# TRANSEPITHELIAL TRANSPORT OF LUNASIN AND DERIVED PEPTIDES: INHIBITORY EFFECTS ON THE GASTROINTESTINAL CANCER CELLS VIABILITY

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**ABSTRACT**

Lunasin is a soybean peptide with demonstrated chemopreventive properties. Upon its oral intake, studies dealing with the effect of the digestive process on lunasin's properties are crucial. The present study describes, for the first time, the behavior of lunasin and fragments derived from its digestion in the Caco-2 cell monolayer. The sequences SKWQHQQDSC and KIQGRGDDDDDDDDD showed a notable resistance against the epithelial brush-border peptidases, although some fragments were generated as cellular hydrolysis products. Lunasin and RKQLQGVN were absorbed intact across the intestinal epithelium. The tight junction disruptor cytochalasin D increased their transport, suggesting that the paracellular passive diffusion was the main mechanism involved. The study on the cancer cells viability showed that lunasin and SKWQHQQDSC exerted the highest effects on colorectal cancer HT-29 cells. The stability assay suggested that the cell line type was determinant in the behavior of lunasin added to the culture medium, and therefore in the anti-proliferative activity of released fragments.

**KEYWORDS:** peptide lunasin; lunasin-fragments; gastrointestinal digestion; Caco-2 cells transport; cancer cells; cell viability; food analysis; food composition

## 1. INTRODUCTION

The gastrointestinal epithelium acts not only as a physical barrier against the external environment, but also is the place where food digestion and absorption of nutrients occur. The gastrointestinal tract is known to play a key role on the physiological relevance of orally administered bioactive components (Shimizu and Hachimura, 2011). Firstly, the resistance of bioactive peptides to pepsin and pancreatic enzymes in gastric and intestinal fluids, respectively, must be one of the primary aspects to be addressed before their potential application into functional foods or drugs (Segura-Campos et al., 2011). Moreover, other factors such as the epithelial brush-border membrane peptidases, the absorption rate through the intestinal barrier, the possibility of active intracellular peptidases in case of transcellular transport, as well as the potential action of serum peptidases can be determinant leading to the formation and/or degradation of bioactive peptides upon oral administration. These peptides might be absorbed and reach the target tissues acting at systemic level or exert their effects locally in the gastrointestinal tract.

In the last years, several studies have employed *in vitro* gastrointestinal digestion and Caco-2 cell absorption models to estimate the bioavailability of bioactive peptides (Sánchez-Rivera et al., 2014). The human Caco-2 cells are able to spontaneously differentiate into enterocyte-like monolayers with morphological polarity with an apical and basolateral side, and develop feature characteristics such as apical microvillus and brush-border hydrolases, intercellular tight junctions (TJs), and active receptors and transport systems, including those for peptide transport (Deferme et al., 2008). Among the several routes described for the transport of peptides in the gut, the main mechanisms include the PepT1, a proton-coupled membrane transporter (Brodin et al., 2002), the paracellular passive pathway through intercellular TJs, the transcellular passive diffusion, and the vesicle-mediated transcytosis

(Ziv and Bendayan, 2000). For oligopeptides, susceptibility to brush-border peptidases has been recognized as the primary factor affecting to the apical-to-basolateral transport rate, with both the paracellular and transcytosis transports identified as the principal involved mechanisms (Shmizu et al., 1997).

Lunasin is a 43-amino acid peptide naturally present in soybean, with a variety of biological functions demonstrated by cell cultures and animal models (Fernández-Tomé and Hernández-Ledesma, 2016). *In vitro* studies simulating the gastrointestinal digestion have proposed that this peptide was able to resist the action of digestive enzymes due to naturally present protease inhibitors in foods, such as Bowman-Birk protease inhibitor (BBI) and Kunitz trypsin inhibitor (Jeong et al., 2007; Park et al., 2007). Moreover, Hsieh and co-workers (2010) showed that lunasin was bioavailable when orally administered to mice and rats, and Dia et al. (2009) reported the presence of this peptide in human plasma after consumption of soy protein. These findings suggest that lunasin is absorbed in the intestine and reaches the bloodstream. Although brush-border intestinal peptidases are key factors limiting the half-life and leading to the transformation of dietary peptides in the digestive tract (Picariello et al., 2015), to our knowledge, the behaviour of the lunasin sequence in contact with this epithelial membrane has not been previously evaluated.

Our group has recently identified the peptide patterns of gastric and gastrointestinal digests of lunasin by *in vitro* digestion and reverse phase-high liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) analysis, confirming the protective role played by the major Bowman-Birk family isoinhibitor 1 (IBB1) on the digestion of this peptide. Moreover, the final digests were found to exhibit anti-proliferative properties against the growth of colorectal cancer HT-29 and Caco-2 cells (Cruz-Huerta et al., 2015).

Thus, the aims of the present study are i) to investigate whether lunasin and selected lunasin-derived fragments arising from its digestion are resistant to brush-border peptidases and susceptible to intestinal transepithelial transport in Caco-2 monolayers, identifying the potential mechanism involved in the intestinal absorption by using selective inhibitors. and ii) to evaluate the anti-proliferative effect of lunasin and released peptides on human adenocarcinoma gastric (AGS) and colorectal (HT-29 and Caco-2) cells.

## 2. MATERIALS AND METHODS

### 2.1. Reagents and peptides

Hanks´ balanced salt solution (HBSS), trifluoroacetic acid, Lucifer yellow, cytochalasin D, wortmannin, dimethylsulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (Madrid, Spain). The rest of chemicals used were of HPLC grade.

Lunasin and lunasin-derived peptides used in this study (Table 1) were provided by Chengdu KaiJie Biopharm Co., Ltd (Chengdu, Sichuan, P. R. China) that synthesized them by the conventional Fmoc solid-phase synthesis method. The purity of synthetic peptides was determined by HPLC-MS analysis through peptide peak area integration.

### 2.2. Cell cultures

The human gastric adenocarcinoma cell line (AGS) and two human colorectal adenocarcinoma cell lines (HT-29 and Caco-2) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). AGS, HT-29 and Caco-2 cells were grown in

RPMI medium (Biowest, Nuaillé, France), McCoy´s medium (Lonza Group Ltd, Basel, Switzerland), and Dulbecco´s Modified Eagle Medium (DMEM, Biowest), respectively, supplemented with 10% (v:v) fetal bovine serum (FBS, Biowest) and 1% (v:v) penicillin/streptomycin/amphotericin B solution (Biowest). A non-essential amino acid solution (Lonza Group Ltd) was also added to DMEM medium (1%, v:v) for the culture of Caco-2 cells. Cells were maintained in plastic 75-$cm^2$ culture flasks at 37 ºC in a humidified incubator containing 5% $CO_2$ and 95% air. The culture medium was changed every two days, and cells were kept sub-confluent by using trypsin/EDTA (Lonza Group Ltd) weekly.

## 2.3. Transport studies

### *2.3.1. Culture of Caco-2 monolayers*

Caco-2 cells were used to evaluate the transepithelial transport of peptides following the recommendations described by Hubatsch et al. (2007). Cells were seeded onto 12-well Transwell polycarbonate permeable membrane supports (12 mm diameter, 0.4 µm pore size, 1.12 $cm^2$ growth surface area, Costar, Corning, NY, USA) at a density of $1.5 \times 10^5$ cells/filter, with 0.5 mL of suspended cells in the apical chamber and 1.5 mL of DMEM in the basolateral side. Filters were pre-wet with 0.1 mL of DMEM before seeding. Medium was replaced at days four and seven after seeding, and 24 h before the experiment. To allow transport studies, cells were used nine days after seeding. Previous to the assay, the integrity of the monolayer of differentiated cells was evaluated by measuring the transepithelial electrical resistance (TEER) value with an EVOM epithelial volt/ohm meter (World Precision Instruments, Sarasota, FL, USA). Cells monolayers with values higher than 400 $\Omega \times cm^2$ were considered confluent and used in the assays (García-Nebot et al., 2014).

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139 *2.3.2. Transepithelial transport of peptides*

140 Transport experiments were performed as described by Quirós and co-workers
141 (2008), with some modifications. Cell monolayers were rinsed with DMEM and transferred
142 into new 12-well plates (Costar) containing HBSS (1.5 mL) in the basolateral side to remove
143 interferences with medium components. HBSS (0.5 mL) was carefully added to the apical
144 side, and monolayers were equilibrated at 37 ºC for 20 min. Then, peptides were dissolved in
145 HBSS [lunasin at 10 µM, and fragments f(1-10), f(11-18), f(19-28), and f(29-43) at 1000 µM],
146 and added to the apical chamber. Transwell cultures were incubated at 37 ºC for 60 min, and
147 apical and basolateral samples were withdrawn, freeze-dried, and kept at – 20 ºC until
148 analysis by HPLC-MS/MS. At the end of transport experiments, TEER values were measured
149 again and the flux of Lucifer Yellow (a marker for paracellular permeability) was monitored.
150 Chambers were rinsed with HBSS and incubated at 37 ºC for 30 min. Afterwards, Lucifer
151 Yelow in HBSS (50 µM) was added to the apical compartment and incubated at 37 ºC for 60
152 min. Aliquots from both the apical and the basolateral sides were taken, and fluorescence
153 levels were determined (excitation 485 nm and emission 520 nm) in a FLUOstar OPTIMA
154 plate reader (BMG Labtech, Offenburg, Germany). The post-assay TEER values and the
155 Lucifer Yellow transport (<1% added to apical chamber) were used as parameters to confirm
156 the Caco-2 monolayer integrity during experiments (Broeders et al., 2012). At least, three
157 independent replicates per peptide were evaluated, and the apparent permeability
158 coefficient ($P_{app}$, cm/s) was calculated according to Contreras et al. (2012) as $P_{app}$ =
159 $\frac{\Delta Q}{\Delta t} \times \frac{1}{A} \times \frac{1}{C_0}$; where $\frac{\Delta Q}{\Delta t}$ is the transport rate (µmol/s), *A* is the surface area of the membrane
160 (1.12 $cm^2$), and $C_0$ is the initial peptide concentration in the apical chamber (µmol/mL).

161 To study the mechanism involved in the transepithelial transport of peptides, prior to
162 the experiments described above, cell monolayers were incubated with cytochalasin D (a TJs
163 disruptor, 0.5 μg/mL) and wortmannin (a transcytosis inhibitor, 500 nM) for 30 min before
164 addition of peptides (Quirós et al., 2008). Cytochalasin D and wortmannin were dissolved in
165 DMSO (final concentration in HBSS 0.044%). As control, DMSO supplementation was used.
166 Experiments were carried out in duplicate, and peptide quantification in the basolateral side
167 was expressed as relative percentage of control.
168
169 **2.4. Analysis by HPLC-MS/MS**
170 Analysis of synthetic peptides and samples from apical and basolateral solutions was
171 carried out as previously described (Cruz-Huerta et al., 2015), with minor modifications.
172 Peptides were eluted with a linear gradient of solvent B (acetonitrile:trifluoroacetic acid,
173 1000:0.27, v/v) in solvent A (water:trifluoroacetic acid, 1000:0.37, v/v) going from 0% to 45%
174 in 60 min. Spectra were recorded over the mass/charge (*m/z*) 200-2000, selecting the
175 molecular ion of peptides as the target mass, *m/z* 1258 (lunasin), *m/z* 1247 [f(1-10)], *m/z* 943
176 [f(11-18)], *m/z* 1201 [f(19-28)], and *m/z* 1694 [f(29-43)]. Peptides were identified by their
177 retention times and fragmentation profiles. Data obtained were processed and transformed
178 to spectra representing mass values using the Data Analysis program (version 4.0, Bruker
179 Daltonik). To process the MS/MS spectra and to perform peptide sequencing BioTools
180 (version 3.2, Bruker Daltonik) was used.
181 Previous to the analysis, for lunasin transport experiments (10 μM), freeze-dried
182 samples from apical and basolateral solutions were reconstituted in the initial volume, or
183 concentrated twenty times, respectively, in Milli-Q water. In the case of the four lunasin-

derived peptides (1000 µM), apical samples were reconstituted in the initial volume and then diluted 1:24 (v:v), and basolateral samples were concentrated ten times. Five-point external calibration curves were prepared with synthetic lunasin (from 0.5 to 10.0 µM), and lunasin-derived fragments (from 0.9 to 46.4 µM). Duplicate injections were performed for each point of the calibration curve. The area under the curve of the extracted molecular ions of peptides and their sodium and potassium adducts, when formed, was measured and linear or polynomial regression curves were estimated depending on the fit: lunasin, $y = -4 \times 10^8 + 3 \times 10^9 x$ ($R^2$=0.998); f(1-10), $y = 1 \times 10^8 + 7 \times 10^7 x$ ($R^2$=0.994); f(11-18), $y = 1 \times 10^8 + 1 \times 10^8 x$ ($R^2$=0.976); f(19-28), $y = 2 \times 10^8 + 1 \times 10^8 x$ ($R^2$=0.992); and f(29-43), $y = -8 \times 10^6 + 1 \times 10^8 x - 2 \times 10^6 x^2$ ($R^2$=0.996).

## 2.5. Cell viability experiments

### *2.5.1. MTT assay*

The MTT assay was performed to evaluate the effect of peptides on the viability of AGS, Caco-2 and HT-29 cells. Cells were seeded in 96-well plates (Costar) at a density of 5 × $10^3$ cells/well (AGS) (Wang et al., 2016), and 5 × $10^4$ cells/well (Caco-2 and HT-29) (Dia and de Mejia, 2010). After 24 h incubation, cells were treated with lunasin and all lunasin-derived fragments (Table 1) at different concentrations (10, 50, 100, and 200 µM) for 24, 48, and 72 h. After the different treatment times, cells were washed with phosphate buffer saline (PBS, Lonza Group Ltd), and incubated for 2 h with a MTT solution (0.5 mg/mL final concentration). The supernatants were discarded, and insoluble formazan crystals formed were dissolved in DMSO:ethanol (1:1, v:v). After gently mixing, the absorbance was measured at 570 nm in a FLUOstar OPTIMA plate reader (BMG Labtech). Experiments were

carried out in triplicate and results were expressed as percentage of the control, non-treated cells.

*2.5.2. Morphological analysis*

AGS, Caco-2 and HT-29 cells were plated in 6-well plates (Costar) at a density of $5.0 \times 10^5$, $3.5 \times 10^4$ and $6.7 \times 10^5$ cells/well, respectively. After 24 h incubation, cells contained in four of six wells were treated with lunasin (100 and 200 µM) for 48 h. Two wells were used as control (untreated) cells. Afterwards, cell images were taken by using an optical microscope Leica DM2500 (Leica Lasertechnik GmbH, Mannheim, Germany) at 10X magnification, coupled to a camera Leica DFC420 C (Leica Lasertechnik GmbH). This assay was carried out in duplicate.

*2.5.3. Stability of lunasin*

AGS, Caco-2 and HT-29 cells were plated as described above for morphological analysis, and treated with 10 µM lunasin for 72 h, taking aliquots of the medium at 0, 2, 24, 48, and 72 h of incubation. Aliquots of cultures with non-treated medium were also taken at these times. Samples were evaluated in duplicate and analysed by HPLC-MS/MS as indicated above for transepithelial transport experiments.

**2.6. Statistics**

Data were analyzed using GraphPad Prism 5.03 software (San Diego, CA, USA) by a one-way ANOVA, followed by the Bonferroni *post hoc* test. Differences with a $P$ value < 0.05 were considered significant.

## 3. RESULTS AND DISCUSSION

### 3.1. Effect of epithelial peptidases on lunasin and lunasin-peptides

In a previous study, our group had demonstrated that more than 5% of synthetic lunasin resisted at the end of an *in vitro* simulated pepsin-pancreatin digestion when IBB1 was present (lunasin:IBB1 ratio of 1:2, w:w). Moreover, the peptides released in the gastric and gastrointestinal digests of lunasin were identified by HPLC-MS/MS (Cruz-Huerta et al., 2015). Therefore, in this transepithelial study, the lunasin-derived peptides f(1-10), f(11-18), f(19-28), and f(29-43) arising from its digestion were selected according to their proven resistance to the gastrointestinal enzymes. In order to evaluate a possible structure/activity relationship, peptide lunasin and these four fragments integrating the complete 43-amino acid sequence were assessed for their resistance to brush-border peptidases and their intestinal transport by using Caco-2 monolayers.

The integrity of the monolayers was confirmed by measuring TEER values and the flux of Lucifer Yellow before and after the experiments. Cell monolayers maintained intact along the incubation with lunasin-derived peptides at 1000 μM while lunasin at 50-1000 μM resulted in a reduction of the TJs strength indicating altered integrity of the monolayers . However, lunasin at 10 μM did not exert a drop on TEER values. Previous studies carried out in our group had demonstrated that lunasin at concentrations ranged from 0.5 to 25 μM did not damage cell integrity during the period of incubation of this peptide with differentiated human Caco-2 (García-Nebot et al., 2014), and liver HepG2 cells (Fernández-Tomé et al., 2014). Thus, 10 μM was the lunasin´s concentration selected to carry out the following transport studies. As illustrated in Figure 1A, lunasin was shown to reduce its concentration in a 42.8% ± 1.3, while lunasin-peptides f(11-18) and f(19-28) remained intact in a 79.7 ± 2.1

and 78.7% ± 8.4 of the initial peptide, respectively. However, peptides f(1-10) and f(29-43) maintained nearly intact after 60 min incubation with Caco-2 peptidases. This confers further resistance for these peptides in the intestine epithelium, beyond that was previously shown against pepsin and pancreatic proteases (Cruz-Huerta et al., 2015). Similarly, some food-derived antihypertensive peptides from milk caseins such as VPP and IPP (Ohsawa et al., 2008), and HLPLP (Quirós et al., 2008), and from egg white QIGLF (Ding et al., 2014), and the immunomodulatory peptide β-casein f(193-209) (Regazzo et al., 2010) have shown remarkable resistance against intestinal brush-border peptidases. As shown in Figure 1B, when lunasin was added to the apical side of Caco-2 monolayers, brush-border intestinal peptidases were found to cleave at the central region at the peptide bonds $^{15}$Q-G$^{16}$, $^{18}$N-L$^{19}$, $^{19}$L-T$^{20}$, $^{22}$C-E$^{23}$, and $^{23}$E-K$^{24}$. These findings suggest a higher susceptibility to epithelial peptidases for the central region of lunasin sequence than the N- and C-terminal domains. Interestingly, this central region sited between amino acids 11 and 28 was also described to be predominantly cleaved by pepsin and pancreatin when lunasin was subjected to a two-stage hydrolysis process simulating gastrointestinal digestion in absence of IBB1, but enclosed and protected from the enzymatic action when the protease inhibitor was present (Cruz-Huerta et al., 2015).

In the case of the lunasin-derived peptides, the fragments found were mostly formed as consequence of cleavages at the N-terminus (Figure 1B). Caco-2 cells have been demonstrated to express up to eight membrane peptidases on the apical side. Among them, the serine protease dipeptidyl peptidase IV (DPPIV) that predominantly cleaves at the N-terminal position has shown to have the highest activity, especially when Caco-2 cells are completely differentiated (Howell et al., 1992). Besides, N-terminal hydrophobic or basic

amino acid residues represented a preferred substrate for the enzymatic action of DPPIV, while proline residue was fairly resistant (Mentlein, 1999). In our study, degradation by DPPIV presumably occurred at the peptide bonds $^{1}$S-K$^{2}$ in f(1-10), $^{11}$R-K$^{12}$ in f(11-18), $^{19}$L-T$^{20}$ in f(19-28), and $^{29}$K-I$^{30}$ in f(29-43), while the peptide bond $^{20}$T-P$^{21}$ maintained its integrity. The effect of DPPIV enzyme had been also suggested to be responsible for the N-terminal degradation of bioactive peptides LHLPLP (Quirós et al., 2008), FRADHPFL (Miguel et al., 2008), RYLGY and AYFYPEL (Contreras et al., 2012), and RVPSL (Ding et al., 2015) in Caco-2 monolayers.

Despite the enzymatic attack shown on lunasin and derived fragments prior to their possible transport, these peptides have shown a notable resistance against Caco-2 peptidases, especially f(1-10) and f(29-43), when compared to other bioactives peptides such as the μ-opioid receptor agonists β-casomorphin-5 and -7 from both bovine (Sienkiewicz-Szlapka et al., 2009), and human (Iwan et al., 2008) origins that nearly disappeared after incubation in the apical side of Caco-2 monolayers. These results imply the relevance of studying the resistance of bioactive peptides to the epithelial brush-border membrane as first attempt to evaluate their *in vivo* bioavailability.

### 3.2. Transepithelial transport of lunasin and lunasin-fragment f(11-18)

Analysis by HPLC-MS/MS of standard curves prepared with five-point dilutions of synthetic peptides allowed the quantification of peptides in the basolateral solutions. Only lunasin and derived-peptide f(11-18) were absorbed through the cell monolayer, while the rest of lunasin-fragments were not detected at the basolateral side. Any of the released fragments by the action of brush-border peptidases were able to flux across the Caco-2

monolayer. After 60 min incubation in the apical compartment, the $P_{app}$ values calculated for the apical-to-basolateral transport of lunasin and f(11-18) were $3.32 \times 10^{-7}$ cm/s and $2.50 \times 10^{-7}$ cm/s, respectively (Figure 2A). These values were similar to those found for other bioactive peptides on this transepithelial model such as AYFYPEL ($2.60 \times 10^{-7}$ cm/s) and RYLGY ($2.20 \times 10^{-7}$ cm/s) (Contreras et al., 2012), and VLPVP ($2.78 \times 10^{-7}$ cm/s) (Lei et al., 2008). Several studies have been focused on the *in vitro* transport of bioactive peptides through Caco-2 monolayers, with different permeability values reported for peptides VPP and IPP (0.50 and $1.00 \times 10^{-8}$ cm/s, respectively) (Foltz et al., 2008), RVPSL ($6.97 \times 10^{-6}$ cm/s) (Ding et al., 2015), and SRYPSY ($9.21 \times 10^{-6}$ cm/s) (Sienkiewicz-Szlapka et al., 2009).

Further experiments were conducted in order to evaluate the mechanism involved in the transepithelial transport of intact peptides lunasin and f(11-18). Three main mechanisms have been described for the flux of peptides across intestinal epithelium, the PepT1-mediated transport, the TJs-mediated paracellular pathway, and the vesicle-mediated transcytosis route (Shimizu and Son, 2007). Peptides longer than three amino acids residues have been described as not substrates for the PepT1 transporter (Vig et al., 2006). Therefore, in this study, Caco-2 monolayers were pre-incubated with wortmannin (a transcytosis inhibitor), and cytochalasin D (a TJs disruptor) for 30 min before the transepithelial experiments (Quirós et al., 2008). As illustrated in Figure 2B, the presence of wortmannin hardly modified the intensity of neither peptides lunasin nor f(11-18) in the basolateral chamber, suggesting that the vesicle-mediated mechanism was not involved on the transport of these peptides, in disagreement with other peptides such as the β-casein f(193-209) (Regazzo et al., 2010). However, cytochalasin D was shown to induce an approximately 1.5-fold increase in the presence of both lunasin and f(11-18) compared to

controls. The enhanced transport was accompanied by a TEER value decrease (21.2% average), which also pointed toward the involvement of an expanded intercellular effect on the peptides flux (Regazzo et al., 2010). The paracellular route is characterized by the passive diffusion of molecules between adjacent cells, and is regulated by the intercellular TJs forming a biological barrier with selective permeation function (Segura-Campos et al., 2011). The aqueous nature of this pathway makes it favourable for the absorption of water-soluble substances including oligopeptides (Salamat-Miller and Johnston, 2005), and allows the flux to the basolateral side avoiding the enzymatic role of intracellular peptidases. Together, these findings suggested that the paracellular pathway via TJs was the mainly route for the transepithelial transport of lunasin and f(11-18), consistent with the transport of many peptides, such as VPP (Satake et al., 2002), GGYR (Shimizu et al., 1997), HLPLP (Quirós et al., 2008), QIGLF (Ding et al., 2014), RVPSL (Ding et al., 2015), TNGIIR (Ding et al., 2016), and VLPLP (Lei et al., 2008). In addition, the permeability of paracellular-transported compounds has been considered to be underestimated in Caco-2 monolayers because of the colonic origin of these cells that present a relatively higher TJs tightness in comparison with human or animal small intestinal epithelial cells (Antunes et al., 2013), which ultimately might imply a higher *in vivo* transepithelial transport for the peptides lunasin and derived fragment f(11-18) than that found in this study.

This is the first study that evaluates the behaviour of lunasin and some digestion-fragments on the Caco-2 monolayer. Artursson and Karlsson (1991) established an association between the $P_{app}$ coefficient in Caco-2 cells and the oral drug *in vivo* absorption. Later on, numerous studies on this cellular model have also found a high correlation for several compounds compared to the human intestinal transport (Cheng et al., 2008; Press et

345 al., 2008). In this sense, Dia et al. (2009) estimated in humans an average of 4.5% lunasin
346 absorption from the total lunasin ingested in 50 g of soy protein, which is in agreement with
347 the $P_{app}$ coefficient found in this study ($3.32 \times 10^{-7}$ cm/s). Regarding the absorption of
348 lunasin, a relative long 43-amino acid peptide, it is worthy to mention that other
349 polypeptides in this size range such as the therapeutics 32-amino acid calcitonin, and 51-
350 amino acid insulin have been also reported to be transportable in the Caco-2 model
351 (Antunes et al., 2013). This is similar to the flux found for some dietary whey protein-derived
352 peptides in the β-lactoglobulin sequence f(114-146) (Picariello et al., 2013). The RGD-motif
353 found in lunasin sequence at amino acids 33-35 had been demonstrated to allow the
354 attachment of this peptide to the extracellular matrix, suggesting its role in the
355 internalization into mammalian cells (Galvez et al., 2001). Besides, RGD-motif was suggested
356 as the recognizing sequence for the $\alpha_5\beta_1$ integrin-mediated antiproliferative activity of
357 lunasin on colorectal cancer cells (Dia and de Mejia, 2011). Nevertheless, in this study, the
358 RGD-containing peptide f(29-43) was not transported across the Caco-2 monolayer. The
359 peptide f(11-18) was the only fragment resembling the transepithelial behaviour of the
360 parent peptide, thus it may be hypothesized whether this peptide is the presumable target
361 sequence for the intact absorption of complete lunasin across the intestinal epithelium.
362 However, more studies by modifying this region of the sequence are thus required to
363 confirm these findings.
364
365 **3.3. Effects of lunasin and derived peptides on the viability of gastric and colorectal cancer**
366 **cells**

Several food-derived peptides have been described to exert potential chemo-preventive properties against the proliferation of malignant cells (Hernández-Ledesma and Hsieh, 2015). Peptide lunasin has demonstrated anti-proliferative activity in colorectal cancer HCT-116, HT-29, KM12L4, and RKO cells (Dia and de Mejia, 2010, 2011). Cruz-Huerta et al. (2015) recently showed an inhibitory effect for the gastrointestinal digests of lunasin:IBB1 mixtures in colorectal cancer HT-29 and Caco-2 cells. Since the amount of lunasin in these digests was low (ranged between 0.1% and 5.3% from initial lunasin), peptides released during digestion of lunasin might contribute on the anti-proliferative effects observed. Thus, in this study, lunasin and some new derived fragments (Table 1) were evaluated for their potential anti-proliferative effect in the gastrointestinal tract by the MTT protocol.

As shown in Figure 3, peptide lunasin affected the viability of the three cell lines, with relatively higher effects on HT-29 cells. After 24 h treatment, lunasin provoked a ca. 10% significant induction of non-viable cells at all concentrations assessed (Figure 3A). However, after 48 and 72 h treatment, a dose-dependent statistical trend was found for lunasin activity with the maximum inhibitory effect found at 200 µM (19.1 and 23.8%, respectively). Moreover, a time-dependent lunasin´s effect was revealed, showing a significant enhanced activity for this peptide at 50-200 µM from 24 to 72 h (12.4% average). Dia and de Mejia (2010) had previously found that lunasin induced a cytotoxic effect ranging from 19.3% (10 µM) to 62.8% (100 µM) on HT-29 cells. While these authors assessed the activity of purified lunasin (~90%) from defatted soybean flour, we have evaluated the effect of the synthetic peptide. The lower activity found in this study might be due to differences in the secondary and tertiary structures between plant-purified lunasin and the synthetic peptide. In Caco-2

cells, lunasin 50-200 μM, and 10-200 μM was able to induce an average ca. 10% significant increase on the number of non-viable cells after 24 and 48 h treatment, respectively (Figure 3B). In these cells, a dose-dependent trend was not clearly shown; however a notable time-dependent difference was demonstrated at 72 h. At this time, any anti-proliferative outcome was shown, suggesting that lunasin´s effects at 24 and 48 h were insufficient to induce a marked inhibition on treated cells, which were found to recover and grow at the same ratio as control cells after 72 h. As shown in Figure 3C, lunasin showed a significant dose-dependent activity in AGS cells. At 10 and 50 μM, this peptide did not present any statistical difference from control cells. However, lunasin 100 and 200 μM modestly induced an average inhibitory effect ca. 7% and 15%, respectively. Figure 4 illustrates the morphological analysis of non-treated and lunasin-treated cells, which showed a visual change in viable cells as compared with the homogenous growth of control cells over the plate. Together these findings indicated the higher susceptibility of HT-29 cells than both Caco-2 and AGS cells to peptide lunasin, in agreement with previous results that found a more evident anti-proliferative effect for lunasin:IBB1 gastrointestinal digests on the former colorectal cancer cells (Cruz-Huerta et al., 2015). Likewise, the cytotoxic activity of this peptide has been proposed to be dependent on the type of cell line treated with inhibitory concentration ($IC_{50}$) values ranging from 13 μM in colorectal cancer KM12L4 cells (Dia and de Mejia, 2011) to 181 μM in breast cancer MDA-MB-231 cells (Hernández-Ledesma et al., 2011).

Following the same methodology, gastric and colorectal cancer cells were treated with seven lunasin-derived peptides (10-200 μM, 24-72 h). In HT-29 cells, peptide f(1-10) induced a time-dependent trend on the percentage of non-viable cells compared to control non-treated cells (Figure 5A). This peptide showed an increasing effect with the dose at 24 h

413 from 9.4% to 15.1% inhibition, while no statistical differences between concentrations were
414 demonstrated at 48 and 72 h. The highest effect, 25.8% inhibition, was found at 72 h for the
415 dose of 200 μM. As shown in Figure 5B, peptide f(17-28) reproduced these anti-proliferative
416 properties, but to a lower extent, with a dose-dependent trend (10-100 μM) at 24 and 48 h,
417 and an enhanced activity along the treatment time. The maximum inhibitory effect found for
418 this fragment was observed at 72 h for all the concentrations evaluated (17.3% average).
419 Lunasin-peptides f(19-28), f(29-41), and f(29-43) (10-200 μM) showed a ca. 10% inhibitory
420 activity at 48 and 72 h, while f(11-18) and f(34-43) did not cause significant effect at any
421 condition evaluated in HT-29 cells (data not shown). In Caco-2 cells, f(1-10) and f(17-28)
422 were also the most active peptides among all lunasin-fragments assessed. Lunasin-peptides
423 f(1-10) exhibited the highest effect after 24 h treatment with 200 μM (18.6%) (Figure 5C),
424 and f(17-28) after 48 h treatment with 200 μM (22.8%) with a marked dose-dependent
425 statistically different activity at this time, but not at 24 h (Figure 5D). As it was shown for
426 lunasin treatment on Caco-2 cells, the activity of both fragments was notably decreased
427 after 48 h, with any statistical difference from control cells at 72 h. While peptide f(29-41)
428 showed a ca. 8% inhibitory activity at 24 and 48 h, none of the rest lunasin-peptides induced
429 cytotoxic effects on these cells (data not shown). Therefore, in colorectal HT-29 cells,
430 peptide f(1-10) might be the main contributor to the anti-proliferative activity proven for the
431 complete lunasin. Despite the inhibitory activity exerted by lunasin and its fragments f(1-10)
432 and f(17-28), the functionality of these peptides on Caco-2 cells was not absolutely
433 established due to the higher resistance of these cells, showing weaker effects that lead to a
434 recovery to control levels after 72 h treatment. Similarly to that was observed for lunasin in
435 gastric AGS cells, all derived fragments showed modest results in the range of 10-15%

436 inhibition, especially at 100 and 200 µM (data not shown). Thus, the effect of lunasin on
437 these cells seems to be unspecific and independent of the assessed region within the
438 complete 43-amino acid peptide. The chemo-preventive properties of lunasin had been
439 attributed to its C-terminal domain that presents: (i) a predicted helix with structural
440 homology to a conserved region of chromatin-binding proteins, (ii) an RGD cell adhesion
441 motif, and (iii) a poly-D tail, with a high negatively charge, known to preferentially bind to
442 positively charged deacetylated histones and hypoacetylated chromatin (Galvez et al., 2001).
443 To date, to the best of our knowledge, only one study had evaluated a possible structure-
444 activity relationship with different lunasin fragments on breast cancer MDA-MB-231 cells
445 (Hernández-Ledesma et al., 2011). Whereas peptide lunasin inhibited the malignant growth
446 with an $IC_{50}$ value of 181 µM, these authors proved that the C-terminal region was more
447 active than the complete peptide with $IC_{50}$ values of 175 and 138 µM for lunasin-fragments
448 EKHIMEKIQGRG f(23-34), and EKHIMEKIQGRGDDDDDDDDD f(23-43), respectively, while the
449 fragment SKWQHQQDSCRKQLQGVNLTPC f(1-22) at the N-terminal domain was less potent
450 ($IC_{50}$: 323 µM). Therefore, this is the first study suggesting a protective role for the N-
451 terminal region of lunasin, especially for f(1-10) in HT-29 cells. In this line, Chang et al. (2014)
452 recently found that a truncated peptide lacking the RGD-motif and the poly-D was equally
453 active than the complete lunasin sequence on the immunomodulatory activation of natural
454 killer cells. However, in the present study, neither lunasin nor lunasin-derived peptides
455 exerted the protective activity previously shown for lunasin:IBB1 digests (Cruz-Huerta et al.,
456 2015). Therefore, the inhibitory properties of other compounds in the digests or a possible
457 synergistic effect between the peptides identified could not be discarded in this study.
458 Moreover, it should be highlighted that culture conditions have been recently found to

notably modulate the anti-proliferative activity of lunasin on non-small cell lung cancer cells (McConnell et al., 2015). These authors demonstrated that anchorage-independent growth conditions, more closely mimicking the *in vivo* tumor environment, increased the sensitivity of cancer cells to lunasin. In our study under anchorage-dependent growth conditions, the moderate biological MTT-activity would be presumably meaningful in a more physiological state.

### 3.4. Stability of lunasin on gastric and colorectal cancer cells

As shown in Table 2, by HPLC-MS/MS analysis, the residual intact lunasin in the cell cultures was quantified and the derived fragments released by cellular enzymatic action were identified. In AGS cells, lunasin remained intact for up to 24 h incubation, and showed a 13.2% and 27.0% reduction in its content after 48 and 72 h, respectively. Furthermore, at 48 h incubation, the lunasin fragments EKHIMEKIQGRGDDDDDDDDD f(23-43), HIMEKIQGRGDDDDDDDDD f(25-43), and KIQGRGDDDDDDDDD f(29-43) were generated and remained in the culture after 72 h. In HT-29 cells, a relatively higher cellular enzymatic activity was evidenced, with residual lunasin percentages being decreased in a time-dependent manner from 2 h (97.3%) to 72 h (68.0%), and the identification of the same three lunasin-derived peptides from 24 h incubation, as well as the new fragment GRGDDDDDDDDD f(32-43) at 72 h. In addition to peptide hydrolysis, degradation by the enzymatic response of cells to incubation with peptide explained the decrease in lunasin concentration. It might be also due to peptide´s internalization into AGS and HT-29 cells, as it has been demonstrated in murine fibroblast C3H10T1/2 cells (Galvez et al., 2001), and human THP-1 macrophages (Cam et al., 2013), colorectal cancer KM12L4 cells (Dia and de

Mejia, 2011), and prostate epithelial RWPE-2 cells (Galvez et al., 2011). Four lunasin-derived fragments were formed corresponding to the C-terminal region of the sequence from the amino acid residue 23. Among them, fragment f(29-43) coincided with the sequence of one lunasin-peptide that showed a ca. 10% inhibitory activity on AGS and HT-29 cells in this study, and had been identified in *in vitro* gastrointestinal digests of lunasin (Cruz-Huerta et al., 2015). In this sense, it is worthy to mention that the lunasin-derived peptide SKWQHQQDSC f(1-10) was not formed during the stability assay, although it was the most active in the MTT assay. The enzymatic action of digestive peptidases would be thus required to release this fragment. Moreover, the fragments f(25-43), f(29-43), and f(32-43) had been already identified in a previous study of lunasin´s stability in human liver HepG2 cells (Fernández-Tomé et al., 2014), which suggested that these cultures might share enzymatic activities upon incubation with lunasin peptide. In contrast, neither lunasin decrease nor derived fragments were demonstrated in Caco-2 cells along the incubation time (data not shown). Lunasin degradation in this culture was thus not the cause of the anti-proliferative activity loss showed after 72 h treatment. Therefore, it might be suggested that the cell line type is determinant in the behavior of lunasin added to the culture medium.

## 4. Conclusions

The present study has been focused on the analysis of peptides lunasin and lunasin-digestion fragments at the intestinal epithelium. The results pointed out for the first time a notable resistance against the epithelial brush-border membrane of Caco-2 monolayers, especially for peptides f(1-10) and f(29-43). Lunasin and f(11-18) were demonstrated to cross the intestinal epithelial barrier, with the paracellular route found as the main transport

mechanism involved. The f(11-18) might be proposed as preferred target sequence for the transepithelial transport of lunasin peptide. These findings might imply biological relevance of lunasin and derived-fragments, suggesting potential to exert bioactive effects both locally in the gastrointestinal tract as well as at a systemic level. Studies focused on detecting lunasin-derived peptides in plasma and target tissues should be needed to confirm their demonstrated *in vitro* bioavailability. Lunasin and lunasin-released peptides after simulated *in vitro* digestion were shown to play moderate protective properties against the growth of gastric and colorectal cancer cells, with the maximum inhibitory effects shown for lunasin and f(1-10) in HT-29 cells. Therefore, this is the first study that postulates a preventive role for the N-terminal region of lunasin. It has been suggested that the cell line type is determinant for the different behaviour of lunasin-derived sequences.

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**FIGURE CAPTIONS**

**Figure 1.** Behaviour of lunasin and lunasin-fragments added to Caco-2 monolayers. Analysis by HPLC-MS/MS of peptides in apical solutions after incubation of lunasin 10 µM and lunasin-fragments 1000 µM with Caco-2 monolayers for 60 min. (A) Remaining intact peptide in apical solutions was evaluated by the extracted molecular ions of lunasin mass/charge (*m/z*) 1258.0 (charge +4) and *m/z* 1677.2 (charge +3), f(1-10) *m/z* 1246.3 (charge +1), f(11-18) *m/z* 942.3 (charge +1), f(19-28) *m/z* 1200.7 (charge +1), and f(29-43) *m/z* 1694.0 (charge +1). Sodium and potassium adducts were considered, when formed. Data are represented as relative percentage of initial peptide added to the chamber, and expressed as mean ± standard error of the mean (SEM) of at least three independent replicates. (B) Intact peptides presented in the apical chamber and derived fragments released by brush-border intestinal peptidases are shown. Sequence of peptide added to the apical solution is marked with an arrow: Lunasin, SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDD; f(1-10), SKWQHQQDSC; f(11-18), RKQLQGVN; f(19-28), LTPCEKHIME; and f(29-43), KIQGRGDDDDDDDDD.

**Figure 2.** Transepithelial transport of peptides lunasin and f(11-18), RKQLQGVN. (A) Peptides were analyzed by HPLC-MS/MS in the basolateral solutions after incubation of 10 µM lunasin and 1000 µM lunasin-fragment f(11-18). The apparent permeability coefficient ($P_{app}$) was calculated as described in Materials and methods section, and represented as mean ± standard error of the mean (SEM) of at least three independent replicates per assayed peptide. (B) Effects of cytochalasin D and wortmannin on the transepithelial transport of peptides. Cells were pre-incubated with cytochalasin D (0.5 µg/mL) and wortmannin (500

689 nM) for 30 min, or Hanks' Balanced Salt Solution (HBSS) with 0.044% dimethylsulfoxide
690 (DMSO) (control), before addition of 10 µM lunasin and 1000 µM lunasin-fragment f(11-18).
691 Experiments were carried out in duplicate, and results were represented as relative peptide
692 intensity in the basolateral chamber (% of control, mean ± SEM). (*, $P < 0.05$) denotes
693 statistically significant differences versus control by a one-way ANOVA followed by the
694 Bonferroni *post hoc* test.
695
696 **Figure 3.** Effects of lunasin on cancer cells viability. Cell viability was evaluated by the 3-(4,5-
697 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay after treatment of
698 human adenocarcinoma colorectal (A) HT-29 and (B) Caco-2 cells and (C) gastric cancer AGS
699 cells with lunasin (10-200 µM, 24-72 h). Results were expressed as percentage of non-viable
700 cells compared to control, considered as 0% (% of control, mean ± standard error of the
701 mean, SEM). Experiments were carried out in triplicate, and analysed by a one-way ANOVA
702 followed by the Bonferroni *post hoc* test. Lower case letters denote statistically significant
703 differences ($P < 0.05$) between doses and control, considered as (a). Capital letters denote
704 statistically significant differences ($P < 0.05$) for the same dose between treatment times.
705
706 **Figure 4.** Morphological analysis. Representative images, taken by using an optical
707 microscope at 10X magnification, of HT-29, Caco-2, and AGS (A-C, respectively) non-treated
708 cells, and (D-F, respectively) lunasin-treated cells (200 µM, 48 h). Lunasin-treated cells
709 showed areas with minor cellular density (arrows), compared to non-treated control cells
710 presenting a continuous growth over the plate.
711

712 **Figure 5.** Effects of lunasin-derived fragments on cancer cells viability. Cell viability was
713 evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay
714 after treatment of human adenocarcinoma colorectal (A and B) HT-29 and (C and D) Caco-2
715 cells with (A and C) peptide SKWQHQQDSC, f(1-10) and (B and D) peptide VNLTPCEKHIME,
716 f(17-28) at concentrations ranging from 10 to 200 μM for 24-72 h. Results were expressed as
717 percentage of non-viable cells compared to control, considered as 0% (% of control, mean ±
718 standard error of the mean, SEM). Experiments were carried out in triplicate, and analysed
719 by a one-way ANOVA followed by the Bonferroni *post hoc* test. Lower case letters denote
720 statistically significant differences ($P < 0.05$) between doses and control, considered as (a).
721 Capital letters denote statistically significant differences ($P < 0.05$) for the same dose
722 between treatment times.