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LUNASIN REDUCES THE MELANOMA STEM CELL POPULATION *IN*
VITRO AND INHIBITS TUMOR PROLIFERATION *IN VIVO*

By

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B.S., University of Kentucky, 2008

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ABSTRACT

LUNASIN REDUCES THE MELANOMA STEM CELL POPULATION *IN VITRO* AND INHIBITS TUMOR PROLIFERATION *IN VIVO*

Christopher P. Shidal

October 30, 2014

Lunasin is a 44 amino acid peptide derived from the soybean seed that has been shown to have cancer chemopreventive and chemotherapeutic properties. In this study, we investigated the potential utility of lunasin as a chemotherapeutic in a melanoma model. Initial studies showed that lunasin has little activity against established melanoma cell lines *in vitro* using adherent culture methods; however, lunasin's *in vitro* activity was significantly higher in non-adherent colony-forming assays in soft agar and oncosphere assays. These results led us to investigate whether lunasin selectively affects cancer initiating cells (CIC) that are known to be present in these melanoma cell lines. We found that lunasin treatment did selectively inhibit the proliferation of high-ALDH-expressing malignant melanoma initiating cells (MMIC) *in vitro*, and had the striking effect of preventing oncosphere formation under non-adherent culture conditions. These *in vitro* results were extended into mouse xenograft studies using both bulk melanoma cells and isolated CICs. Lunasin significantly inhibited tumor growth in both cases, with the highest inhibition being observed in tumors initiated by MMICs. Mechanistic studies suggested that lunasin inhibits CIC proliferation *in vitro* through interactions with

integrins and disruption of integrin signaling by inhibiting the activity of integrin binding partners such as integrin-linked kinase (ILK) and focal adhesion kinase (FAK). These studies demonstrate for the first time that lunasin has activity against putative CICs and that lunasin may have utility as a therapeutic agent for the treatment of melanoma.

TABLE OF CONTENTS

	PAGE
ACKNOWLEDGEMENTS.....	v
ABSTRACT.....	vi
LIST OF FIGURES.....	viii
INTRODUCTION.....	1
Profiling the Soy-derived Peptide Lunasin as an Anticancer Therapeutic.....	1
Integrins as a Targeted Therapy in Melanoma Patients.....	4
Melanoma and the Presence of Melanoma Stem Cells.....	7
METHODS AND MATERIALS.....	11
RESULTS.....	17
<i>In vitro</i> Effects of Lunasin on Established Melanoma Cell Lines.....	17
<i>In vitro</i> Effects of Lunasin on ALDH Sorted Melanoma Cells.....	20
<i>In vivo</i> Xenograft Models for Bulk and Sorted Tumor Samples.....	25
DISCUSSION.....	28
SUMMARY AND CONCLUSIONS.....	33
REFERENCES.....	35
CURRICULUM VITAE.....	41

LIST OF FIGURES

FIGURE	PAGE
1. Lunasin Inhibits HAT Binding to Core Histones.....	42
2. Integrin Signaling through FAK and ILK.....	43
3. Fluorescence Assisted Cell Sorting Based on the ALDH Biomarker.....	44
4. Proliferation Assays of Melanoma Cell Lines.....	45
5. Colony Formation in Soft Agar.....	46
6. ALDH Staining of Melanoma Cells Treated with Lunasin and Vemurafenib.....	47
7. Representative Histogram for Lunasin Treated A375 Cells.....	48
8. Flow Cytometry for ALDH in Lunasin Treated Samples.....	49
9. Cell Cycle Analysis by PI Staining.....	50
10. Annexin V Binding Assay for Melanomas Treated with Lunasin and Vemurafenib.....	51
11. Apoptotic Profiles of Melanomas in Response to Lunasin Treatment.....	52
12. Immunoblot Analysis for FAK and AKT	53
13. Oncosphere Formation in Low Adherent Conditions	54
14. ALDH Positive Cell Growth as Colony Formation in Soft Agar	55
15. Xenograft Model for A375 Melanoma Cells (IV Injection).....	56
16. Xenograft Model for A375 Melanoma Cells (IP Injection).....	57
17. NSAID Toxicological Screen and CBC for Safety of Extended Lunasin Treatment.....	58

18. Xenograft Model for ALDH Positive Melanoma Stem Cells.....	59
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INTRODUCTION

Lunasin as an Anticancer Agent

Lunasin is a soy derived peptide that has demonstrated anticancer, anti-inflammatory, antioxidant, and immunomodulatory activity [1-4]. Lunasin has been reported as a 43 amino acid fragment present in processed 2S albumin protein [5]; however, we recently identified a native 44 amino acid sequence of lunasin isolated from defatted soy flour consisting of the sequence:

SKWQHQQDSCRKQLQGVNLTPEKHIMEKIQGRGDDDDDDDDDDN [6]. Lunasin has been proposed to have three distinct domains that are responsible for its therapeutic and chemopreventive activity: an RGD sequence involved in internalization of the peptide via integrin binding, a poly-aspartic acid tail that binds lysine residues present in H3 and H4 histone tails, and a hypothesized chromatin binding domain [7, 8]. Limited studies describe the direct chemotherapeutic effects of lunasin against cancer as it is generally defined as a chemopreventive agent based on earlier studies by de Lumen and coworkers [7, 9-12]. Thus, many questions remain about the number of cancer types sensitive to lunasin, the possible mechanisms of lunasin's anticancer effects, and to what extent lunasin is involved with the tight correlation of soy consumption with a protective effect against certain cancer types [13-15].

Lunasin has been found to inhibit transformation induced by multiple carcinogens and viral oncogenes [7, 9, 16-18]. Moreover, studies in our lab indicate lunasin is able to inhibit transformation of mouse fibroblast cells induced by carcinogens present in

cigarette smoke including cadmium and nicotine-derived nitrosamine ketones (data not shown). The most discussed mechanism of action of lunasin is the inhibition of histone acetyltransferases (HATs) and modulation of histone acetylation.

The acetylation of core histones initiates the unwinding of tightly packed DNA from the nucleosome complex allowing for transcription of target genes (Figure 1). HAT inhibition alters normal acetylation patterns leading to hypoacetylation of histone tails, repressing transcription and can account for global cellular effects including proliferation, cell cycling, and apoptosis [19-21]. Although some evidence supports HAT inhibition as lunasin's primary mechanism of action, to date, there have been no functional studies to support this hypothesis. Moreover, as new principal mechanisms of lunasin action are still being discovered, it is not clear in the different experimental systems that have been studied whether histone acetylation is involved in all cases.

Studies in our lab confirm a significant antiproliferative effect of lunasin on non-small cell lung cancer (NSCLC), an effect which is mediated by disrupting cell cycle signaling (McConnell, 2014). Previous studies have acknowledged that lunasin reduces cyclin-dependent kinase (CDK) levels, and may accommodate aspirin-induced apoptosis in breast cancer models [22, 23]. Additionally, lunasin was reported to induce apoptosis and alter expression of matrix adhesion proteins in metastatic colon cancer [24]. Lunasin was recently shown to suppress FAK/ERK/NF- κ B signaling in human colon cancer as well as potentiate the antiproliferative and antimetastatic effects of oxaliplatin [25].

Sadly, many plant-derived compounds (e.g. curcumin) are quickly metabolized or excreted resulting in poor bioavailability, however, lunasin is active and bioavailable in humans consuming physiologically relevant amounts of soy [26]. In this study,

volunteers were orally dosed with lunasin (155.5 mg/day) in 50 grams (g) soy protein for 5 consecutive days. De Meija et al. revealed lunasin is orally bioavailable; however, incomplete GI absorption resulted in a low concentration (71.0 ng/mL or approximately 14 μ M) lunasin in plasma samples [26].

Prior studies suggest that lunasin interacts with a specific subset of integrin subunits, as supported by a 2012 study by De Meija and coworkers [2]. Furthermore, recent studies suggest that internalization of lunasin is mediated by $\alpha_v\beta_3$ integrins via clathrin and caveolin-mediated endocytosis [27]. Proximity ligation assays (PLA) verify that the specific integrin subunits α_v , α_5 , β_1 and β_3 network with the lunasin peptide (Inaba and Davis, unpublished data). In normal cells, integrins mediate cell-cell and cell-matrix adhesions by recognizing binding motifs (i.e. RGD) as well as cooperating with growth factor receptors to induce proliferative and survival signaling [28, 29]. The ubiquitous nature of integrin signaling provides an interesting target for cancer prevention and treatment because many of these pathways are deregulated in cancer and result in uncontrolled proliferation and metastasis. More specifically, we are interested in elucidating the effects of lunasin treatment on downstream pathways associated with integrin signaling and how disrupting these pathways can therapeutically benefit melanoma patients.

Integrins as a targeted therapy in melanoma patients

Integrins are vital to most cellular processes, and remain an important and underexplored target for cancer therapies. Recent studies utilizing RGD peptides have shown targeting of integrins as a viable treatment alternative in melanoma therapy by inhibiting tumor angiogenesis, growth, and metastasis [30-32]. Integrins are heterodimeric membrane proteins primarily implicated in cell adhesion and migration [33, 34]; yet, integrins have also been reported to be intimately involved in cell growth, differentiation, and survival [35, 36]. Two families consisting of 18 α -subunits and 8 β -subunits comprise the 24 heterodimeric proteins known in humans [37], combined with complex conformational states and overlapping ligand specificities, make integrin signaling flexible yet highly intricate [38]. Furthermore, integrin expression profiles are largely dependent upon two dimensional (i.e. adherent conditions) adhesion versus three dimensional (i.e. non-adherent) adhesion [39], and can result in recruitment of very different subsets of proteins. Proteins attracted to different adhesion structures (e.g. paxillin) can produce diverse yet specific signaling cascades.

By categorizing NSCLC lines based on integrin expression profiles, we associated explicit integrin subunits with lunasin sensitivity (McConnell and Davis, unpublished data). Moreover, we showed lunasin exerts its anticancer effects in NSCLC by reducing activating phosphorylations of v-akt murine thymoma viral oncogene homolog 1 (AKT), Focal adhesion kinase (FAK), and interactions of β -subunits with integrin-linked kinase

(ILK), thereby altering signaling pathways downstream of integrin-ligand binding (unpublished data).

In melanoma models, the integrin $\alpha\text{v}\beta 3$ is currently the predominant target for therapeutic applications of integrin antagonists. Integrin $\alpha\text{v}\beta 3$ is expressed at low levels in non-transformed epithelial cells relative to melanoma cells [40], as $\alpha\text{v}\beta 3$ expression has been related to metastatic potential and dissemination of melanoma neoplasms to a metastatic phenotype [41, 42]. Crosstalk between integrins and growth factor receptors has been well documented [43, 44]. Enhanced cancer cell survival has been attributed to a number of interactions between integrin signaling and other pathways including increased BCL-2 expression, PI3K-AKT activation, or NF- κ B signaling [45-47]. We predict that lunasin binds $\alpha\text{v}\beta 3$ integrins through its RGD domain and inhibits proliferative and prosurvival signaling.

Certain integrins have specific roles in melanoma; for instance, it has been shown that $\alpha_4\beta_1$, a homing molecule on leukocytes that binds VCAM-1, is absent on melanocytes, yet present in melanoma cultures [48]. Consequently, $\alpha_4\beta_1$ may help melanomas mimic hematopoietic cells by enabling melanoma migration into tissues that express sing VCAM-1 [49]. Other integrins implicated in melanoma are $\alpha_3\beta_1$ and $\alpha_5\beta_1$, which were elevated in metastatic melanoma tissue. Furthermore, $\alpha_1\beta_1$, $\alpha_2\beta_1$ and $\alpha_6\beta_1$ integrin subunits were found to be reduced in metastatic versus primary melanoma [48].

Roles of integrins in CICs are somewhat ambiguous. Recent studies show that integrin subunits may be a viable marker for CICs and are responsible for stem cell pool maintenance and differentiation mediated by FAK [50, 51]. CICs are proposed to be more tumorigenic based on properties such as chemoresistance, immune evasion, and

self-renewal capabilities [52, 53]. Cilengitide (Merck Co.), a cyclic RGD (cRGD) peptide, has been used to treat glioblastoma and is the first integrin inhibitor to be used in Phase III clinical trials [54]. By selectively targeting melanoma stem cells and altering integrin signaling, we may provide a novel treatment for malignant metastatic melanoma. Furthermore, targeting of integrin subunits explicitly expressed on cancer cells may represent a dynamic solution to reducing off-site, adverse side effects generally seen with traditional chemotherapy.

Melanoma and the presence of melanoma stem cells

Skin cancers account for nearly half of all diagnosed cancer cases in the United States and have increased in frequency over the last thirty years [55]. Melanoma is estimated to account for 76,000 new cancer cases in 2014 [56]. Despite being less frequent than other skin cancers, nearly 75% of skin cancer deaths are attributed to melanoma [56]. Even more unnerving, NCI's Surveillance, Epidemiology, and End Results (SEER) program estimates cases of melanoma have nearly tripled in the past thirty years increasing from 7.9 (per 100,000) in 1975 to 22.7 in 2011, while 5-year survival rates remain constant. Early detection and diagnosis is paramount for overall survival with 5-year survival rates of 98%, 62%, and 16% for localized, regional, and distant diseases, respectively [56]. Continued research of melanoma has provided several "cracks in the armor" of metastatic melanoma leading to the development of several targeted therapies that aim to inhibit proliferation, metastasis, and angiogenesis of primary and secondary tumors. One such targeted therapy is vemurafenib, which decreases melanoma cell viability and proliferation resulting in tumor regression and increasing overall mean survival time [57, 58].

Vemurafenib targets a mutated form of the B-Raf protein found in approximately 60% of melanomas in which a V600E substitution leads to constitutive Raf signaling within the mitogen-activated protein kinase (MAPK) cascade [59]. In the majority of patients harboring this mutation, mean survival time has been improved with vemurafenib; however, after initial tumor regression, many patients experience

recurrence of tumors that are vemurafenib-resistant [60-62]. Conferred resistance to vemurafenib may occur through a number of mechanisms including, but not limited to, feedback activation of epithelial growth factor receptor (EGFR), upregulation of other Raf proteins, or upregulation of N-Ras [62-64]. Our present studies focus on developing lunasin as an adjuvant therapy to targeted therapies such as vemurafenib. We found that lunasin interacts additively with vemurafenib to reduce the ability of melanoma cells to proliferate and form oncospheres *in vitro*, and may sensitize cell lines with acquired resistance to subsequent vemurafenib treatments via inhibition of PI3K/AKT signaling (data not shown).

One explanation for the reformation of palpable tumors with chemoresistance is the presence of CICs within the bulk tumor population. The presence of CICs and their origin have become a topic of debate [65-69]. According to the cancer stem cell (CSC) theory, a subset of cells within the tumor population have properties that resemble physiological stem cells including the ability to self-renew while also giving rise to daughter cells that differentiate to reform heterogeneous tumor populations [65]. In this study, we will show that CICs exist within bulk tumor populations at a relatively high rate, and that this subset of cells displays enhanced tumorigenicity.

Discovery of melanoma cells with stem-like plasticity was initially found in patient tumors overexpressing CD20 and CD133 [70, 71]. CD20 is a membrane-spanning surface molecule generally found on B lymphocytes; per se, it is the molecular target for monoclonal antibodies (e.g. rituximab) for treatment of select leukemias and lymphomas. CD133 (also called prominin-1) is a novel, membrane-spanning protein of no known function that is classified as a marker for primitive hematopoietic and neural

stem cells. These subsets of cells were found to have properties of stem cells as well as enhanced ability to form palpable tumors in immunodeficient mice. Ensuing studies verify ATP-binding cassette sub-family B member 5 (ABCB5), a drug transporter playing a key role in chemoresistance, and Low-Affinity Nerve Growth Factor Receptor (LNGFR/CD271), a member of the tumor necrosis factor (TNF) receptor family involved in survival and differentiation of neurons, as viable MMIC biomarkers [53, 72]. However, scientists are slow to embrace this concept for a number of valid reasons. One concern remains the standardization of techniques for identifying and propagating cancer stem cells. Serial dilution and transplantation of CICs into NOD/SCID mice has long been the gold standard for determining stem cell populations; however, spheroid assays in addition to genetic lineage tracing provide *in vitro* assays for CIC classification [66].

To make matters more complicated, some evidence supports plasticity of differentiated cancer cells in a breast cancer model [73]. By reverting to a dedifferentiated phenotype, stem-like cells arise *de novo* in response to environmental cues [73]. These data support the theory of bidirectional movement between stem and non-stem compartments, and have serious implications on the plasticity of cells in cancer models as well as subsequent therapeutic strategies.

Although populations of stem-like cells are recognized in melanoma cell lines, the frequency of these cells is highly variable, ranging from less than 1% up to nearly 25% [53, 74]. The incidence of cancer stem cells seems dependent upon the *in vivo* model, the biomarker used for identification, and the tumor microenvironment [49, 75]. Conflicting reports indicate that tumor samples enriched for MMIC markers have enhanced tumor forming capacity. Quintana [74] showed tumorigenic cells are phenotypically

heterogeneous in melanomas, as significant *in vivo* tumor growth was marginal based on select CSC biomarkers. Despite these findings, numerous studies report superior tumor forming capabilities of cells enriched for melanoma stem cell biomarkers including ABCB5 [76], CD133 [71], CD271 [72], and ALDH [77].

Aldehyde dehydrogenase (ALDH) is a family of detoxifying enzymes responsible for metabolism of certain alkylating agents such as cyclophosphamide. Enrichment for melanoma initiating cells by intracellular ALDH staining has come with mixed reviews; however, most evidence supports ALDH as a MMIC biomarker [77-80]. ALDH expression has also successfully been used to detect CICs in breast and colon cancer models [81, 82]. In this study, we implement a commercially available ALDH detection kit to identify and sort melanoma cells expressing elevated levels of ALDH from ALDH negative fractions.

Unfortunately, current therapeutics heavily rely on efficacy against bulk tumor cells and the ability of the drug to reduce primary or secondary tumor size. We suggest that lunasin specifically targets MMICs and reduces this aggressive subpopulation of cells. We are currently elucidating mechanisms for this effect, but believe lunasin treatment may induce terminal differentiation of cancer stem cells, inhibit self-renewal capacity, or alter integrin signaling leading to antiproliferative effects. By reducing CIC populations, we hypothesize lunasin alters the ability of melanoma tumor cells to establish palpable tumors *in vivo*. By selectively reducing the population of melanoma cells with the ability to form solid tumors and metastasize, lunasin has a potential therapeutic application.

METHODS

Lunasin Isolation and Purification

Lunasin was isolated from “white flake,” a product resulting from the flaking and defatting of soybeans via hexane extraction. The extraction and purification was scaled and performed by Kentucky BioProcessing (KBP) as previously described [6]. Briefly, lunasin was extracted from defatted soy flour and purified using a combination of Q-Sepharose FF chromatography, ultrafiltration utilizing a 30 kDa membrane, and reverse-phase chromatography. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis indicate these lunasin preparations have >99% purity.

Cell Culture and Reagents

SKMEL-28 and A375 cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cell lines were grown in Dulbeccos Modified Eagles Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at 37°C at 5% CO₂ and subcultivated every 72 hours. Vemurafenib was obtained from Selleck Chemicals (Houston, TX). DMEM media (Invitrogen) was reconstituted in 500mL ultrapure H₂O and supplemented with 20% FBS, 200 U/mL penicillin, and 200 µg/mL streptomycin for use in soft agar assays.

Proliferation Assays

Manufacturer protocols were followed to determine effect of lunasin on melanoma cell proliferation (Promega Cell titer-96 Aqueous Reagent). Initial seeding

densities were standardized at 7.5×10^3 cells/cm² in 100 μ L culture media. Briefly, cells were plated and incubated at 37°C, 5% CO₂ for 4 hours. Media were drained from each well and replaced with media containing varying concentrations of lunasin. Treatment media were replaced every 24 hours during the 72 hour treatment period. After 72 hours of treatment, wells were drained of expired media and refilled with fresh media. 20 μ L [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent was added to each well and the plate was incubated for 2-3 hours. Absorbance was read at 490 nm on a plate reader (Biotek Instruments, Winooski, VT). Average absorbance of media containing no cells was subtracted from all absorbance values. Absorbance values were then normalized to control and expressed as percent control.

Soft Agar Colony Forming Assay

In order to assess the ability of lunasin to suppress anchorage-independent growth of cancer cell lines, cells were suspended in soft agar. Solutions of 1% and 0.7% w/v Bacto Agar were mixed with equal volumes (1:1) of 2x DMEM media containing 20% FBS and 2% Pen-Strep. 2 mL of 0.5% agar solution was used to coat the bottom of each well in a 6-well plate and allowed to solidify in a laminar flow hood. 1×10^3 cells were suspended in a 1 mL top layer containing 0.3% agar with and without lunasin present and placed on top of the bottom layer of agar. Plates were allowed to fully solidify and were placed in the incubator. Generally, colony formation occurred within 2-3 weeks. Colonies were stained with a 1 mg/mL iodonitrotetrazolium chloride (Sigma-Aldrich, St. Louis, MO) solution and counted using a light microscope. Images from each well were analyzed using ImageJ software (National Institutes of Health, Bethesda, Maryland).

ALDEFLUOR Staining and Flow Cytometry

The ALDEFLUOR kit (Stem Cell Technologies, Vancouver, BC) was used to identify cells with high ALDH activity. Briefly, cells were suspended in assay buffer containing a fluorescent ALDH substrate and incubated for 30 min at 37 °C. The ALDH substrate passively diffuses into live cells and is then converted by ALDH into a fluorescent product detectible by FL-1 (FITC) signal. A specific ALDH inhibitor, diethylaminobenzaldehyde (DEAB) served as a negative control and allowed gating of ALDH-positive cells. Becton, Dickinson, and Co. FACS Caliber (BD Biosciences San Jose, CA) was used for all flow cytometry experiments.

Annexin V binding assays were conducted using FITC conjugated antibodies (BD Bioscience) and propidium iodide to measure rates of apoptosis/cell death. Cells were harvested and resuspended in 1x binding buffer (0.1 M HEPES, pH 7.4; 1.4 M NaCl; 25 mM CaCl₂) at a concentration of 1x10⁶ cells per mL. Staining was conducted following manufacturer protocols. Briefly, 1x10⁵ melanoma cells were incubated with 100 µM lunasin for 24, 48, and 72 hours. Cells were suspended in 0.5 mL binding buffer and stained with 5 µL PI, 5 µL Annexin antibody, or both for 15 minutes at room temperature. Gates were set based on controls (unstained, PI only, Annexin only). 1x10⁴ events were collected per run.

Cell cycle analysis was performed on synchronized melanoma cells; cells were serum starved for 72 hours and then released by addition of FBS during lunasin or vehicle treatment. 1x10⁶ cells were harvested and resuspended in 200 µL PBS. Cells were slowly added to 4 mL ice cold 70% ethanol for overnight fixation at -20°C. After fixation, cells were spun down at 300g for 10 minutes and resuspended in 0.5 mL of PI

master mix (40 $\mu\text{g/mL}$ PI, 100 $\mu\text{g/mL}$ DNase in PBS) and incubated at 37°C for 30 minutes prior to flow analysis.

Fluorescence-assisted Cell Sorting

A MoFlo cell sorter (Beckman Coulter, Brea, CA) was used for all fluorescence assisted cell sorting (FACS). ALDH-positive and ALDH-negative fractions were isolated using FL1 signal and collected for subsequent *in vitro* and *in vivo* experiments. Sorted cells were confirmed to be positively stained for ALDH by fluorescent microscopy under blue laser (illumination at 488 nm). Because a relatively large population of melanoma cells expressed at least some basal level of ALDH, only ALDH-positive cells with high FL1 intensity were collected. A representative histogram for FACS based on the ALDH biomarker is shown in Figure 3.

Formation of Multicellular Oncospheres

Stem-like cells isolated by FACS were cultured in low adherent T-25 flasks (Corning, Corning, NY) in DMEM culture media supplemented with 2% FBS at a density of 1×10^4 cells/mL. Cultures were grown for 28 days and treated every 48 hours during this time period with lunasin or vehicle in DMEM culture media. Oncospheres began to form around 14 days and continued to grow until they were harvested, dissociated, and counted. Visual counts of oncospheres were performed by light microscopy and analyzed using ImageJ software.

In vivo Xenograft Model

In vivo experiments using 6-8 week old, male athymic nude mice (Jackson labs Stock# 002019, Bar Harbor, Maine) illustrated the anticancer effects of lunasin in a xenograft model. Briefly, mice were injected subcutaneously (s.c.) with 2.5×10^6 A375

cells reconstituted in 100 μ L phosphate buffered saline (PBS) on the right flank.

Intraperitoneal (IP) injections of 30 mg/kg lunasin reconstituted in 50 mM phosphate buffer, pH 7.4 (PB) were administered starting immediately after s.c. injection of tumor cells and repeated every 24 hours. Noticeable tumor formation was observed approximately 14 days post-injection and measured every other day thereafter.

Experimental endpoint was set at tumor volumes exceeding 20 mm in diameter or ulceration of tumor tissues. At endpoint, mice were sacrificed and organs were resected for slide preparations.

For MMIC studies, 1×10^4 ALDH⁺ cells were reconstituted in Hank's Balanced Salt Solution (HBSS) +calcium +magnesium (Invitrogen) with an equal volume of Matrigel (BD Biosciences) and injected on the dorsal side of the mice at a total volume of 100 μ L. Palpable tumor formation was observed at approximately 16 days post-injection and measured every other day as described above. Again, organs were resected and blood was drawn via cardiac puncture upon sacrifice for future processing.

SDS-PAGE and Immunoblot

Cultured cells were harvested by using enzyme free dissociation buffer to minimize protein degradation, spun down, and washed in ice-cold PBS. After pelleting, cells were resuspended in appropriate amounts of RIPA buffer (250 mM Tris-HCl, pH 7.5, 5 mM EDTA, 750 mM NaCl, 0.5% Lauryl sulfate, 2.5% Deoxycholic acid, 5% Igepal CA-630, Protease inhibitor cocktail containing 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatin A, bestatin, leupeptin, aprotinin and trans-epoxysuccinyl-L-leucyl-amido(4-guanidino)-butane (E-64) (Sigma-Aldrich). Protein concentrations of cell lysates were determined by a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific,

Waltham, MA). 20 µg of protein were loaded into 10% polyacrilamide gels (BioRad, Hercules, CA) and run at 100 volts for 1 hour. The protein was then transferred to a polyvinylidene difluoride (PVDF) membrane (BioRad) at 350 milliamps for 1 hour. The PVDF membrane was blocked with 5% bovine serum albumin (BSA) or non-fat dry milk for 1 hour. After several washing steps, primary antibodies were incubated with membrane at 4°C overnight. FAK (Cell Signaling, Danvers, Massachusetts), AKT (Cell Signaling), phospho-FAK (Cell Signaling), and phospho-AKT (Cell Signaling) monoclonal antibodies were diluted in Tris Buffered Saline with 0.1% Tween (TTBS) at 1:1000 – 1:2000 v/v. After three washes, secondary antibodies at 1:10,000 dilutions (Cell Signaling) were incubated with the membrane for 1 hour at room temp.

Electrochemiluminescent (ECL) substrate/enhancer solutions (Thermo Fisher) were allowed to activate horseradish peroxidase (HRP) signal on membrane for 2-3 minutes; Chemiluminescence was developed on x-ray film and/or detected using a ChemiDoc station (BioRad).

Non-steroidal anti-inflammatory drug (NSAID) Toxicological Panel and Complete Blood Count (CBC)

Whole blood was drawn from athymic nude mice by cardiac puncture immediately following CO₂ asphyxiation and collected in serum separator tubes (BD Biosciences) or EDTA coated collection tubes (BD Biosciences). 25 µL of whole blood were collected in EDTA coated tubes and send to the RRC facility at the University of Louisville for CBC analysis. After 1 hour post-collection, whole blood collected in serum separator tubes were centrifuged for 10 minutes at 10,000 g. 250 µL of serum was removed from each sample, collected in a 1.5 mL eppendorf tube, and send to the RRC

facility for NSAID toxicological analysis. Liver damage was assessed by levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALKP). Kidney damage was assessed by level of blood urea nitrogen (BUN) and creatinine (CREA).

Statistical Analysis

All data were analyzed in three independent experiments. *In vitro* results were analyzed using GraphPad Prism (v 5.0) software and shown as mean \pm standard deviation (SD). Comparison of results from treated versus control cells was done using unpaired Student's t test. A p-value of less than 0.05 was considered statistically significant. *In vivo* experiments were statistically analyzed using GraphPad Prism ANOVA analysis tool ($p < 0.05$), and shown as mean \pm standard error (SEM). Individual data points were compared by unpaired Student's t test ($p < 0.05$) to determine significance. Interactions between lunasin and vemurafenib were determined to be antagonistic (< 1), additive (1), or synergistic (> 1) by calculating the Drewinko Index. All samples were normalized to appropriate controls and applied to the formula $DI = \frac{(SF^1)(SF^2)}{SF^3}$ in which SF^1 is equal to the surviving fraction of drug¹, effect² is equal to the surviving fraction of drug², and effect³ is equal to the surviving fraction of the combination of drug¹ and drug².

RESULTS

Lunasin has modest antiproliferative effects on melanoma in adherent cell culture

Proliferation assays remain an effective and high throughput method for screening drug efficacy. We used a tetrazolium based proliferation assay to determine if lunasin displayed a significant antiproliferative effect on melanoma cell lines. MTS results (Figure 4) indicate that lunasin marginally inhibits proliferation in adherent conditions and has an additive interaction with the B-Raf inhibitor vemurafenib as calculated by Drewinko Index assessment ($DI = 1$). We replicated these experiments with cells isolated based on high expression of the ALDH biomarker using FACS. ALDH positive cells showed no significant difference from the parental cell lines in terms of sensitivity to lunasin using this culture method. Figure 4 illustrates that lunasin interacted additively with vemurafenib in both parental and ALDH⁺ cells as assessed by DI.

Lunasin reduced anchorage-independent growth of melanoma cells in soft agar

Soft agar assays represent a robust system for screening cancer cells for drug sensitivity that is thought to be more representative of potential in vivo effects. We observed a heightened sensitivity of melanoma cells to lunasin when plated in soft agar versus adherent culture conditions (Figure 5). This is consistent with previous studies in our lab in which lunasin-insensitive NSCLC lines displayed lunasin sensitivity in a non-adherent format (McConnell and Davis, unpublished data). Lunasin showed a significant antiproliferative effect on A375 melanoma cells plated in soft agar. 30 μ M lunasin reduced colony formation by approximately 25%, while 100 μ M lunasin inhibited colony

formation by 38% (Figure 5A). Along with decreased colony counts, a noticeable reduction in the size and change in phenotype accompanies lunasin treatment (Figure 5D) versus vehicle treated A375 cells (Figure 5E). Cells treated with lunasin formed colonies that tended to be less dense and displayed a somewhat dissociated phenotype. Furthermore, we found that lunasin interacts additively with the specific B-Raf inhibitor vemurafenib. A combination of 100 μ M lunasin plus 300, 700, and 1000 nM vemurafenib yielded a significant decrease in colony formation, illustrating the potential application of lunasin as an adjuvant therapy in melanoma patients.

Lunasin alone shows an antiproliferative effect on the SKMEL-28 melanoma cell line (Figure 5B). Concentrations of 30 μ M and 100 μ M reduced colony formation of SKMEL-28 melanoma cells by 14% and 20%, respectively (Figure 5B). The additive effect of lunasin was again measured with vemurafenib co-treatment; however, a statistically significant additive effect was not obtained at 700 nM vemurafenib. Anchorage independent growth is a hallmark of cancer; these data illustrate the utility of chemotherapeutics in disrupting proliferation of cancer cells in non-adherent conditions. From this, we concluded that concurrent treatment of lunasin and the B-Raf inhibitor vemurafenib could be a legitimate therapeutic strategy in malignant melanomas with the V600E mutation.

Lunasin reduces the melanoma stem cell population *in vitro*

We conducted initial experiments in which A375 and SKMEL-28 cells were treated with 1 μ M vemurafenib, 100 μ M lunasin, and a combination of both agents. Melanoma stem cell markers (e.g. ALDH) in the cell population were analyzed by flow cytometry. ALDH staining has been recognized as a technique to distinguish and isolate

melanoma stem cells from bulk tumor populations. Our results indicated that both vemurafenib and lunasin are able to dramatically reduce the population of cells expressing measureable levels of ALDH (Figure 6). This reduction in ALDH expression is time dependent. An overall reduction in ALDH-positive cells was seen with lunasin treatment, vemurafenib treatment, and in combination treatment groups.

Lunasin alone had a significant effect on ALDH expression in melanoma cells versus vehicle treatment (Figure 8). ALDH positive populations were reduced by nearly 50% in lunasin treated SKMEL-28 cells at 24 hours (Figure 8B). This effect was seen at later time points, albeit, it was not significantly different from controls. In A375 cells, ALDH positive populations were reduced by lunasin treatments; ALDH expressing cell populations were reduced 56%, 35%, 18%, and 22% for 24, 48, 72, and 96 hour time points, respectively (Figure 8A). By effectively reducing the tumor initiating cells in culture, lunasin may inhibit primary tumor formation or alter metastatic growth by inducing terminal differentiation of cancer stem cells or by reducing CIC pools available to invade distant tissues.

Cell cycling and apoptotic effects on melanoma

Trypan blue exclusion tests were performed in concurrence with annexin binding assays to measure apoptotic rates within melanoma cell populations. Initial experiments suggest that lunasin has little effect on cell viability; however, potentiates the apoptotic effects of vemurafenib in combination treatments (Figure 10). At the later time points, it was clear that a number of cells in both A375 and SKMEL-28 treated samples were dead or dying; however, these observations were apparent in both control and lunasin treated samples. Results from later annexin binding assays did not show a definitive increase in

early or late apoptotic populations within either cell line at 24, 48, or 72 hours in response to 100 μ M lunasin (Figure 11). Apoptotic and necrotic profiles for each treatment and time point were generated, but no significant difference was seen in either cell line; although, a clear trend in increasing early and late apoptotic cells was observed with lunasin treatment. We therefore concluded that lunasin does not induce apoptosis or cause a necrotic response in the parental melanoma cell lines; however, we expect to isolate CICs based on the ALDH biomarker and determine if lunasin induces a selective apoptosis response in this subset of cells.

Cell cycle analysis was performed using propidium iodide (PI) staining of DNA (Figure 9). Briefly, cells were permeabilized and fixed in 70% ethanol over night and resuspending in a PI master mix (PI 40 μ g/mL, RNase 100 μ g/mL in PBS). Flow analysis commenced after a 30 minute incubation. Interestingly, lunasin had little effect on cell cycling of either melanoma cell line before the 72 hour time point. However, at 72 hours, a decrease of cells in S-phase was seen. These data may suggest lunasin has a delayed effect on cellular proliferation, and causes accumulation of cells in G0/G1 and G2 phases of the cell cycle. Treatment with vemurafenib caused a decrease in S-phase throughout the time course, and an accumulation of cells in G0/G1 can be seen in both cell lines. Combination treatment curves were similar to treatment with the B-Raf inhibitor, however, S-phase cycling was further reduced, and frequency of sub-G1/super-G2 cells was modestly increased.

Lunasin reduced phosphorylation of Akt and Fak in melanoma cells

Since lunasin was effective at inhibiting the growth of melanoma cells as colonies in soft agar, we studied whether it affects the signaling pathways associated with cell

growth and survival. FAK and AKT are downstream mediators of integrin signaling. Figure 12 illustrates that total FAK (125 kDa) remains unchanged with lunasin treatment; however, tyrosine phosphorylation (Y397) is significantly impaired. We next examined the serine phosphorylation (Ser473) of Akt (60 kDa), a downstream signal molecule of FAK. As expected, the phosphorylation of Akt was observed in untreated cells and inhibited by treatment with lunasin. These results confirm that downstream mediators of integrin signaling are severely affected by lunasin. By effectively reducing active forms of Akt and Fak, signal transduction is interrupted, which may stimulate antiproliferative and antisurvival cell signaling. Inhibiting these pathways may sensitize CICs with superior survival signaling and chemoresistance to traditional or targeted therapies (e.g. vemurafenib).

In vitro effects of lunasin were potentiated in melanoma stem cells

In order to determine whether or not lunasin has a selective apoptotic or antiproliferative effect on cancer stem cells, in vitro assays measuring proliferation and anchorage-dependent growth were performed, and the results from the ALDH-positive populations were compared to the parental lines. Overall, lunasin had an increased effect in the ALDH⁺ sorted cells. 100 μ M lunasin reduced colony forming ability in soft agar by approximately 40% and 75% in A375 and SKMEL-28 cell lines, respectively (Figure 14A). Interestingly, a reduction in colony size accompanied lunasin treatment in both cell lines versus vehicle treatment (Figure 14B-E)

CICs have been reported to have enhanced ability to form oncospheres in low adherent conditions. We assessed the ability of lunasin to disrupt formation of oncospheres derived from sorted samples based on the ALDH biomarker. 100 μ M

lunasin almost completely inhibited spheroid formation in the A375 cell line versus control (Figure 13B and Figure 13C), while reducing spheroid counts and size in SKMEL-28 cells (Figure 13A). When spheres were dissociated and counted, viability did not significantly change due to lunasin treatment; however, slightly reduced cell counts as determined by trypan blue exclusion assays were observed (data not shown). Furthermore, lunasin inhibited foci formation when A375 CICs were dissociated and replated at low density in adherent conditions (Figure 13D and Figure 13E). ALDH positive cells quickly grew in tightly packed clusters of cells and formed foci within a matter of days. Lunasin restores growth of these cells as a monolayer and inhibits foci formation in adherent culture. These data suggest lunasin may convert the more tumorigenic ALDH expressing cells to the less aggressive phenotype of a terminally differentiated bulk tumor cell. More research into possible mechanisms for this effect is warranted as little is known about the movement of melanoma cells in and out of the stem cell compartment. Furthermore, a definitive link between cancer stem cells and integrin signaling has yet to be established in the roles of CSC differentiation, metastasis, and overall patient outcome.

Lunasin had significant antimelanoma effects in vivo:

Our results indicate lunasin substantially inhibits tumor formation and growth in an athymic nude mouse xenograft model. 30 mg/kg injections of lunasin reduced tumor volume by 35% and 55% with intravenous (Figure 15) and intraperitoneal (Figure 16) injections, respectively. IV injections were administered every other day; IP every day. Tumor mass was also significantly decreased by lunasin treatments. Upon necroscopy, whole tumors were resected and a wet tumor weigh was measured. IP and IV lunasin

treatments resulted in a 46% and 34% decrease in wet tumor weights upon endpoint of the experiment. These data delineate lunasin's bioavailability and anticancer effects in an *in vivo* model.

Non-steroidal Anti-inflammatory Drug (NSAID) toxicological panels showed lunasin treatments did not alter circulating plasma levels of liver enzymes ALKP, ALT, or AST. These data suggest lunasin does not induce a toxicological response in mice and is safe for future therapeutic applications. Furthermore, renal function was not impaired as measured by BUN and CREA. Complete blood count (CBC) analysis revealed lunasin does not significantly alter levels of leukocytes, erythrocytes, or thrombocytes compared to control.

Lunasin selectively inhibits MSC proliferation *in vivo*

Male athymic nude mice (6-8 weeks old) were transplanted with 1×10^4 cells that had previously been sorted using the ALDEFLUOR staining kit in an equal volume of HBSS (with calcium and magnesium) and Matrigel. Tumor formation was established at 16 days after injection and measured every other day thereafter. Control vehicle and 30 mg/kg lunasin were injected IP every 24 hours for the duration of the experiment. Our results demonstrate the selective effect of lunasin on melanoma stem cells expressing the ALDH biomarker. These cells are generally accepted to have enhanced tumorigenic capacity versus non-sorted bulk tumor cells. Compared to our previous study in which we injected 2.5×10^6 bulk A375 cells, MSCs displayed superior tumor forming ability.

MMICs established palpable tumors in a similar amount of time compared to parental tumor cells, however, this experiment utilized Matrigel in order to keep MMICs localized to subcutaneous tumor growth. Matrigel contains growth factors which may

boost cell proliferation; therefore, we concede that our MMIC and parental models are not comparable in terms of tumor growth rates. Lunasin did not decrease the number of tumors formed, but reduced tumor burden by 74%, a nearly 50% increased effect versus parental A375 cells. Onset of tumor formation was delayed in lunasin treated mice; tumors also displayed linear growth versus the exponential growth seen in control mice. Wet tumor weights for lunasin treated mice were reduced by 66%, thus illustrating the potential for lunasin to specifically target and inhibit growth of tumors derived from CICs.

DISCUSSION

Lunasin has previously been shown to have chemotherapeutic effects in a number of cancer models [3, 23, 25, 83-85]. To our knowledge, this is the first study identifying the anticancer effects of lunasin in a melanoma model. Therapies for treating patients with malignant melanoma remain largely ineffective, despite novel targeted therapies that show clinical improvements over traditional alkylating agents. Here we show that lunasin has potential clinical utility as both an adjuvant therapy as well as a standalone chemotherapeutic against malignant metastatic melanoma. Our data suggest that lunasin interacts with subsets of integrin subunits and interrupts internal signaling cascades induced by integrin activity.

Although HAT inhibition is generally regarded as the prime mechanism for lunasin's chemotherapeutic and chemopreventive attributes, we suggest the interactions between the RGD-peptide and integrin dimers found on the cell surface to be essential mechanisms in the *in vitro* and *in vivo* effects found in this study. Utilizing proximity ligation assays, we have shown that lunasin interacts with specific integrins (Ianaba and Davis, unpublished data), evidence supported by recent studies on integrin mediated endocytosis [27].

We do not postulate that the described effects are due to the epigenetic effects stimulated by lunasin treatment; though, we have found that lunasin does indeed affect patterns of histone acetylation in melanoma cells *in vitro* (data not shown). Because integrins are explicitly involved in cell adhesion and division, we concluded that

reductions in clonogenic capacity in soft agar and formation of multicellular oncospheres in low adherent culture are attributed to the disruption of adhesive signaling mediated by integrins. We agree that HAT inhibition does have global cellular effects; however, we believe that lunasin binding of integrins through its RGD domain is responsible for lunasin's anticancer activity in our melanoma models. However, *in vivo* effects may stem from a variety of cellular effects induced by lunasin including integrin antagonism, epigenetic modifications, and immunomodulatory functions.

Integrin signaling can regulate the stem cell processes of self-renewal, differentiation, and proliferation [86]. We have shown that lunasin treatment altered these processes and diminished intracellular interactions with Akt and Fak *in vitro* and resulted in decreased tumor burden *in vivo*. Though lunasin decreased tumor burden in mice injected with parental A375 melanoma cells, these effects were exacerbated in MMIC populations in our xenograft models. Furthermore, we confirmed that lunasin depleted the stem cell pool by reducing ALDH signal in established melanoma cell lines. These effects may be associated with the ability of lunasin to induce terminal differentiation of CICs or by altering downstream mediators of integrin signaling. The enhanced effects seen in MMIC populations suggests that cancer stem cells may rely on integrin signaling for proliferation and differentiation, more so than bulk tumor populations. These findings are consistent with recent studies that suggest FAK as well as $\beta 1$ integrin subunits regulate stem cell pools in breast cancer [87].

Fak normally functions as a scaffolding protein to mediate signal transduction, however, Fak also serves as a non-receptor tyrosine kinase mediating signal transduction from external stimuli. Several studies correlate Fak overexpression or amplification with

advanced disease resulting in poor prognosis [88, 89], consequently, targeting of FAK may provide a novel therapeutic strategy for inhibiting metastatic disease progression. FAK has been shown to arbitrate stem cell maintenance and differentiation in cancer models [50, 51]. FAK association and activation of PI3K leads to increased production of phospholipids, which can in turn activate Akt kinase [90, 91]. Again, these findings are consistent with the results in our melanoma model as lunasin decreased phosphorylation of Akt. Therefore, integrin signaling through FAK may play an essential role in melanomagenesis or disease progression and metastasis; a pathway targeted by the lunasin peptide.

Crosstalk between PI3K/Akt and NOTCH signaling pathways in melanoma has been well documented [92, 93]. NOTCH signaling has been implicated in stem cell processes such as self-renewal, differentiation, survival, and proliferation [94, 95]. It is plausible that the decreased Akt phosphorylation seen in melanoma cells is mediated through NOTCH signaling. Further studies are necessary to clarify whether or not lunasin induces changes in the PI3K/Akt pathway through NOTCH family receptors.

Another aspect of melanoma development is the theory of direct phenotype switching [96, 97]. To summarize, three distinct compartments exist in cancers; a highly proliferative cell with low invasive capability, a differentiated phenotype derived from daughter cells of divided stem-like cells, and an invasive, stem-like, quiescent phenotype that regenerates stem cell pools. Switching between these subsets may rely on certain genes such as microphthalmia-associated transcription factor (Mitf) and NOTCH. NOTCH activity is frequently elevated in melanomas; it also maintains melanocyte stem cell populations [98]. As discussed previously, NOTCH can mediate PI3K/Akt

signaling; overexpression of AKT by NOTCH1 provides a key step in promoting an invasive phenotype [99]. It has been shown that NOTCH1 is able to hyperactivate PI3K/Akt signaling through NF- κ B [93], a pathway previously revealed to be inhibited in human macrophages by lunasin treatment [2].

Mitf has been established as a differentiation marker in melanocytes; however, Mitf has also been reported to mediate proliferation, differentiation, and plasticity of melanoma cells [100]. Insightful studies have addressed that melanomas with a negative Mitf phenotype are generally quiescent, yet highly invasive [101]. Inversely, melanoma cells highly expressing MITF proliferate rapidly, but do not seem to metastasize. In support of these findings, inhibition of MITF increased expression of stem cell markers Nanog and Oct4 resulting in enhanced tumor growth in C57/B6 mice [102]. Again, culture conditions played a major role in expression of differentiation and stem markers [100, 103]. Hoek and Goding summarize: “If the combination favours the proliferative phenotype over the invasive, a tumour may grow rapidly, but for a given size, it will seed fewer metastases than a tumour whose cells are not subject to a proliferative phenotype bias.” [97] This hypothesis highlights the potential of selectively targeting cancer stem cells instead of relying on drugs targeting rapidly dividing cell populations to increase the overall efficacy of treatment in metastatic melanomas.

Clearly, these signaling pathways are implicated in both physiological and cancer stem cell processes. Our results show an apparent decline in cancer stem cell pools that may be influenced by these pathways; however, the potentiated effect in ALDH positive MSCs cannot be fully explained until further research elucidates lunasin’s effects on certain genes and gene products responsible for maintenance, proliferation, and

differentiation of stem-like cancer cells. Our data hints that lunasin may alter stem pool maintenance and proliferation, and therefore, has clinical utility in cases of late stage melanoma in which traditional therapies have failed. Future studies in our lab will focus on exploring the alterations induced by lunasin on cancer stem cell populations in established melanoma lines.

SUMMARY AND CONCLUSIONS

The soy-derived peptide lunasin has significant activity as a chemopreventive [7]; however, lunasin also serves as an anticancer agent in established cancer models [24, 83]. Lunasin treatment reduces the capacity of melanoma cells to form oncospheres in low adherent culture as well as inhibits clonal growth in soft agar. We believe these results stem from an interaction between lunasin and integrins on the ECM through its RGD domain, and results in decreased phosphorylation of intracellular signaling proteins such as Fak and Akt. *In vivo* studies show lunasin significantly decreased tumor growth in athymic nude mice injected subcutaneously with A375 melanoma cells. Tumor volume and weight were reduced by 55% and 46%, respectively. These data illustrate the potential clinical utility of lunasin against malignant melanoma.

Furthermore, we present a unique study showing the enhanced effects of lunasin against stem-like melanoma cells which display superior tumor forming capability compared to bulk tumor cells. Using ALDH, a recognized MMIC biomarker [77], we isolated cells expressing distinctively higher levels of the enzyme and subjected them to further *in vitro* testing. We found that MMIC response to lunasin treatment was potentiated versus the parental cell lines both *in vitro* and *in vivo*. 30 mg/kg lunasin treatment reduced tumor burden in our melanoma xenograft model by approximately 75%. Despite using appreciably fewer cells to initiate tumor formation, ALDH expressing cells formed palpable tumors within a comparable amount of time versus bulk tumor populations.

The functional assays in this study illustrate the prospective use of lunasin as a chemotherapeutic against melanomas in which traditional therapy has failed.

Additionally, we showed that extended lunasin treatment displayed no toxicological effects versus control in our animal model. However, we have only begun to elucidate mechanisms in which lunasin acts as an anticancer agent. While epigenetic effects undoubtedly play a major role in tumorigenesis, we feel that lunasin has several critical effects on integrin signaling and further, CIC maintenance and proliferation resulting in the augmented effects seen in MMICs.

It must be taken into consideration that several theories exist in which cells are able to move between stem and differentiated cell compartments; theories in which melanoma serves as a prime example. If this is the case, more research is warranted to determine if A) lunasin is inducing terminal differentiation of melanoma cells, B) confirm ALDH as a viable biomarkers for MMIC populations by utilizing alternative markers, and C) analyze the alterations in gene expression patterns induced by lunasin treatment to further advance our understanding of the peptides anticancer effects.

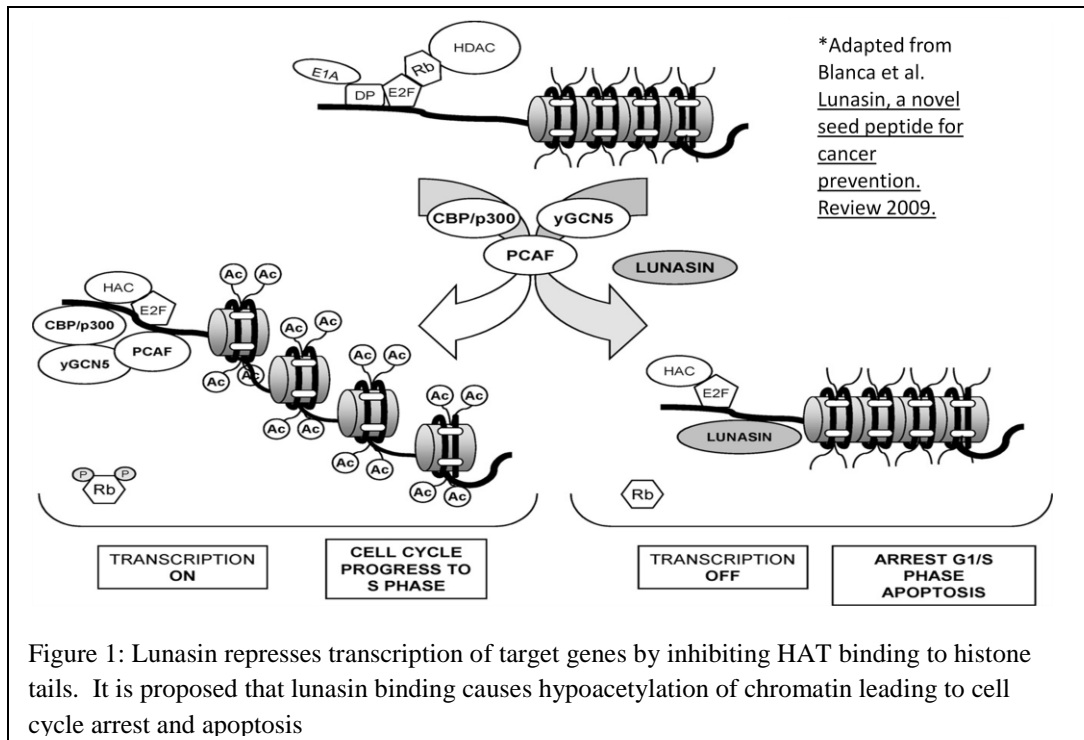
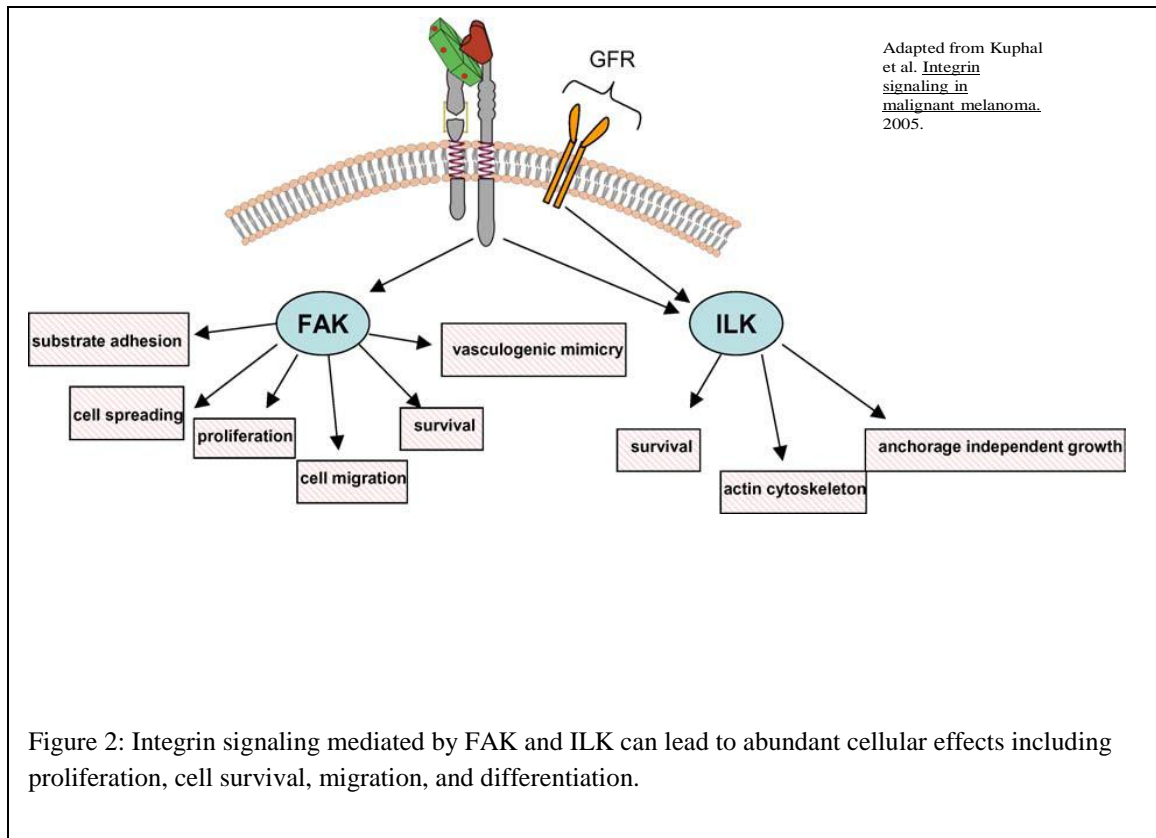
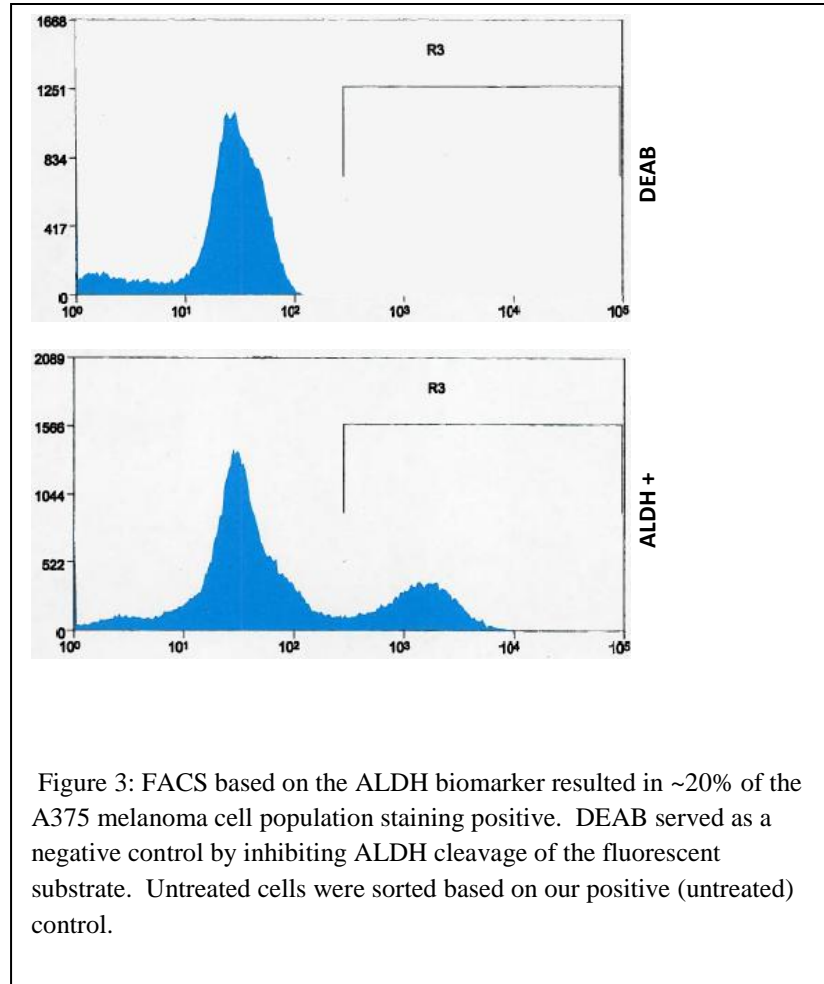
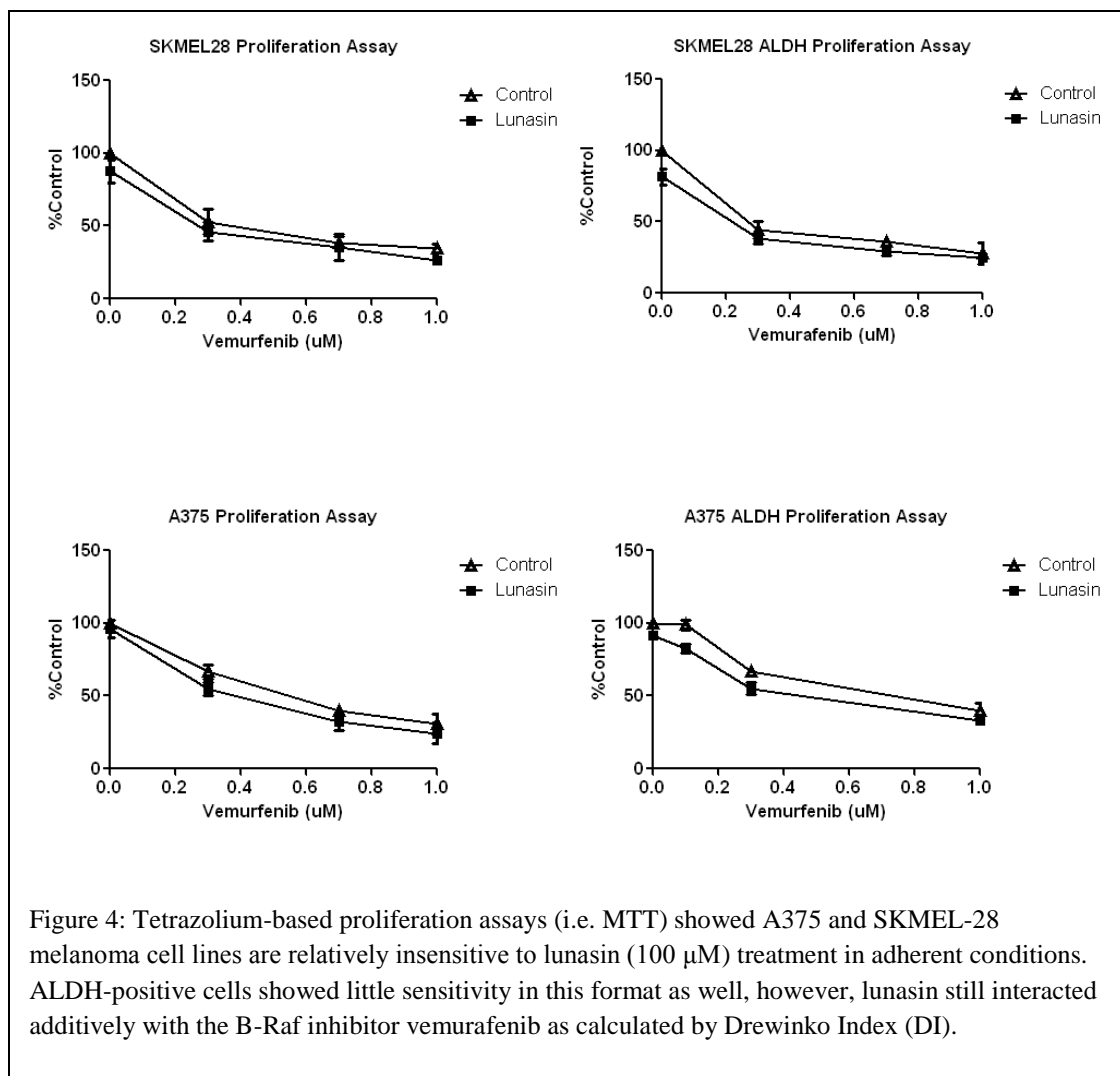


Figure 1: Lunasin represses transcription of target genes by inhibiting HAT binding to histone tails. It is proposed that lunasin binding causes hypoacetylation of chromatin leading to cell cycle arrest and apoptosis







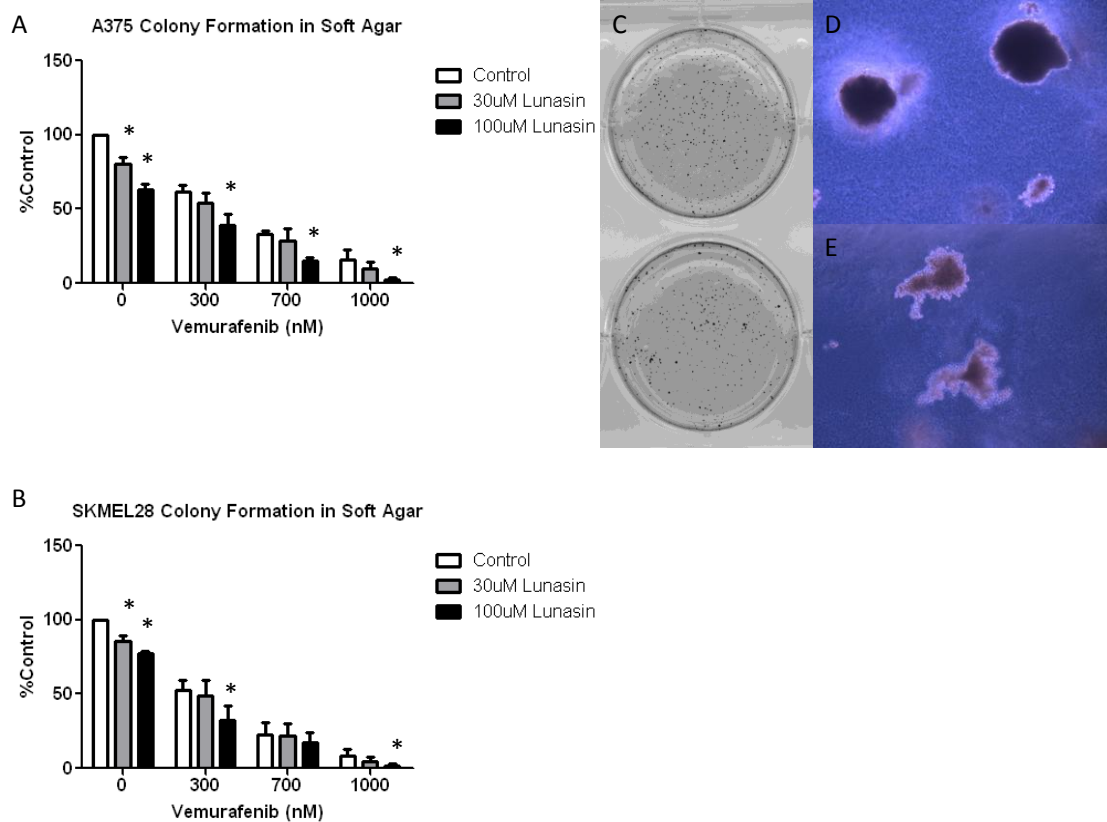


Figure 5: Lunasin and vemurafenib combination treatments show the potential of lunasin as an adjuvant treatment of (A) A375 and (B) SKMEL-28 melanoma cells by reducing colony forming ability in soft agar. (C) 100 μ M lunasin (bottom well) reduced colony formation by approximately 40% and 20% in A375 and SKMEL-28 melanoma cell, respectively. (D) Control A375 colonies formed tight, well defined colonies in soft agar that were larger than (E) lunasin treated colonies. Significance denoted by asterisk as assessed by unpaired Student's T test ($p < 0.05$).

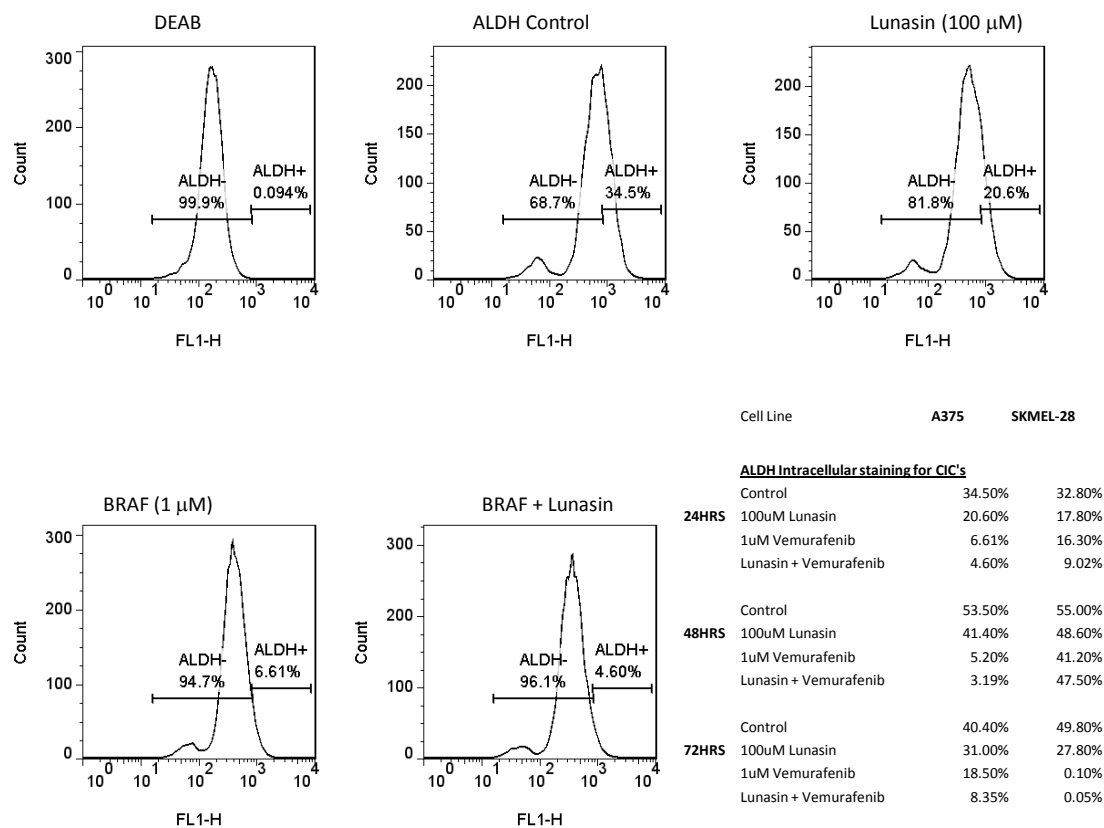


Figure 6: Lunasin and vemurafenib treatment decreased the number of ALDH^{high} cell populations in A375 and SKMEL-28 melanoma cells. Combination treatment further reduced ALDH expression in both cell lines, however, this effect was less pronounced at later time points. (n = 1)

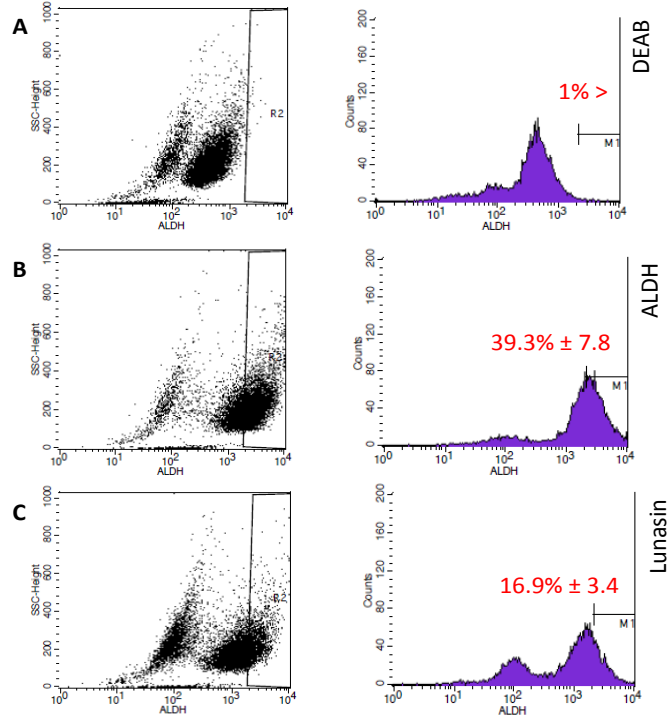


Figure 7: ALDH represents a unique biomarker to identify and isolate melanoma stem cells. Here we use the specific ALDH inhibitor DEAB as a negative control (A) to determine parameters for ALDH-positive (B) cells. We show that 100 μ M lunasin (C) treatments for 24 hours reduced ALDH expressing populations in melanoma cell lines, while concurrently

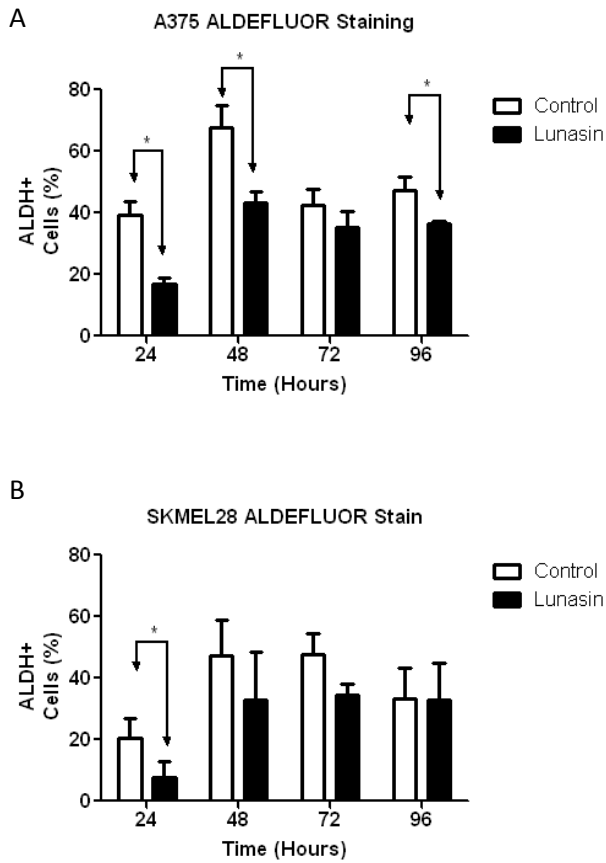
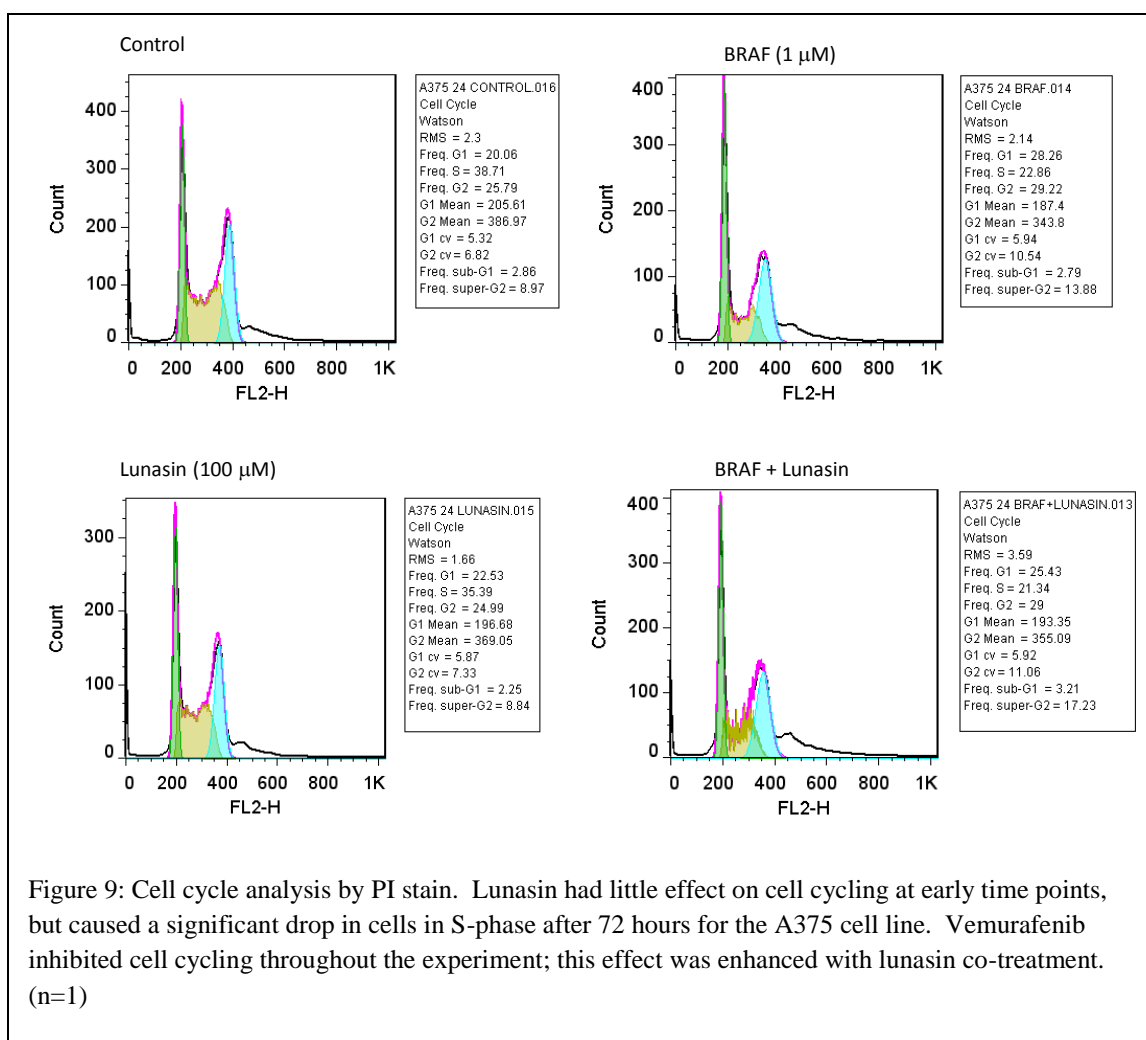


Figure 8: ALDH expression was significantly reduced by 100 μ M lunasin treatments (A) lunasin reduced ALDH expressing cell populations by 56%, 35%, 18%, and 22% for 24, 48, 72, and 96 hour timepoints, respectively. (B) ALDH expressing cell populations were significantly reduced at 24 hours in SKMEL28 melanoma cells, but only marginally at later time points. Statistical significance was determined by Student's T test with p values set at 0.05.

	A375					SKMEL-28				
	G1	S	G2	Sub-G1	Super-G2	G1	S	G2	Sub-G1	Super-G2
Control	20.06	36.71	25.79	2.86	8.97	52.5	36.85	8.18	0	0
100uM Lunasin	22.03	35.39	24.99	2.25	8.84	41.24	35.61	9.49	1.58	3.95
1uM Vemurafenib	28.26	22.86	29.22	2.79	13.88	64.99	15.18	4.75	8.84	6.24
Lunasin + Vemurafenib	25.43	21.04	29	3.21	17.23	44.83	15.6	39.44	8.52	24.83
Control	40.8	32.6	29.6	1.31	3.19	53.7	26.2	8.1	0.74	0.73
100uM Lunasin	38.5	32.8	20.1	2.16	3.09	51.9	25.6	11.9	1.56	0.36
1uM Vemurafenib	77.4	14.2	3.6	2.22	0.04	72.7	15.7	2.8	4.79	1.27
Lunasin + Vemurafenib	70.3	13.1	9.6	2.62	3.73	63.7	18.2	4.6	6.64	3.04
Control	42.2	36.5	14.3	4.81	2.2	45	21.8	6.1	4.71	0.06
100uM Lunasin	37.3	8.1	22.7	4.27	24.2	36.6	18.9	8.7	2.34	4.24
1uM Vemurafenib	38	6.6	20.8	9.99	22.23	69.2	7.4	0.5	13.73	0.03
Lunasin + Vemurafenib	18.4	6.6	10.9	23.5	32.97	55.5	11.4	1	14.69	0.38



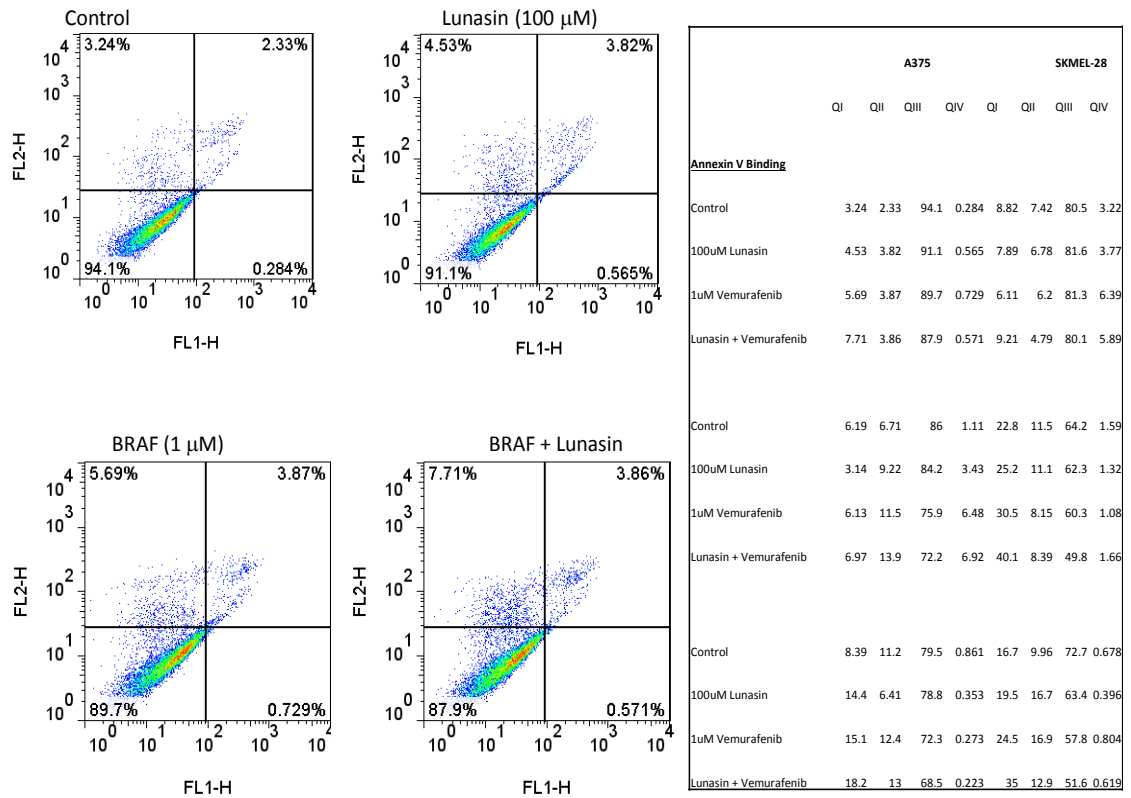
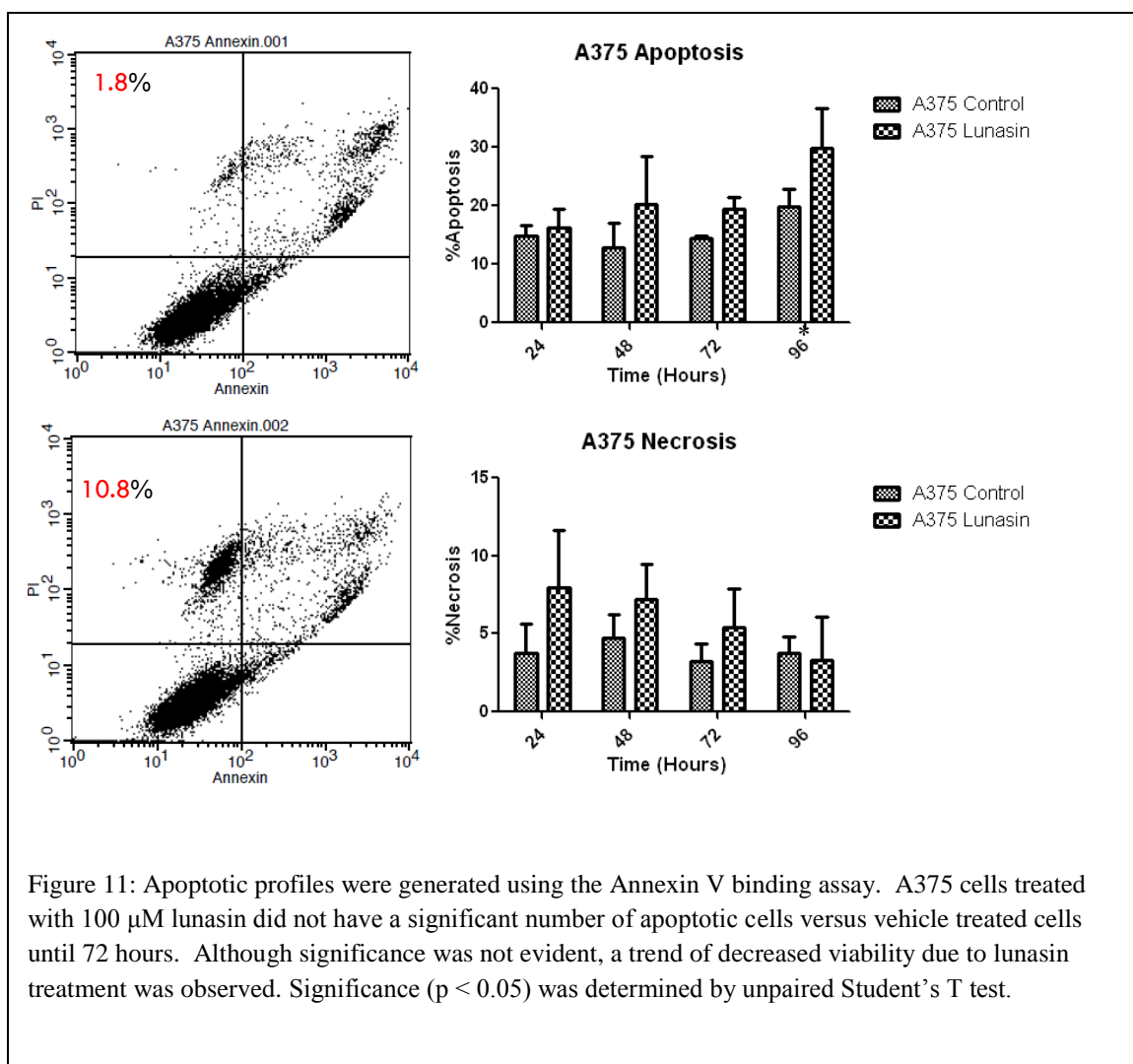
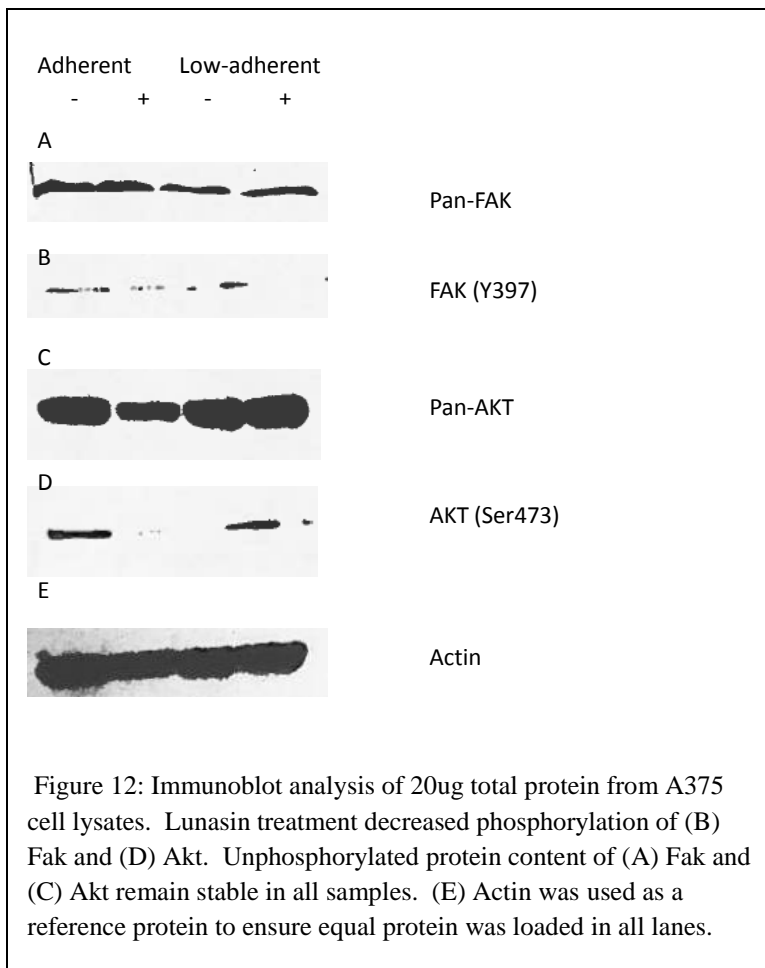


Figure 10: Annexin V binding assays were used to assess apoptotic profiles for A375 and SKMEL-28 melanoma cell lines in response to vemurafenib and lunasin treatments. Lunasin again potentiated the effects of vemurafenib in both cell lines resulting in decreased viability and a concomitant increase in late apoptotic cells.





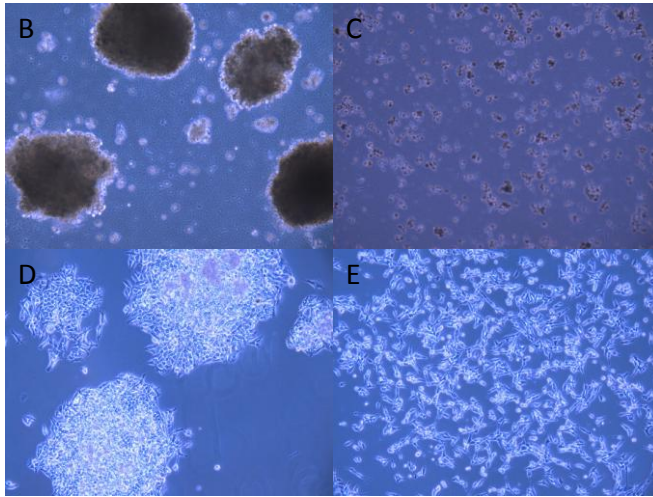
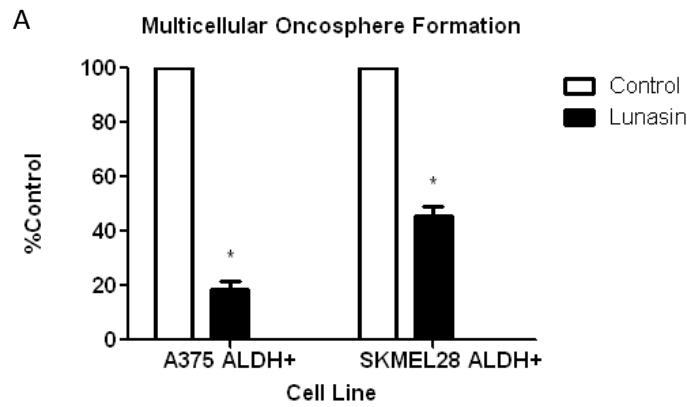


Figure 13: 100 μ M lunasin reduced formation of oncospheres in A375 and SKMEL28 cell lines. (A) lunasin inhibited melanoma stem cells from forming oncospheres in low adherent culture. (B) Control wells contained A375 spheroids cultured for 28 days (C) lunasin treatment inhibited A375 oncosphere formation in low adherent culture (D) A375 ALDH+ MSCs were replated into adherent T-25 flasks and allowed to form foci on the plate (E) lunasin treatment completely inhibited foci formation. Significance is denoted by asterisk as assessed by Student's T test ($p < 0.05$).

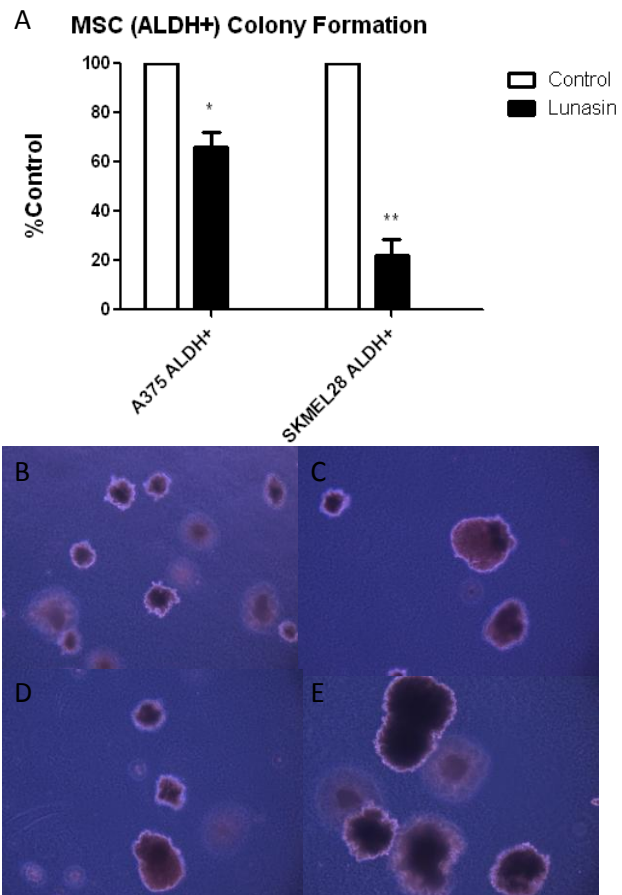
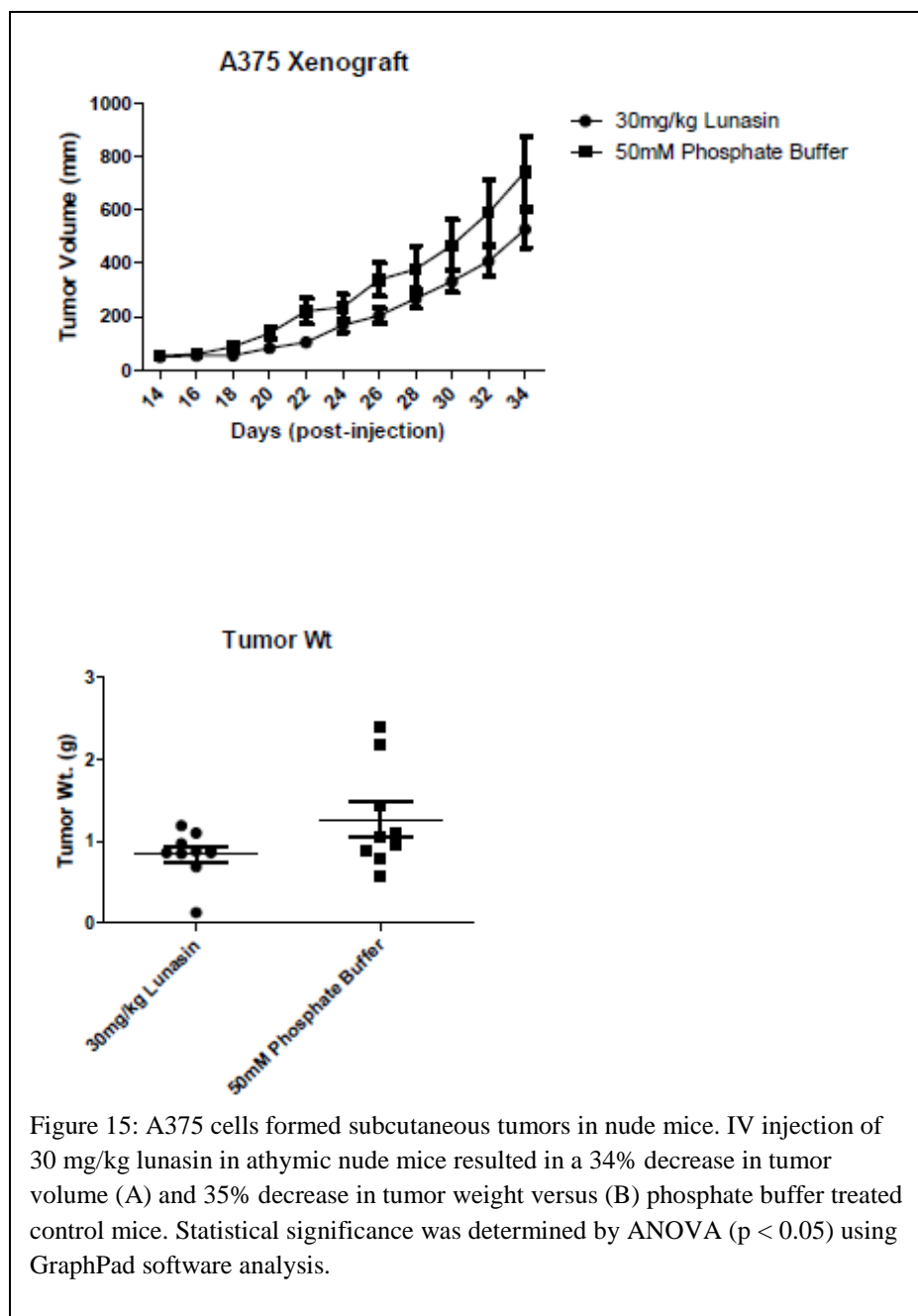


Figure 14: Colony formation in soft agar was significantly reduced with 100 μ M lunasin treatment. (A) Colony formation in soft agar was significantly reduced in both A375 and SKMEL-28 cell lines. Cell proliferation was reduced as seen by the smaller colony size in lunasin treated A375 (B) and SKMEL-28 (D) cell lines versus phosphate buffer controls (C,E). Significance is denoted by an asterisk and assessed by Student's T test ($p < 0.05$).



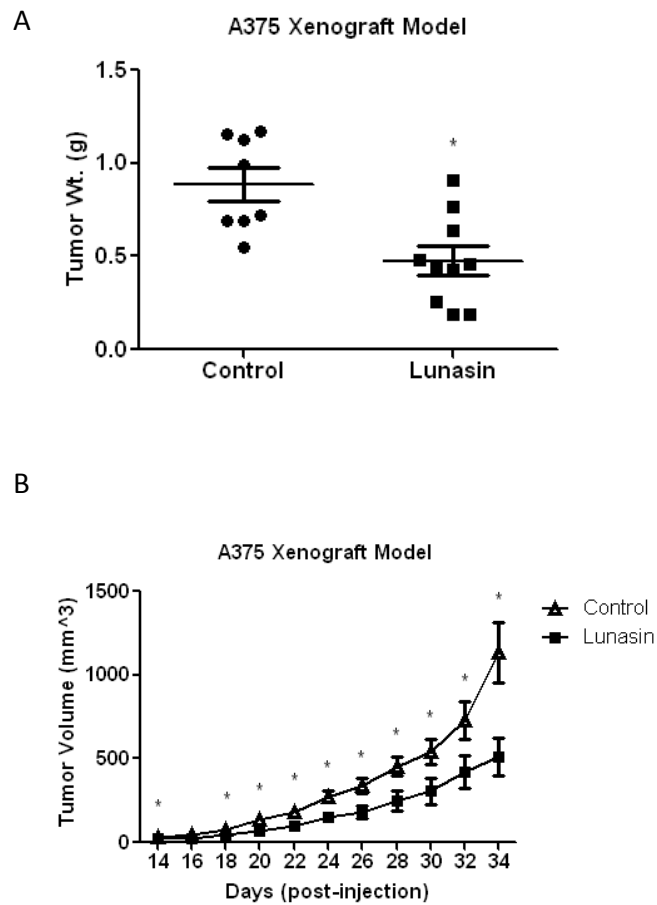


Figure 16: In-vivo xenograft models utilizing athymic nude mice illustrate the potential therapeutic advantage to the soy-derived peptide lunasin. (A) Wet tumor weight from resect tumor tissues. 30 mg/kg lunasin treatment reduced tumor weights by 46%. (B) Tumor volume was reduced by 55% and represented a significant difference from phosphate buffer control as determined by one-way ANOVA ($p < 0.05$)

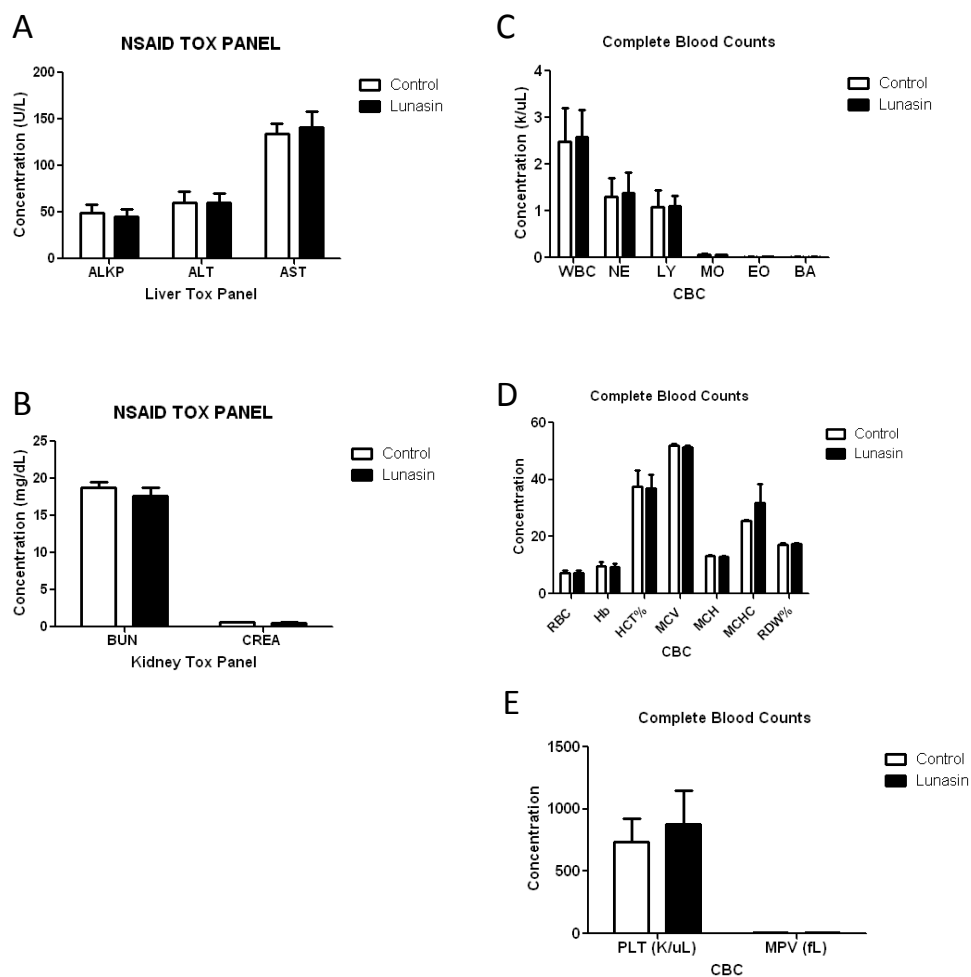


Figure 17: Lunasin showed no apparent liver (A) or kidney (B) damage as assessed by NSAID toxicological panels. Complete blood counts (C,D,E) also illustrate the safety of continual lunasin treatment. No significance was determined by Student's T test ($p < 0.05$) between treatment groups.

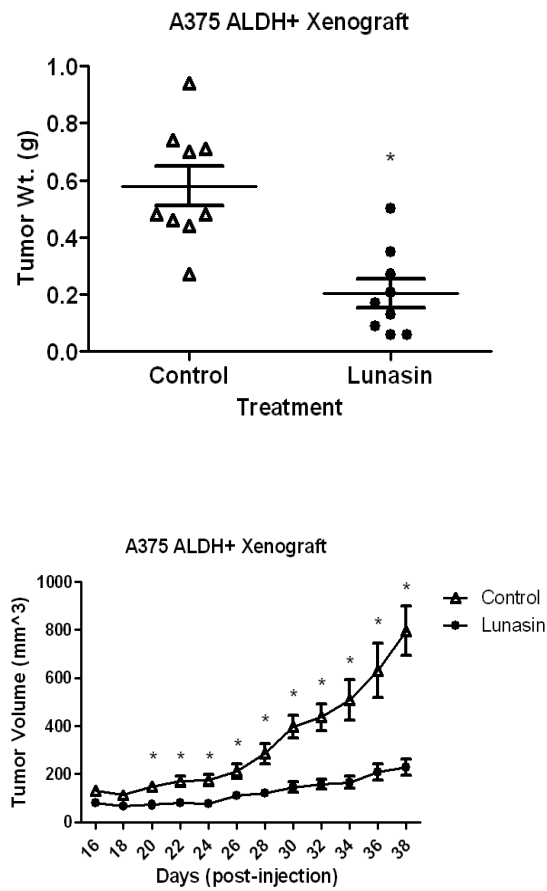


Figure 18: Melanoma stem cell xenograft model (A) 30 mg/kg lunasin treatment reduced tumor weight by 64% compared to phosphate buffer control. (B) Tumor volume was also significantly reduced 74% versus control vehicle. Statistical significance was determined by ANOVA ($p < 0.05$) using GraphPad software analysis.

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CURRICULUM VITAE

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Personal Statement:

My goals as a research fellow are to define the molecular interactions between specific carcinogens and to characterize the roles of these interactions in the initiation of cancer development. Furthermore, I expect to profile and advance the soy-derived peptide lunasin as a direct chemotherapeutic in melanoma and lung cancers. Previous academic history as well as direct work experiences has provided the basic knowledge and confidence necessary to succeed on these projects. Background experience in the field of pharmacology has allowed me to develop concepts and critical thinking skills necessary to excel throughout my career. While I was an undergraduate at UK, I tested domestic well water samples for coliform and coliphage, which established good laboratory practices and techniques. My time as a quality assurance technician allowed me to demonstrate my ability to work in a group. Furthermore, I was given the opportunity to advance a number of projects independently and in a timely manner. Although it was more geared toward production, my work in quality assurance permitted me to learn a number of wet chemistry protocols and utilize various analytical instruments (i.e. HPLC). This project will sharpen these skills and hopefully become a solid base for future work in the field of pharmacology and toxicology.

Education:

INSTITUTION AND LOCATION	DEGREE (if applicable)	DATES ATTENDED	FIELD OF STUDY
Paducah Community College (Paducah, KY)	N/A	08/04 – 08/05	Biology
University of Kentucky (Lexington, KY)	B.S.	08/05 – 08/08	Biology
University of Louisville (Louisville, KY) (<i>expected</i>)	M.S.	08-11 - Present	Toxicology

Positions and Honors:

Employment

- ❖ 2003 - 2006 Pharmacy Technician, Lourdes Hospital, (Paducah, KY)
- ❖ 2008 Research Technician, University of Kentucky (Lexington, KY)
- ❖ 2008 - 2010 Quality Assurance Technician, Air Products (Calvert City, KY)
- ❖ 2011 - present Predoctoral Researcher, University of Louisville (Louisville, KY)
- ❖ 2014 – present Visiting Research Associate, Indiana University

Volunteerism and Memberships

- ❖ 2001 - present Volunteer, Relay for Life (Sponsored by ACS)
- ❖ 2004 Volunteer, Habitat for Humanity
- ❖ 2008 - 2011 Volunteer, ASPCA
- ❖ 2011 - present Member, Society of Toxicology (SOT)

Honors

- ❖ 2004 – 2005 Academic Scholarship, Paducah Community College
- ❖ 2004 – 2005 Deans List, Paducah Community College
- ❖ 2009-2010 Governor's Safety and Health Award, Air Products (Company Award)
- ❖ 2011-2013 Graduate Fellowship, University of Louisville (IPIBS)

Publications:

Submitted Abstracts

Arrowood, Karen, et al. (2008) *Characterizing the health risks associated with domestic well water use in rural Western Kentucky leading to an intervention study*. University of Kentucky.

Shidal, Chris and Keith R. Davis (2012). *Lunasin inhibits proliferation of non-small cell lung cancer (NSCLC) in vitro*. Research Louisville, University of Louisville.

Shidal, Chris and Keith R. Davis (2012). *Lunasin inhibits proliferation of non-small cell lung cancer (NSCLC) in vitro*. J.G. Brown Cancer Center Retreat, University of Louisville.

Shidal, Chris and Keith R. Davis (2013). *Molecular interactions between cadmium and nitrosamine ketone potentiate carcinogenic potential of cigarette smoke*. OVSOT 2013 Spring Meeting, University of Louisville.

Shidal, Chris and Keith R. Davis (2013). *Profiling lunasin as an adjuvant treatment in malignant melanoma*. J.G. Brown Cancer Center Retreat, University of Louisville

Presentations

Shidal, Chris (2011). *The soy peptide lunasin has antiproliferative effects on non-small cell lung cancer*. Departmental seminar, University of Louisville Pharmacology and Toxicology Department.

Shidal, Chris (2012). *Lunasin inhibits proliferation of non-small cell lung cancer (NSCLC) in vitro*. Research Louisville, University of Louisville.

Shidal, Chris (2012). *Lunasin inhibits proliferation of non-small cell lung cancer (NSCLC) in vitro*. J.G. Brown Cancer Retreat, University of Louisville.

Shidal, Chris and Keith R. Davis (2013). *Molecular interactions between cadmium and nitrosamine ketone potentiate carcinogenic potential of cigarette smoke*. OVSOT 2013 Spring Meeting, University of Louisville.

Shidal, Chris and Keith R. Davis (2013). *Profiling lunasin as an adjuvant treatment in malignant melanoma*. J.G. Brown Cancer Center Retreat, University of Louisville

Skills:

Cell Culture Work (monolayer and 3D cell cultures, along with co-cultures), Assays for Cancer Cell Growth (Soft Agar, Proliferation, Oncosphere Formation), General Lab Protocols (Slide Preparation of Cultured Cells/Tissues, Confocal/Fluorescent Microscopy, Immunoblot, Transient Transfection of Plasmids, Functional knock-down using siRNA, Flow Cytometry/FACS, GC, Microarray), Animal Work (IV/IP Injections in Mice, Resection of Organ/Tumor Tissues), Statistical Analysis (GraphPad Prism, CalcuSyn, MS Databases)