell lines all containing wild type B-Raf were cultured as described (21). Human melanoma cell lines WM35, WM115, WM278.1, SK-MEL-24, 1205 Lu provided by Dr. Herlyn (between 2003-2005), Wistar Institute, Philadelphia, PA and UACC 903 provided by Dr. Mark Nelson (1995-1999), University of Arizona, Tucson, AZ, all containing mutant \(^{V600E}\) B-Raf were cultured as described (8, 21-23). Wild type B-Raf containing SbCl\(_2\) provided by Dr. Herlyn (between 2003-2005), Wistar Institute, Philadelphia, PA, C8161.Cl9 provided by Dr. Danny Welch (2003), University of Kansas, Kansas City, KS and MelJuSo provided by Dr. Judith Johnson (between 1995-1999), Institute for Immunology, Germany) cell lines were cultured as described (23, 24). All the cell lines were maintained in a 37°C humidified 5% CO\(_2\) atmosphere incubator and periodically monitored for genotypic characteristics, phenotypic behavior and tumorigenic potential of melanoma cell lines.

**Natural product screening.** Natural product library NPL- 480 (TimTec Inc., Newark, DE) consists of 480 compounds derived from plants, animals, bacteria and fungus. The library was screened to identify compounds inhibiting melanoma cell survival and subsequently for those inhibiting multiple key pathways important in melanoma development. Powders or oils constituting the library were dissolved in DMSO to a stock concentration of 10 mM and stored at -20°C. DMSO concentrations in the reaction mixture did not exceed 0.5% (vol/vol). For the initial screen, 5\(\times\)10\(^3\) UACC 903 melanoma cells were plated in 96-well plates for 24 hours followed by treatment with each compound at a concentration of 5 \(\mu\)mol/L. Viability was measured using MTS assay. The screen was repeated thrice and data represent averages; bars, ± S.E.M.

**Cell viability, proliferation, apoptosis and cell cycle analysis.** Cell viability and IC\(_{50}\) of normal human melanocytes, keratinocyte, fibroblasts and cell lines derived from melanoma and other malignancies following treatment with leelamine were measured using MTS assay (Promega, Madison, WI). In brief, 5\(\times\)10\(^3\) cells per well in 100 \(\mu\)L of media were plated and grown in a 96-well plate for 48 or 72 hours respectively for those representing normal cells (FOM103, FF2441 and HFK)...
and melanoma cell lines (WM35, SbCl2, WM115, WM278.1, SK-MEL-24, 1205 Lu and UACC 903). Each cell line was treated with either DMSO vehicle control or 0.62 to 40 µmol/L of leelamine for 24, 48 or 72 hours. IC₅₀ values for each cell line for each compound in µmol/L were measured from three independent experiments using GraphPad Prism version 4.01 (GraphPad Software, La Jolla, CA).

Rates of proliferation and apoptosis were measured by seeding 5X10³ cells in 96-well plates, followed by treatment with 0.62 to 10 µmol/L of leelamine for 24 hours. Proliferating and apoptotic cells were quantified using a colorimetric cell proliferation ELISA BrdU kit (Roche diagnostics, Indianapolis, IN) or Apo-ONE Homogenous caspase-3/7 assay kit (Promega, Madison, WI), respectively. Data represent averages of at least 3 independent experiments; bars, S.E.M.

Cell cycle analysis was undertaken by growing UACC 903 and 1205 Lu melanoma cells in 100-mm culture dishes followed by treatment with 2 or 3 µmol/L of leelamine for 24 hours. The samples were processed as described previously (25). Stained cells were analyzed using the FACScan analyzer (Becton Dickinson, Franklin lakes, NJ) and data processed utilizing ModFit LT software (Verity Software House, Topsham, ME). Data represent averages of at least 3 independent experiments; bars, S.E.M.

Identification and validation of pathways targeted by leelamine using the Kinexus antibody microarray and Ingenuity Pathway Analysis. A Kinexus antibody microarray was used to identify the pathways targeted by leelamine using the protocols provided by the Kinexus company (http://www.kinexus.ca/). In brief, 1.5X10⁶ UACC 903 cells were plated in 100 mm dishes and 48 hours later, treated with 3 µmol/L leelamine for 3-24 hours, lysates collected and processed by Kinexus using 812-antibody microarray analysis. Kinexus 812-antibody microarray results were analyzed using Ingenuity Pathway Analysis software. Significantly up-regulated or down-regulated pan or phospho specific proteins or unaffected proteins with corresponding Swiss-Prot accession numbers and ratio changes were uploaded as an Excel spreadsheet file to the Ingenuity Pathway Analysis server and pathways identified. Involvement or lack of involvement of signaling pathways was validated by independent Western blot analysis.
Western blot analysis. Cell lysates were harvested by addition of RIPA lysis buffer and samples were processed as described. Briefly, 1.5X10^6 melanoma cells were plated in 100 mm culture dishes and 48 hours later, treated with leelamine (3-6 µmol/L) for 3 to 24 hours. Protein lysates were collected for Western blotting and targets validated. Blots were probed with antibodies according to each supplier’s recommendations: antibodies to total Akt, phospho-Akt (Ser473), total AURKB, phospho-AURKB (Thr232), β-catenin, total CDK2, phospho-CDK2 (Thr160), total GSK3α, total GSK3β, phospho-GSK3α/β (Tyr279/Tyr216) active, total glycogen synthase, phospho-glycogen synthase (Ser641), phospho-IKKα/β (Ser 176/Ser180), Bcl2, phopho-p38MAPK (Thr180/Tyr182), α-tubulin, phospho-TAK (Thr 184), total PRAS40, phospho-PRAS40 (Thr246), total CREB, phospho-CREB (Ser133), phospho-p70 S6 kinase (Thr389), total Erk1/2, phospho-Erk1/2 (Thr202/Tyr 204), total Stat1, phospho-Stat1 (Tyr701), phospho-Stat2 (Tyr690), total Stat3, phospho-Stat3 (Tyr705) and cleaved PARP from Cell Signaling Technology (Danvers, MA); total PRAS40 from Invitrogen (Carlsbad, CA); cyclin D1, α-enolase and secondary antibodies conjugated with horseradish peroxidase from Santa Cruz Biotechnology (Santa Cruz, CA). Immunoblots were developed using the enhanced chemiluminescence (ECL) detection system (Thermo Fisher Scientific, Rockford, IL).

Tumorigenicity assessments. Animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee at Penn State University. Tumor kinetics were measured by subcutaneous injection of 1X10^6 UACC 903 or 1205 Lu cells in 0.2 mL of DMEM supplemented with 10% FBS and 1% Glutamax subcutaneously injected above both left and right rib cages of 3- to 4-wk-old female Athymic Nude-Foxn1nu mice (Harlan Sprague Dawley, IN). Six days later, when a fully vascularized 50-75 mm^3 tumor had formed, mice were randomly divided into DMSO vehicle control and experimental groups (5 mice/group; 2 tumors/mouse) and treated intraperitoneally with 2.5-7.5 mg/kg body weight leelamine depending on the cell line daily for 3 to 4 weeks. Body weight in grams and dimensions of the developing tumors in mm^3 were measured at the time of drug treatment (25).

Size and time matched tumors for analysis of biological processes regulating tumor development. Pathways targeted by leelamine and mechanism by which leelamine inhibited tumor development was established by comparing size and time matched melanoma tumors treated with leelamine compared to DMSO vehicle treated animals. 2.5X10^6 UACC 903 cells were injected s.c.
into nude mice, generating tumors of the same size developing at parallel time points. Six days later, mice were treated i.p. with DMSO vehicle or 7.5 mg/kg body weight of leelamine 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 immunohistochemistry and Western blotting analysis (8, 24). Cell proliferation was calculated using mouse anti-human Ki-67 staining from Pharmingen (San Diego, CA). Apoptosis rates were scored using “terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)” TMR Red Apoptosis kit from Roche (Mannheim, Germany). Vessel density indicative of apoptosis was estimated using a purified rat anti-mouse CD31 (PECAM-1) monoclonal antibody immunostaining (Pharmingen). Number of Ki-67, TUNEL and CD31 stained cells were quantified as the percentage of total cells in tumors using the IP Lab imaging software program. For all tumor analyses, a minimum of 6 different tumors with 4-6 fields per tumor was analyzed and data represent averages; bars, ± S.E.M. Western blot analysis of size and time matched tumors lysates harvested at days 11, 13 and 15 from animals treated with leelamine were analyzed for pAkt (Ser473) and pStat3 (Tyr705) compared with vehicle DMSO control treated animals.

**Subchronic toxicity assessments.** Swiss Webster (n=5) mice were i. p. injected with 5 or 10 mg/kg body weights every day for 22 days. Animals were weighed daily to ascertain toxicity leading to changes in body weight. At the end of treatment, blood was collected from each sacrificed animal in a serum separator tube with lithium heparin (BD Microtainer) following cardiac puncture and levels of ALP (Alkaline phosphatase), ALT (Alanine aminotransferase), AST (Aspartate aminotransferase), CK (Creatine kinase), CREA (Creatinine) and GLU (Glucose) measured (25). Vital organs including liver, spleen, kidney, intestine, lung and heart from control and experimental animals were collected on day 22, formalin fixed, paraffin-embedded, H&E stained and analyzed microscopically for changes in cellular morphology or tissue architecture (25).

**Statistical analysis.** Statistical analysis was performed using Prism 4.01 GraphPad Software and R version 2.15.1. One-way or Two-way Analysis Of Variance (ANOVA) was used for groupwise comparisons, followed by the Tukey’s or Bonferroni’s post hoc tests (26). For comparison between two groups, Student t test was used. Results represent at least two to three independent experiments and are shown as averages ± S.E.M. Results with a P value less than 0.05 (95% CI)
were considered significant. Number of asterisks in the figures indicates the level of statistical significance as follows: * P < 0.05, ** P < 0.01, *** P < 0.001.
RESULTS

Natural product library screen identified leelamine as a potent anti-melanoma agent. Natural product library NPL-480 (from TimTec Inc.) consisting of 480 compounds derived from plants, animal, bacteria and fungus was screened using MTS assay to identify agents inhibiting melanoma cell survival. The primary screen was conducted using the UACC 903 melanoma cell line following treatment with a concentration of 5 µmol/L for 24 hours. Compounds showing a minimum of 70% inhibition were considered as potential hits (Fig. 1A). Leelamine was the most potent inhibitory compound (Fig. 1B), decreasing cell viability by approximately 95% at 5 µmol/L (Fig. 1A). Next, efficacy of leelamine for killing melanoma cells isolated from various stages of melanoma development was compared to normal cells (Table 1). On average leelamine was 4.5-fold less toxic to normal control cells compared to melanoma cells (Fig. 1C). Leelamine concentration of 5 to 8 µmol/L killed 50% of normal cells following 72 hours exposure compared with 1 to 2 µmol/L for cell lines derived from advanced stage melanomas, suggesting potential cancer therapeutic utility at concentrations <2 µmol/L (Table 1). Furthermore, leelamine inhibited the growth of melanoma cell lines at IC50s of 3-7 µmol/L irrespective of B-Raf mutational status (Table 1).

Leelamine decreased the proliferative potential and increased apoptotic rates of cultured melanoma cells. To determine the mechanism through which leelamine inhibited cultured melanoma cell survival, proliferation and apoptosis rates were assessed (25). Leelamine inhibited the viability of UACC 903 and 1205 Lu cells as measured by MTS assay in a dose dependent manner (Fig. 2A). Increasing concentrations of leelamine from 0.62 to 10 µmol/L decreased the cellular proliferative potential as measured by BrdU incorporation (Fig. 2B) and increased cellular apoptosis measured by caspase-3/7 activity (Fig. 2C) of UACC 903 and 1205 Lu cells with similar inhibitory patterns following treatment. Cell cycle analysis of propidium iodide stained UACC 903 and 1205 Lu cells following 24 hours leelamine treatment, showed an increase in the sub-G0/G1 and G0/G1 cell populations, with a corresponding decrease in the S-phase population (Fig. 2D). Thus, leelamine 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.
Leelamine inhibits the activity of three driver pathways promoting melanoma development.

Pathways targeted by leelamine in melanoma cells were identified using a Kinexus antibody microarray and Ingenuity Pathway Analysis followed by Western blot confirmation. Leelamine altered the expression/activity of some but not all proteins or pathways, which was due to its unique mechanism of action, detailed in the manuscript by Kuzu OF et al., in the current issue of this journal. There was a consistent decreases in the members of the PI3K, MAPK and STAT3 signaling pathways following leelamine treatment, which are the major signaling cascades promoting melanoma development (5-8, 27). Decreased signaling through each pathway following treatment with 3 to 6 μmol/L of leelamine for 3 to 24 hours is shown for PI3K-Akt (Fig. 3A, Supplementary Figures 1 and 2), MAPK (Fig. 3B, Supplementary Figures 1 and 2) and STAT3 pathways (Fig. 3C and Supplementary Figure 2). Similar signaling inhibition was observed for both UACC 903 and 1205 Lu cell lines, with inhibition of PI3K-Akt and MAPK pathways occurring at 3 to 6 hours while inhibition of the STAT3 pathway occurred from 12 hours of treatment (Fig. 3C). The mechanism leading to the simultaneous inhibition of the PI3K-Akt, MAPK and STAT3 occurs through inhibition of intracellular cholesterol transport and is detailed in the manuscript by Kuzu OF et al., in the current issue of this journal. Pathway or protein expression or activity not regulated by leelamine is listed in Supplementary Table 1 and Supplementary Figure 3.

Leelamine reduced melanoma tumor development with negligible toxicity. Efficacy of leelamine for inhibiting melanoma tumor growth was evaluated on preexisting tumors following subcutaneous injection of cells into nude mice (25). UACC 903 or 1205 Lu melanoma cells were injected subcutaneously and 6 days later when a vascularized tumor had formed, mice were treated with i.p. injections of leelamine or control DMSO vehicle alone on a daily basis and tumor development measured at 2-day intervals for 3 to 4 weeks (Figs. 4A & 4B). Leelamine at 5-7.5 mg/kg led to significantly reduced tumor volume by 61% and 57% for UACC 903 (Fig. 4A) and 1205 Lu (Fig. 4B) cell lines respectively, compared to DMSO vehicle control (Fig. 4A). Body weights of mice at these concentrations of leelamine showed no significant differences between leelamine or control groups, again suggesting negligible toxicity (Figs. 4A & 4B; inset). Furthermore, subchronic toxicity was further assessed following daily i.p. treatment of Swiss Webster mice with 5 or 10 mg/kg body weights every day for 22 days. Body weights of these mice showed no significant differences between groups suggesting negligible toxicity (Fig. 4C). Next, blood parameters (Alkaline phosphatase, alanine...
aminotransferase, aspartate aminotransferase, creatine kinase, creatinine and glucose) indicative of organ related toxicity were measured following systemic administration of 10 mg/kg-body weight of leelamine after 22 days of treatment to further demonstrate negligible toxicity mediated by the agent (Fig. 4D). No significant differences between controls or leelamine treated animals for any of these parameters were observed. Furthermore, histological examination of H&E stained vital organ sections showed no change in cellular morphology or overall structure of the liver, spleen, kidney, intestine, lung or heart following treatment with 10 mg/kg body weight of leelamine after 22 days of treatment (Fig. 4E).

Leelamine decreased the proliferative potential of melanoma tumor cells leading to increased apoptosis and decreased vascular development. To identify the underlying mechanism by which leelamine inhibited melanoma tumor growth, an established published approach was used (8, 24, 25). It involved quantifying the rates of cellular proliferation (using Ki-67 staining), apoptosis (using TUNEL staining) and tumor angiogenesis (using CD31 staining) occurring in time and size matched tumors treated with leelamine compared with DMSO exposed control animals. Size and time matched tumors at days 11, 13 and 15 were compared to identify the first statistically quantifiable difference in cell proliferation, apoptosis or vascular development affected by leelamine treatment (8, 24, 25). At day 11, a statistically significant 50% reduction in proliferating cells (Fig. 5A) was observed after the leelamine treatment but not in cellular apoptosis or vascular development rates compared with DMSO control treated animals (Figs. 5B & 5C). Similar significant differences in cellular proliferation, apoptosis and vascular development were detected in all tumors compared with DMSO controls at days 13 and 15, suggesting that lack of proliferation subsequently triggered apoptosis and decreased vascular development (Figs. 5A, B & C). Western blot analysis of size and time matched tumor lysates harvested at days 11, 13 and 15 from animals treated from day 6 with leelamine showed decreased active pAkt (Ser473) and pStat3 (Tyr705) compared with vehicle DMSO control treated animals indicating the compound was acting on the pathways inhibited by leelamine to mediate the effects observed (Fig. 5D). However, no significant changes in the pErk1/2 levels were observed in the tumor lysates (Supplementary Figure 4).
DISCUSSION

According to the American Cancer Society, incidence and mortality rates for malignant melanoma continue to increase annually and it remains one of the most invasive as well as drug resistant cancers (28). Early stage disease can be treated with surgery or radiation, while guidelines for later stage disease recommend chemotherapy, interferon, interleukin-2 or targeted inhibitors (29). Although efforts have been made to design structurally well-defined small molecule targeted inhibitors that interact with single deregulated proteins in melanoma cells (30), these efforts have failed due to the development of resistant disease, suggesting a problem for any targeted agent inhibiting a single protein or pathway (31, 32).

Zelboraf and Yervoy were recently approved by the FDA for treating advanced melanoma (4, 33). Zelboraf has been evaluated in the 50% of the patients having mutant V600E-B-Raf protein with an approximately 80% partial or complete anti-tumor response rate during the first 2-month treatment cycle (4, 30, 34). However, as observed with molecularly targeted agents in other malignancies, tumors initially responsive to Zelboraf with an average regression period of 2 to 18 months and 6.2 months progression-free survival, developed drug resistance and invasive recurrent tumors (34-36). Resistance to Zelboraf illustrates the drug resistance hurdle faced by melanoma drugs inhibiting single targets (35). Development of resistance in cultured cells, animal models or in tumors from patients was mediated by secondary B-Raf mutations, alternate pathways of MAPK reactivation, or activation of compensating alternative survival pathways (32, 35, 37). In clinical studies, survival was extended by approximately 50% (3 to 5 months) and nearly all Zelboraf treated patients eventually relapsed after a period of progression-free survival with drug resistant invasive disease (2, 38).

Yervoy may be effective in 10 to 20 % of melanoma patients and has side effects that might limit its use (33). While initially effective, the formation of tumors resistant to this agent is likely to occur with other immune system modulators (39). These observations underscore the plasticity of melanoma in acquiring resistance to targeted chemotherapeutic or immune modulating agents and the need to identify agents targeting multiple important pathways involved in melanoma to circumvent the development of resistance (40, 41). This may be achievable through the use of drug cocktails or a single drug simultaneously inhibiting multiple key signaling pathways implicated in melanoma, which is detailed in this report through the discovery of leelamine.
Leelamine is the novel anticancer agent described in this manuscript that targets multiple key pathways important in melanoma. Using a combination of protein arrays and systems biology followed by validation studies, leelamine was found to inhibit the PI3K (pAkt), MAPK (pErk) and STAT (pStat3) signaling pathways deregulated in melanoma through inhibition of intracellular cholesterol transport, detailed in the manuscript by Kuzu OF et al., in the current issue of this journal. This makes leelamine a first-in-class multi-target inhibitor for the treatment of melanoma, which uniquely attacks melanoma tumor development by inhibiting three major signaling cascades regulating the development of this disease.

The PI3K, MAPK and STAT3 signaling pathways are constitutively activated in melanoma and play a prominent role in the development of recurrent resistant disease (5-7, 27). The MAP kinase pathway through B-Raf mutation is activated in ~50% of melanomas with 90% of these mutations leading to $V^{600E}$-B-Raf protein that is 10.7-fold more active than wild type protein (42). This occurs due to a conformational change in protein structure, where glutamic acid acts as a phosphomimetic between the Thr$^{598}$ and Ser$^{601}$ phosphorylation sites (42). Pharmacological or genetic approaches inhibiting the MAP kinase cascade reduce tumor development and decrease metastasis development (23). The PI3 kinase is equally important in melanoma development and has been shown to be upregulated in up to 70% of sporadic melanoma through copy number increases of the Akt3 gene and preferential activation in this cancer type (7, 43). Targeted inhibition of Akt3 or its downstream target PRAS40 has been shown to retard melanoma growth in animals and sensitize cells to various therapeutic agents (9, 44). Akt3 has also been found to mediate resistance to B-Raf inhibitor (Zelboraf) treatment (45). Recent studies evaluating the therapeutic efficacy of targeting STAT3 pathways also showed melanoma growth inhibition when STAT3 signaling is downregulated (27, 46). Results from these studies demonstrate the pivotal role played by these signaling cascades in regulating melanoma tumorigenesis and metastasis (43).

Mechanistically, leelamine inhibits the P13K, MAPK and STAT signaling pathways reducing phosphorylation of Akt, Erk and Stat3 without affecting total protein levels in a dose and time dependent manner. The consequence is inhibition of melanoma xenograft tumor development without affecting animal weight or organ function. Leelamine had a minor effect on the ERK signaling pathway in cultured cells and no measurable effect in tumors in mice treated with the drug, likely due
to the drug mechanism of action that involves inhibition of receptor-mediated endocytosis that shuts down receptor tyrosine kinase (RTK) signaling and inhibits the activation of downstream Akt, MAPK, and Stat3 signaling cascades. Since, mutant B-Raf activates the MAP kinase cascade downstream of the RTKs, leelamine only moderately inhibits the ERK signaling pathway. Furthermore, B-Raf mutation is not able to trigger melanoma development alone and requires cooperation with other cellular alterations, which might account for this observation (47). The detailed mechanism of action of leelamine is outlined in the manuscript by Kuzu OF et al., in the current issue of this journal.

Targeting multiple key pathways involved in melanoma by combining existing agents could also help prevent recurrent resistant disease (40, 41). Preclinical and clinical studies have tested whether combining Zelboraf with agents targeting the PI3K pathway or MEK1/2 would cooperatively inhibit melanoma tumor growth (48). Combining B-Raf inhibitor (GSK2118436) with a selective MEK inhibitor (GSK1120212) has been shown in preclinical studies to cooperatively decrease xenografted melanoma tumor development (49). Preclinical observations of agents inhibiting MAPK (U0126, PD98059 and PD325901) and mTORC1 (using rapamycin) more effectively reduced melanoma cells growth compared to either of the agents tested singly (43). Delivering siRNAs inhibiting Akt3 and V600EB-Raf in nanoparticle based agents, synergistically inhibited melanoma tumor cells growth in culture and in xenografted melanoma tumors (23). Topical application of LY-294002 and U0126 in combination also effectively decreased melanoma tumor incidence in the transgenic TPRas mouse model when compared to either of these agents alone (50). These results lead to the conclusion that inhibiting multiple targets is the next approach in the search for more effective strategies for treating melanoma. Single agents inhibiting multiple key pathways occurs could also be effective with leelamine being the prototype for this class of compounds. Leelamine associated cell death was mediated by its lysosomotropic properties, which triggered cholesterol accumulation in lysosomal/endosomal cell compartments disrupting the autophagic flux, endocytosis and receptor tyrosine kinase signaling pathways (see manuscript by Kuzu OF et al., in the current issue of this journal). Depletion of accumulated cholesterol using beta-cyclodextrin eliminated leelamine activity and restored pathways inhibited by the drug.

In conclusion, this study demonstrates the tumor inhibitory activity of leelamine by targeting three important driver pathways involved in melanoma development through inhibition of cholesterol
transport. Thus, a potentially viable drug has been identified that can decrease melanoma development by targeting the PI3K (pAkt), MAPK (pErk) and STAT (pStat3) signaling cascades in melanoma with negligible toxicity.
ACKNOWLEDGEMENTS

We thank Anton Mulder and Virginia Robertson for technical assistance.
REFERENCES

Table 1

IC50 (μmol/L) of leelamine treated cultured normal and melanoma cell lines

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<th>Vertical Growth Phase (RGP)</th>
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<th>Treatment time (h)</th>
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</table>

Table 1. Leelamine kills melanoma cells more effectively than normal cells. Normal and melanomas cell lines were seeded in to a 96-well plate and after 36 to 72 h, treated with increasing concentrations of leelamine for the indicated time period. Number of viable cells was measured using MTS and percentage decrease in viability calculated. IC50 values for each inhibitor in μmol/L for respective cell lines were measured from three independent experiments using GraphPad Prism version 4.01 (GraphPad Software, La Jolla, CA).
LEGENDS

Figure 1. Identification of leelamine as a therapeutic agent for melanoma treatment. 1A. Natural product library NPL-480 was screened to identify compounds that kill UACC 903 melanoma cells. Leelamine was identified as a candidate in the screen. Data represent averages of at least 3 independent experiments; bars, S.E.M. 1B. Structure of leelamine. 1C. Average IC50 of melanoma compared to normal cells treated with leelamine. Data represent averages of at least 3 independent experiments; bars, S.E.M.

Figure 2. Leelamine inhibits melanoma cell growth by reducing cellular proliferation, triggering apoptosis, and arresting melanoma cells in the G0/G1 phase of cell cycle. 2A, 2B and 2C. UACC 903 and 1205 Lu cells treated with increasing concentrations of leelamine for 24 hours showed decreased cell viability and proliferation with increased apoptosis rates determined by MTS, BrdU incorporation and caspase-3/7 assays, respectively. Data represent averages of at least 3 independent experiments; bars, S.E.M. 2D. Cell cycle analysis of cultured melanoma cells treated with leelamine showed a G0/G1 cell cycle block by decreasing the percentage of cells in the S-phase of the cell cycle and increasing levels of cellular apoptosis. Data represent averages of at least 3 independent experiments; bars, S.E.M.

Figure 3. Leelamine inhibits three key signaling pathways regulating melanoma development. 3A, 3B and 3C. Treatment with 3 to 6 μmol/L of leelamine for 3 to 24 hours decreased PI3K/Akt (3A), MAPK (3B) and STAT3 pathways (3C). Signaling inhibition was observed for both UACC 903 and 1205 Lu cell lines, with inhibition of PI3 and MAP kinase pathways occurring at 3 to 6 hours while inhibition of the STAT3 pathway occurred from 12 hours of treatment. Alpha-enolase served as a control for equal protein loading.

Figure 4. Leelamine inhibits melanoma tumor development with negligible toxicity. 4A and 4B. Leelamine inhibits melanoma tumor development by an average of 60%. Athymic Nude-Foxn1nu mice were s.c. injected with 1X10^6 UACC 903 or 1205 Lu melanoma cells and six days later, when a fully vascularized tumor 50-75 mm^3 had formed, mice were randomly divided into DMSO vehicle control and experimental groups (5 mice/group; 2 tumors/mouse) and treated intra
peritoneally with 2.5-7.5 mg/kg body weight leelamine daily for 3 to 4 weeks. **4C.** No significant difference was observed in body weight of Swiss Webster mice following 22 days of daily treatment with leelamine, indicating negligible subchronic toxicity. **4D.** Levels of blood biomarkers used to indicate major organ related toxicity was measured, indicating negligible toxicity at the concentrations examined in Swiss Webster mice. **4E.** H&E stained sections of liver, heart, lung, kidney and spleen from Swiss Webster mice treated for 22 days with leelamine showed no differences in cell morphology or organ structure following treatment Swiss Webster mice with leelamine (Magnification; 200X).

**Figure 5. Leelamine inhibits cellular proliferation leading to increased apoptosis and decreased vascular development.** **5A, 5B and 5C.** Analysis of proliferation, apoptosis and vascular development in size and time match tumors showed decreased cellular proliferation triggering increased apoptosis and reduced vascular development. 2.5×10⁶ UACC 903 cells were injected s.c. into nude mice, generating tumors of the same size developing at parallel time points. Six days later, mice were treated i.p. with DMSO vehicle or leelamine (7.5 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 (Ki-67 staining), apoptosis (TUNEL staining) and vessel density (CD31 staining) by immunohistochemistry. For all tumor analyses, a minimum of 6 different tumors with 4 to 6 fields per tumor was analyzed and results represented as the average ± SEM. **5D.** Western blot analysis of size and time matched tumor lysates treated with leelamine or DMSO vehicle at days 11, 13 and 15, showed inhibition of pAkt (Ser473) and pStat3 (Tyr705) levels.
Figure 2

2A

Cell viability (% of control)

Concentration (μmol/L)

UACC 903
1205 Lu

2B

2C

Concentration (μmol/L)

Differentiating cells (% of control)

Fold increase in expression 3-T

Concentration (μmol/L)

UACC 903
1205 Lu

2D

Sub-G0/G1

G0/G1

S-phase

% of cell population

UACC 903
1205 Lu

UACC 903
1205 Lu

UACC 903
1205 Lu

UACC 903
1205 Lu
Figure 5

5A
Fold decrease in Ki-67 positive cells over DMSO control
Day 11  Day 13  Day 15
NS  NS  NS

5B
Fold increase in Tim3 positive cells over DMSO control
Day 11  Day 13  Day 15
NS  NS  NS

5C
Area occupied by tumor xenograft
Day 11  Day 13  Day 15
NS  NS  NS

5D
Size and time matched xenografted UACC 903 tumors
DMSO  Leptomine  DMSO  Leptomine  DMSO  Leptomine
pAkt  Total Akt  pStat3  Total Stat  TIM3
Day 11  Day 13  Day 15
Molecular Cancer Therapeutics

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Mol Cancer Ther Published OnlineFirst March 31, 2014.

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