



RESEARCH NOTE

**REVISED** Validation of syngeneic mouse models of melanoma and non-small cell lung cancer for investigating the anticancer effects of the soy-derived peptide Lunasin [version 2; referees: 1 approved, 2 approved with reservations]

Bharat Devapatla<sup>1</sup>, Chris Shidal<sup>1,3</sup>, Kavitha Yaddanapudi<sup>2</sup>, Keith R. Davis<sup>3</sup>

<sup>1</sup>Department of Pharmacology and Toxicology, University of Louisville School of Medicine, , Louisville, KY, 40402, USA

<sup>2</sup>Department of Medicine and James Graham Brown Cancer Center, University of Louisville School of Medicine, Louisville, KY, 40402, USA

<sup>3</sup>Department of Biology and Biotechnology Program, Indiana University, Bloomington, IN, 47405, USA

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**Abstract**

**Background:** Lunasin is a naturally occurring peptide present in soybean that has both chemopreventive and therapeutic activities that can prevent cellular transformation and inhibit the growth of several human cancer types. Recent studies indicate that Lunasin has several distinct potential modes of action including suppressing integrin signaling and epigenetic effects driven by modulation of histone acetylation. In addition to direct effects on cancer cells, Lunasin also has effects on innate immunity that may contribute to its ability to inhibit tumor growth *in vivo*.

**Methods:** Standard assays for cell proliferation and colony formation were used to assess Lunasin’s *in vitro* activity against murine Lewis lung carcinoma (LLC) and B16-F0 melanoma cells. Lunasin’s *in vivo* activity was assessed by comparing the growth of tumors initiated by subcutaneous implantation of LLC or B16-F0 cells in Lunasin-treated and untreated C57BL/6 mice.

**Results:** Lunasin was found to inhibit growth of murine LLC cells and murine B16-F0 melanoma cells *in vitro* and in wild-type C57BL/6 mice. The effects of Lunasin in these two mouse models were very similar to those previously observed in studies of human non-small cell lung cancer and melanoma cell lines.

**Conclusions:** We have now validated two established syngeneic mouse models as being responsive to Lunasin treatment. The validation of these two *in vivo* syngeneic models will allow detailed studies on the combined therapeutic and immune effects of Lunasin in a fully immunocompetent mouse model.

**Open Peer Review**

Referee Status: ? ? ✓

	Invited Referees		
	1	2	3
<b>REVISED</b>			✓
<b>version 2</b> published 22 Feb 2017			report
			↑
<b>version 1</b> published 04 Oct 2016	?	?	?
	report	report	report

- 1 **Jean-Pierre Gillet**, University of Namur Belgium
- 2 **Masaki Terabe**, National Institutes of Health (NIH) USA
- 3 **Elizabeth S. Yeh**, Medical University of South Carolina USA

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**Corresponding author:** Keith R. Davis ([keirdavi@indiana.edu](mailto:keirdavi@indiana.edu))

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**Grant information:** This work was funded by Owensboro Grain Company, Owensboro, Kentucky, USA.

*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

**Competing interests:** KRD is listed as an inventor on two issued patents relating to the expression and purification of Lunasin peptides and may benefit financially if the technologies described in these patents are licensed or sold. BD, CS, and KY declare no conflict of interests.

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**REVISED Amendments from Version 1**

This research note has been updated to address specific questions and suggestions by the reviewers that directly relate to this study. We have not significantly expanded the discussion on what is known about Lunasin and how syngeneic mouse models are used in drug development as it is beyond the scope of this highly focused research note. We have updated [Figure 1](#).

**See referee reports**

## Introduction

Lunasin is a multifunctional bioactive peptide present as a component of the storage protein fraction in soybean seeds and in soy-derived food products<sup>1-4</sup>. Studies from several laboratories have documented that Lunasin has both chemopreventive activity that inhibits cellular transformation by carcinogens or oncogenes<sup>5-7</sup> and chemotherapeutic activity against multiple human cancer types<sup>8-15</sup>. Taken together, these observations suggest that Lunasin may be one of the factors responsible for the lower cancer rates observed in people who consume high-soy diets<sup>1-3</sup>. One intriguing aspect of Lunasin is that this 44 amino acid peptide has at least three potential functional domains; a polyaspartic-acid C-terminal tail that binds Lunasin to the core histones H3 and H4<sup>7,16-18</sup>, a tripeptide Arg-Gly-Asp (RGD) domain that can serve as a recognition signal for specific integrins<sup>9,16,19</sup>, and a putative helical chromatin binding domain<sup>3,11</sup>. An important component of Lunasin uptake appears to be mediated by internalization via the integrin recycling pathway, with the integrin  $\alpha V\beta 3$  being a key factor<sup>15,16,19</sup>.

Our previous studies found that native Lunasin purified from soybean has therapeutic activity against established human non-small cell lung cancer (NSCLC) and melanoma cell lines both *in vitro* and *in vivo*<sup>8,15</sup>. In the case of NSCLC, *in vitro* studies suggested that a primary mechanism of action was the inhibition of proliferation caused by inhibition of integrin signaling and decreased retinoblastoma protein phosphorylation<sup>15,16,20</sup>. In the case of melanoma, Lunasin caused a significant decrease in putative cancer stem cells by causing these cells to switch phenotypes to a cell type expressing higher levels of the transcription factor MITF and one of its downstream targets, tyrosinase. In addition, decreased levels of the stemness protein Nanog were also observed<sup>8</sup>. Our recent unpublished studies suggest that Lunasin effects on melanoma cells are also mediated, at least in part, by effects on integrin signaling<sup>21</sup>. These results, along with a recent report on the effects of Lunasin on colon cancer stem-like cells<sup>10</sup>, suggest the exciting possibility that Lunasin can be used to target cancer stem cells. In addition, the currently available data suggest that Lunasin does possess attributes important for clinical utility including no obvious toxicity<sup>8</sup> and being bioavailable<sup>22,23</sup>.

One of the more recent unexpected and exciting findings regarding Lunasin's anticancer effects is that Lunasin appears to also have immunomodulatory activity<sup>11,24,25</sup>. Interestingly, these effects correlate with epigenetic effects and do not require the RGD domain or the polyaspartic-acid tail, thus implicating the putative chromatin-binding domain as being important<sup>11</sup>. Given that

Lunasin has both direct therapeutic effects on cancer cells as well as the ability to affect immunity, we were prompted to determine if syngeneic mouse cancer models could be identified where both of these activities could be studied in concert so that the relative contribution of these two different effects on the potent *in vivo* activity of Lunasin could be determined. In these studies, we demonstrate that Lunasin has significant *in vitro* and *in vivo* activity in syngeneic mouse models for lung cancer and melanoma. These syngeneic models will provide the ability to pursue studies of Lunasin action in an immunocompetent host and use genetic approaches to understand how specific genetic manipulations affect Lunasin's ability to inhibit tumor growth and metastasis.

## Methods

### Lunasin purification

Lunasin was purified from soybean white flake (Owensboro Grain Company) as previously described<sup>26</sup> by Kentucky BioProcessing (Owensboro, KY). Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis indicated that this Lunasin preparation had >99% purity<sup>8</sup>. The purified Lunasin was diluted to a concentration of 9.3 mg/ml in sterile 50 mM sodium phosphate buffer, pH 7.4 and stored at 4°C.

### Cell lines and treatments

LLC (mouse lung carcinoma) and B16-F0 (mouse melanoma) cell lines were obtained from the American Type Culture Collection (ATCC). LLC and B16-F0 cells were cultured in DMEM medium (Invitrogen). Medium was supplemented with 10% fetal bovine serum (Invitrogen), 100 IU/mL of penicillin, and 100 µg/mL of streptomycin (Invitrogen) and cells grown at 37°C in a humidified incubator containing 5% CO<sub>2</sub>.

### Cell growth assay

*In vitro* cell growth inhibition was measured via a tetrazolium-based [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay (Promega). Briefly, 2 × 10<sup>3</sup> cells were plated into 96-well plates and incubated overnight. The cells were treated with the indicated concentrations of Lunasin for 72 hours in 100 µL fresh medium. Every 24 hours, cell culture media was replaced with fresh culture media amended with the indicated concentrations of Lunasin. After 72 hours, 20 µL of CellTiter 96® Aqueous One reagent (Promega) was added and incubated with the cells for 1 hour. Absorbance was measured at 490 nm using a Synergy HT plate reader (Biotek). Cell growth was estimated from the absorbance readings and has been normalized to vehicle-treated control cells. Averages of three replicates per treatment were used for analysis.

### Soft agar colony-forming assays

These assays were done as previously described<sup>8</sup> except that a 24-well per plate format was used. LLC and B16-F0 cells were plated at a density of 500 and 1,000 cells/well, respectively.

### *In vivo* tumor growth studies

Six-week-old male mice (C57 BL/6) were purchased from Harlan Laboratories (Indianapolis, IN). All procedures involving mice were carried out in accordance with the international guidelines of the Association for Assessment and Accreditation of Laboratory Animals Care with the approval of the University of

Louisville Institutional Animal Care and Use Committee (Protocol # 12091). Mice were maintained in the University of Louisville Health Center animal use facility and maintained by Research Resources Facilities staff using standard approved protocols. Mice were housed in polycarbonate shoebox cages (maximum 5 mice/cage) on a ventilated rack system in a temperature controlled room operating on a timed 12 hour light/dark cycle. Mice were randomly placed into groups (6–10 mice per group) and received subcutaneous injections of LLC ( $1 \times 10^5$ ) or B16-F0 ( $1 \times 10^6$ ) cells suspended in 100  $\mu$ L of phosphate buffered saline (PBS) in the hind flank. Tumors were measured starting 10 days post-injection up to 22 days post-injection. Tumor size was measured twice weekly using digital calipers (Mitutoyo) with an accuracy of  $\pm 0.02$  mm. Tumor volume was calculated as  $\frac{w^2 \times l}{2}$  where  $w$  = width and  $l$  = length. All mice except in control group were treated with Lunasin daily starting from the day of injection. Lunasin was administered by intraperitoneal (IP) injections in 50 mM phosphate buffer at a dose of either 10 or 30 mg Lunasin/kg body weight. In some experiments, cells were pretreated with 100  $\mu$ M Lunasin for 72 hours prior to injection of cells into mice. At the end of the experiments, mice were euthanized by CO<sub>2</sub> asphyxiation followed by cervical dislocation.

## Results and discussion

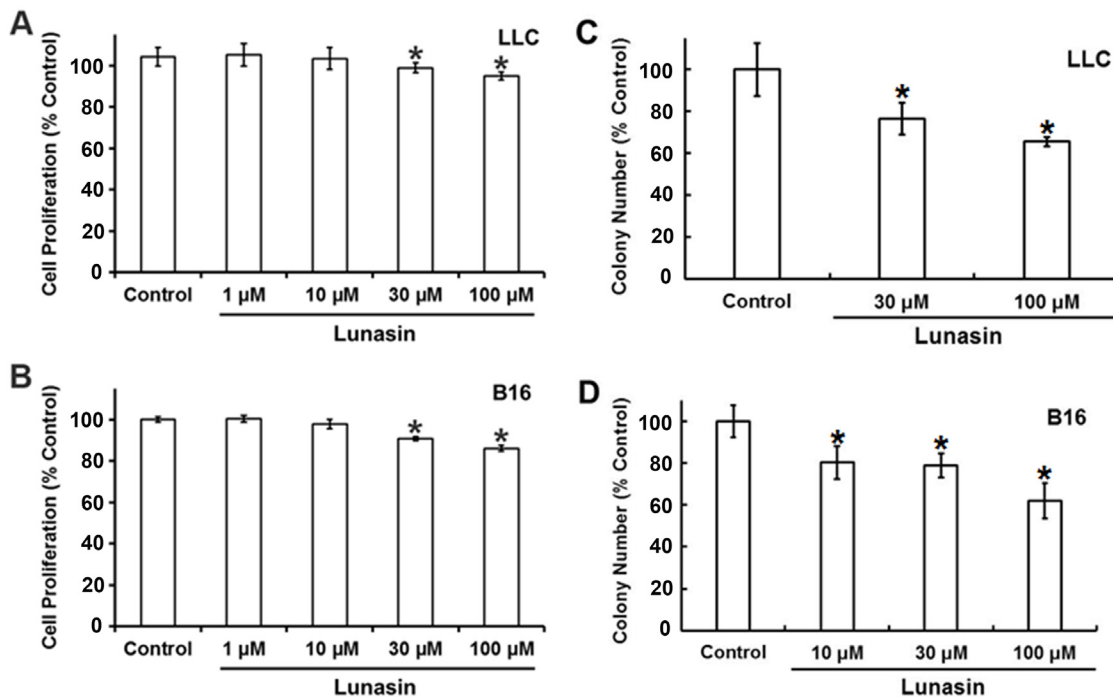
### *In vitro* effects of Lunasin treatment

We tested the ability of Lunasin to inhibit LLC and B16-F0 growth in both adherent and non-adherent assays. In adherent

assays, Lunasin had modest dose-dependent effects on the growth of both LLC and B16-F0 cells; <10% at 30 and 100  $\mu$ M Lunasin (Figure 1A–B, Table 1). In contrast, Lunasin had substantial inhibitory activity in non-adherent colony forming assays. Both LLC and B16-F0 exhibited a dose-dependent reduction in colony formation from ~20% to 40% over a Lunasin concentration range of 10 to 100  $\mu$ M (Figure 1C–D, Table 2). The difference in activity observed in adherent versus non-adherent assays recapitulates our previous results using human NSCLC and melanoma cells and likely reflects differences in integrin expression profiles under these distinct culture conditions<sup>8,15</sup>. The sensitivity of the mouse cell lines were comparable to that observed for human NSCLC and melanoma cells. Growth inhibition under adherent culture conditions was <15% for most NSCLC cell lines and <10% for melanoma cell lines treated with 100  $\mu$ M Lunasin, whereas inhibition of colony formation by human NSCLC and melanoma cell lines treated with 100  $\mu$ M Lunasin ranged from ~65% to 85%, and ~20 to 40%, respectively. These results demonstrate that the Lunasin sensitivity of human and mouse lung cancer and melanoma cells are quite similar *in vitro*.

### *In vivo* effects of Lunasin treatment

We initially tested the ability of Lunasin to inhibit tumor growth initiated by LLC cells at doses of 10 and 30 mg/kg. These doses are comparable to those for several biologic drugs and the cyclic peptide, cilengitide<sup>27,28</sup>. Lunasin inhibited tumor growth in mice treated at the 30 mg/kg dose by 55% at day 22 whereas the

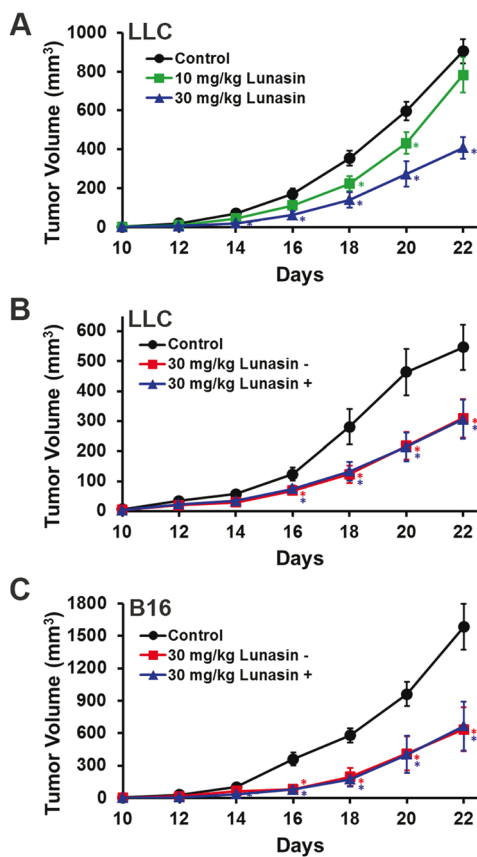


**Figure 1.** Effects of Lunasin on the *in vitro* growth of LLC and B16-F0 cells. Cells were cultured under adherent (A,B) or non-adherent (C,D) culture conditions and treated with the indicated concentrations of Lunasin. For adherent-culture assays, proliferation was assessed after 72 hours of treatment using a MTS assay. For non-adherent-culture assays, colonies were allowed to form over 10–18 days until colonies grew to approximately 100  $\mu$ m in diameter. The number of colonies formed was counted after staining with crystal violet. Data from both assays have been normalized to the vehicle treated control and represent the mean  $\pm$  S.D. An asterisk (\*) indicates that a treatment was significantly different ( $p < 0.05$ ) from the control as determined by an unpaired student's *t*-test.

10 mg/kg dose had only modest effects that were only statistically significant on days 18 and 20 (Figure 2A, Table 3). We next tested whether pre-treating LLC cells with 100  $\mu$ M for 72 h *in vitro* prior to implantation further affected tumor growth. This was prompted by our earlier studies demonstrating that Lunasin reduces the putative cancer initiating cell pool in human melanoma cell lines<sup>8</sup>. The results clearly show that pre-treatment did not enhance inhibition of tumor growth by Lunasin at a dose of 30 mg/kg (Figure 2B, Table 4). In this experiment, tumor growth at day 22 was 43% of the control. The inhibition of LLC tumor growth by Lunasin was somewhat less than that observed

in xenograft studies of NSCLC H1299 where tumor growth was reduced by 63% at 32 days in mice treated with 30 mg/kg Lunasin<sup>15</sup>.

Lunasin at a dose of 30 mg/kg was also found to inhibit tumor growth initiated by B16-F0 melanoma cells, with a reduction in tumor growth of 60% at day 22 (Figure 2C, Table 5). As was the case with LLC, pre-treatment with 100  $\mu$ M Lunasin for 72 h *in vitro* did not enhance inhibition of tumor growth. These results are quite comparable to our xenograft studies using the human melanoma cell line A375 where we observed a 55% reduction in tumor volume 34 days after implantation<sup>8</sup>.



**Figure 2. Lunasin inhibition of LLC and B16-F0 tumor growth in C57BL/6 mice.** (A) Effects of 10 mg/kg and 30 mg/kg Lunasin treatment on LLC tumor growth. (B) Effects of 30 mg/kg Lunasin on the growth of tumors initiated by LLC cells either not pre-treated (Lunasin-, red) or pre-treated with 100  $\mu$ M Lunasin (Lunasin+, blue) for 72 hours prior to injection of cells into mice. (C) Effects of 30 mg/kg Lunasin on the growth of tumors initiated by B16-F0 cells either not pre-treated (Lunasin-, red) or pre-treated with 100  $\mu$ M Lunasin (Lunasin+, blue) for 72 hours prior to injection of cells into mice. LLC ( $1 \times 10^5$ ) or B16-F0 ( $1 \times 10^6$ ) cells were injected subcutaneously in the hind flanks of mice to initiate tumors. Lunasin treatments were initiated on the same day that cells were injected and continued daily until the end of the experiment. Tumor volumes were determined from caliper measurements. Treatment groups contained 6–10 mice per group. The data shown represent the mean  $\pm$  SEM and an asterisk (\*) indicates that an individual treatment was significantly different ( $p < 0.05$ ) from the control as determined by an unpaired student's *t*-test.

**Dataset 1. Raw data of validation of syngeneic mouse models of melanoma and non-small cell lung cancer for investigating the anticancer effects of the soy-derived peptide lunasin**

<http://dx.doi.org/10.5256/f1000research.9661.d136965>

All raw data are available in Table 1–Table 5.

## Conclusion

These studies establish that syngeneic mouse models for lung cancer and melanoma are sensitive to Lunasin and that their sensitivity is comparable to that observed in xenograft studies of human NSCLC and melanoma. Thus, these models may be useful to further elucidate the mechanisms of Lunasin action, particularly potential immune effects, and provide important new information on the feasibility of using Lunasin to treat these two deadly cancers.

## Data availability

F1000Research: Dataset 1. Raw data of validation of syngeneic mouse models of melanoma and non-small cell lung cancer for investigating the anticancer effects of the soy-derived peptide lunasin, [10.5256/f1000research.9661.d136965](https://doi.org/10.5256/f1000research.9661.d136965)<sup>29</sup>

## Author contributions

BD performed the MTS assays and *in vivo* studies, and edited the manuscript; CS performed the colony-forming assays and edited the manuscript; KY assisted in directing the study and editing the manuscript; KRD directed the study and wrote the manuscript. All authors agreed to the final content of this article.

## Competing interests

KRD is listed as an inventor on two issued patents relating to the expression and purification of Lunasin peptides and may benefit financially if the technologies described in these patents are licensed or sold. BD, CS, and KY declare no conflict of interests.

## Grant information

This work was funded by Owensboro Grain Company, Owensboro, Kentucky, USA.

*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

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# Open Peer Review

Current Referee Status:



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## Version 2

Referee Report 22 February 2017

doi:[10.5256/f1000research.11815.r20445](https://doi.org/10.5256/f1000research.11815.r20445)



**Elizabeth S. Yeh**

Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, Charleston, SC, USA

The authors have addressed the concerns raised in the original review.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

**Competing Interests:** No competing interests were disclosed.

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## Version 1

Referee Report 30 November 2016

doi:[10.5256/f1000research.10410.r18085](https://doi.org/10.5256/f1000research.10410.r18085)



**Elizabeth S. Yeh**

Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, Charleston, SC, USA

The manuscript would be improved by addressing the following suggestions:

1. Is it known how Lunasin is penetrating the cell membrane or whether it is acting on an extracellular receptor?
2. With in vivo studies using peptides, it is difficult to assess bioavailability due to lack of stability. The manuscript would benefit from discussing what is known about in vivo delivery. This may take into account why pre-treatment of cells was no more effective than s.c. delivery.

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

**Competing Interests:** No competing interests were disclosed.

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Author Response ( Member of the F1000 Faculty and F1000Research Advisory Board Member ) 13 Feb 2017

**Keith Davis**, The Johnson Center for Entrepreneurship in Biotechnology, Indiana University, USA

We thank Dr. Yeh for her review and comments.

1. Is it known how Lunasin is penetrating the cell membrane or whether it is acting on an extracellular receptor?

*We do not currently know all the precise mechanisms whereby Lunasin may enter the cell. There are data that strongly support the hypothesis that lunasin can enter cells via the recycling of integrin receptors; however, how Lunasin is released internally and makes its way to the nucleus is not known.*

*Our studies suggest that it may not be necessary for Lunasin to enter the cell to exert at least some of its effects given that the inhibition of integrin signaling likely occurs at the cell surface.*

*We have added additional details in the Introduction to address this question.*

2. With in vivo studies using peptides, it is difficult to assess bioavailability due to lack of stability. The manuscript would benefit from discussing what is known about in vivo delivery. This may take into account why pre-treatment of cells was no more effective than s.c. delivery.

*There is very little information available on the pharmacokinetics and bioavailability of Lunasin in vitro or in vivo, particularly with regard to the purified Lunasin peptide. Human subjects fed 50 g of soy protein per day were found to have detectable levels of Lunasin in their blood. Based on the amount of Lunasin thought to be present in the soy protein and estimates of loss due to digestion, the authors concluded that the average absorption rate of Lunasin in these subjects averaged 4.5% [1]. Another study suggested that Lunasin was also orally bioavailable in mice and that as much as 30% of the Lunasin reached the tested tissues intact [2].*

*This is clearly an important area that needs significantly more work as we continue assessing the potential of Lunasin as an anticancer agent.*

*We have added the details of the available data on bioavailability in the Introduction.*

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2. Hsieh, C.C., et al., *Complementary roles in cancer prevention: protease inhibitor makes the cancer preventive peptide lunasin bioavailable*. PLoS One, 2010. **5**(1): p. e8890.

**Competing Interests:** No competing interests to declare.

Referee Report 23 November 2016

doi:10.5256/f1000research.10410.r17556





**Masaki Terabe**

Vaccine Branch, National Cancer Institute (NCI), National Institutes of Health (NIH), Bethesda, MD, USA

Major points:

1. Are the concentrations of Lunasin used physiologically relevant?
2. Does Lunasin have similar effects on the progression of these tumors in immunodeficient mice such as RAG deficient mice and RAG/common gamma chain deficient mice? Comparing the effect of Lunasin in these two models, immunodeficient mice and immunocompetent mice, will provide information as to whether adaptive and innate immune cells are involved. Since there is a direct toxicity of Lunasin to tumor cells, it would be important to show the contribution of immune cells to the anti-tumor effect of Lunasin seen in the in vivo models.

Minor point:

1. Axis labels of Fig 1 may not be correct. Are those really % control?

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

**Competing Interests:** No competing interests were disclosed.

Author Response ( *Member of the F1000 Faculty and F1000Research Advisory Board Member* ) 13 Feb 2017

**Keith Davis**, The Johnson Center for Entrepreneurship in Biotechnology, Indiana University, USA

We thank Dr. Terabe for his review and comments.

1. Are the concentrations of Lunasin used physiologically relevant?

*Our view is that it is difficult to assess the physiological relevance of potential therapeutics in the absence of detailed pharmacokinetic data, particularly when assessing in vitro assays, which often are not very predictive of in vivo effects. We assume that Lunasin is not particularly stable in cell cultures where a number of proteases are present, thus; it is not surprising that concentrations of 100  $\mu$ M are required for significant activity. With respect to the in vivo studies, a dose of 30 mg/kg body weight is similar to that of several biologic drugs, as well as the RGD-peptide drug cilengitide [1]. Given our initial preclinical data in mice showing that daily injection of 30 mg/kg Lunasin does not induce any signs of toxicity while providing a therapeutic effect [2] suggest that the dosage of Lunasin used in vivo is potentially physiologically relevant. Clearly, more studies are needed to validate this conclusion in humans, and it is very likely that substantially more work will need to be done to find appropriate formulations and/or modifications of the Lunasin peptide before it would have potential clinical utility.*

2. Does Lunasin have similar effects on the progression of these tumors in immunodeficient mice such as RAG deficient mice and RAG/common gamma chain deficient mice? Comparing the effect of Lunasin in these two models, immunodeficient mice and immunocompetent mice, will provide information as to whether adaptive and innate immune cells are involved. Since there is a direct toxicity of Lunasin to tumor cells, it would be important to show the contribution of immune cells to the anti-tumor effect of Lunasin seen in the in vivo models.

*We have not done these studies, nor are we aware that they have been done by others. We agree that using mouse genotypes with various levels of immunodeficiency would be an excellent way to address the potential immune modulatory effects of Lunasin. However, this is beyond the scope of this research note.*

Minor point:

1. Axis labels of Fig 1 may not be correct. Are those really % control?

*The original graph did use a decimal representation of the data rather than the percentage. We have modified the axis to represent the percentage values to be consistent with the axis label.*

1. Nabors, L.B., et al., *Two cilengitide regimens in combination with standard treatment for patients with newly diagnosed glioblastoma and unmethylated MGMT gene promoter: results of the open-label, controlled, randomized phase II CORE study*. *Neuro-Oncology*, 2015. **17**(5): p. 708-717.
2. Shidal, C., et al., *Lunasin is a novel therapeutic agent for targeting melanoma cancer stem cells*. *Oncotarget*, 2016 **7**(51): p. 84128-84141.

**Competing Interests:** No competing interests to declare.

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### Jean-Pierre Gillet

Laboratory of Molecular Cancer Biology, Molecular Physiology Research Unit-URPhyM, Namur Research Institute for Life Sciences (NARILIS), Faculty of Medicine, University of Namur, Namur, Belgium

This manuscript would benefit from further discussion on:

1. The clinical relevance of the Lunasin concentration used in the mice studies.
2. The rationale and the relevance of pretreating the cell lines with Lunasin prior to mouse engraftment.
3. The pros/cons of this model compared with GEMM (including regulatory agency recommendations to better appraise the current drug development environment).

**I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

**Competing Interests:** No competing interests were disclosed.

Author Response ( *Member of the F1000 Faculty and F1000Research Advisory Board Member* ) 13 Feb 2017

**Keith Davis**, The Johnson Center for Entrepreneurship in Biotechnology, Indiana University, USA

We thank Dr. Gillet for his review and suggestions.

1. The clinical relevance of the Lunasin concentration used in the mice studies.

*As discussed in our response to Dr. Terabe's comments, it is difficult to assess the clinical relevance of the 30 mg/kg body weight dose used in our studies beyond the fact that this dose is comparable to that used for other protein and/or peptide drugs and that it is not unusually high when compared to a number of other agents being tested at the preclinical stage.*

2. The rationale and the relevance of pretreating the cell lines with Lunasin prior to mouse engraftment.

*The rationale for pretreating the cells prior to engraftment was based on our recent studies demonstrating the ability of Lunasin to reduce the putative cancer initiating cell population of human melanoma cell lines (as identified as being ALDH<sup>high</sup>) [1]. We have subsequently not pursued this avenue of research with the B16-F10 or LLC cells so we cannot provide any further data on whether any ALDH<sup>high</sup> cells that may be present in these cells lines were affected by Lunasin.*

*We have added the rationale for the pretreatment to the Results and Discussion.*

3. The pros/cons of this model compared with GEMM (including regulatory agency recommendations to better appraise the current drug development environment).

*Dr. Gillet raises a good point, given that the landscape of preclinical cancer research has changed significantly with the development of transgenic mouse models for a variety of cancer types, and in some cases, subtypes of specific cancers. It is well known that traditional xenograft studies using established human cell lines are often not predictive of clinical efficacy, which can also be the case for the B16 and LLC syngeneic models. However, these more traditional models do still have a role in early preclinical studies when one requires a rapid and relatively inexpensive method to obtain an initial assessment of a compound's anticancer activity in vivo [2, 3]. In the case of xenograft studies, there has been a resurgence of use as they represent a convenient system for maintaining and studying patient-derived tumors. Our decision to test the syngeneic B16 and LLC models was based primarily on identifying a model where we could use a fully immunocompetent mouse without establishing the rather costly GEMM and humanized mouse models. Indeed, now that we have significant results in human xenograft and mouse syngeneic models, we hope to be able to extend our studies into appropriate GEMM models as the next logical step leading towards clinical testing.*

*We have not significantly modified the manuscript to address this point, other than to modify the Conclusion to limit the likely utility of using these syngeneic models. The purpose of this research note is simply to inform researchers interested in Lunasin that the B16 and LLC models do respond and may be appropriate for their studies, depending on the goal. We feel that a more detailed discussion of the pros and cons and these models compared to GEMM and humanized mice models would be more appropriate for a full research paper.*

1. Shidal, C., et al., *Lunasin is a novel therapeutic agent for targeting melanoma cancer stem cells*. Oncotarget, 2016 7(51): p. 84128-84141.

2. Richmond, A. and Y. Su, *Mouse xenograft models vs GEM models for human cancer therapeutics*. *Dis Model Mech*, 2008. **1**(2-3): p. 78-82.
3. Talmadge, J.E., et al., *Murine models to evaluate novel and conventional therapeutic strategies for cancer*. *Am J Pathol*, 2007. **170**(3): p. 793-804.

**Competing Interests:** No competing interests to declare.

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