RESEARCH ARTICLE

Lunasin induces apoptosis and modifies the expression of genes associated with extracellular matrix and cell adhesion in human metastatic colon cancer cells

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Scope: Lunasin is an arginine-glycine-aspartic acid (RGD) cancer preventive peptide. The objective was to evaluate the potential of lunasin to induce apoptosis in human colon cancer cells and their oxaliplatin-resistant (OxR) variants, and its effect on the expression of human extracellular matrix and adhesion genes.

Methods and results: Various human colon cancer cell lines which underwent metastasis were evaluated *in vitro* using cell flow cytometry and fluorescence microscopy. Lunasin cytotoxicity to different colon cancer cells correlated with the expression of α_5b_1 integrin, being most potent to KM12L4 cells (IC_{50} = 13 μM). Lunasin arrested cell cycle at G2/M phase with concomitant increase in the expression of cyclin-dependent kinase inhibitors p21 and p27. Lunasin (5–25 μM) activated the apoptotic mitochondrial pathway as evidenced by changes in the expressions of Bcl-2, Bax, nuclear clusterin, cytochrome c and caspase-3 in KM12L4 and KM12L4-OxR. Lunasin increased the activity of initiator caspase-9 leading to the activation of caspase-3 and also modified the expression of human extracellular matrix and adhesion genes, downregulating integrin α_5, SELE, MMP10, integrin β_2 and COL6A1 by 5.01-, 6.53-, 7.71-, 8.19- and 10.10-fold, respectively, while upregulating COL12A1 by 11.61-fold.

Conclusion: Lunasin can be used in cases where resistance to chemotherapy developed.

Keywords:
$\alpha_5\beta_1$ Integrin / Apoptosis / Colon cancer / Lunasin / Metastatic

1 Introduction

Lunasin is a chemopreventive peptide originally isolated from soybean [1]. It is also found in other plants such as amaranth [2], barley [3], *Solanum* [4] and wheat [5]. Its chemopreventive and anticancer properties are demonstrated by suppressing lipopolysaccharide-induced inflammation [6–8] and inducing apoptosis in different cancer cell lines [9–11]. These properties are associated with unique amino acid sequences present in lunasin, which included a cell adhesion motif composed of arginine–glycine–aspartic acid (RGD) residues and a polyaspartic acid tail composed of nine aspartic acid residues. The RGD motif is responsible for the internalization of lunasin into the cells and the polyaspartic acid tail affects kinetochore formation, leading to mitotic arrest [1, 12]. Previous studies have also demonstrated the capability of RGD-containing peptides to promote apoptosis to different cell lines [13, 14]. Apoptosis is a natural phenomenon important in the maintenance of homeostasis and normal development. It is characterized by a series of morphological changes including loss of specialized surface structure, reduction in volume, conservation of cytoplasmic organelles, condensation of nuclear chromatin and phagocytosis [15, 16]. Acquired resistance toward apoptosis is a hallmark of almost all types of cancer cells [17].

Colorectal cancer (CRC) is the third most common cancer in the US with estimated 50 000 new cases occurring every year [18]. Its management cost for 2020 has been

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Abbreviations: CDKI, cyclin-dependent kinase inhibitors; **CRC,** colorectal cancer; **ECM,** human extracellular matrix; **nCLU,** nuclear clusterin; **OxR,** oxaliplatin-resistant; **PI,** propidium iodide; **RGD,** arginine–glycine–aspartic acid

estimated in $14 billion [19]. Although significant progress has been made in the treatment of CRC with the use of adjuvant therapies, the 5-year survival success rate for patients with metastatic CRC is still 8% [20]. This poor survival rate for patients with metastatic CRC is, in part, attributed to the development of resistance to chemotherapy. For instance, resistance to oxaliplatin, a commonly used chemotherapeutic drug for patients with metastatic CRC, is associated with altered mitochondrial-mediated apoptosis as evidenced by the complete loss of pro-apoptotic Bax and undetectable expression of active caspase-3 [21]. Moreover, acquired resistance to oxaliplatin is associated with independent defects both in drug uptake by cells as well as in the formation of DNA adducts [22]. In addition to resistance development, the use of chemotherapeutic drugs has been associated with adverse side effects including acute and chronic neuropathy, hypersensitivity reactions, diarrhea, neutropenia and hand-foot syndrome [23]. As such, it is important to find naturally occurring compounds that can enhance the effectiveness of chemotherapeutic drugs, thereby preventing resistance development.

The objective of this study was to evaluate the potential of lunasin to promote apoptosis in human CRC cells and in their oxaliplatin-resistant (OxR) variants. Moreover, we assessed the effect of lunasin on the expression of human extracellular matrix (ECM) and adhesion genes in KM12L4 cells. We showed for the first time the capability of lunasin to induce apoptosis in KM12L4 and KM12L4OxR human metastatic CRC cells. This effect might be attributed to its capability to modify the expression of human ECM and adhesion genes, suggesting the potential use of lunasin as an adjuvant to currently used chemotherapy for patients with metastatic CRC, with the advantage of reduced cytotoxicity.

2 Materials and methods

2.1 Cell lines

HCT-116, HT-29, KM12L4, RKO cells and their OxR variants designated as HCT-116OxR, HT-29OxR, KM12L4OxR and RKOOxR were obtained from Dr Lee M. Ellis (MD Anderson Cancer Center, University of Texas). The metastatic KM12L4 cell line was established by injecting the parental cell line KM12C into the spleen of nude mice as previously reported [24]. The OxR variants were prepared by repeated exposure of the parental cell lines to increasing concentration of oxaliplatin as reported earlier [25]. The normal human colon fibroblasts CCD-33Co were purchased from American Type Culture Collection (Manassas, VA, USA).

2.2 Cell proliferation

Cells were cultured in minimum essential medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, 1% glutamine, 1% vitamin solution and 1% sodium pyruvate at 37°C in 5% CO_2/95% air. CCD-33Co colon fibroblasts were cultured in Eagle's minimum essential medium containing 10% fetal bovine serum and 1% penicillin/streptomycin. The cell proliferation assay was conducted using the CellTiter 96 AQueous One Solution Proliferation assay kit (Promega, Madison, WI, USA). For parental cells, 5×10^3 cells *per* well were seeded in a 96-well plate while 2.5×10^3 cells *per* well were seeded for OxR variants and the total volume was adjusted to 200 μL with growth medium. The cells were incubated for 24 h at 37°C in 5% CO_2/95% air. After 24-h incubation, the cells were treated for another 24 h with different concentrations of lunasin (>90%) purified from defatted soybean flour as reported previously [6]. For CCD-33Co, 1×10^3 cells *per* well were seeded in a 96-well plate and allowed to grow to confluency for 1 wk with replacement of medium every other day. The cells were then treated with different concentrations of lunasin for 24 h (0–100 μM). After lunasin treatment, cell viability was determined by MTS assay as previously reported [6]. An interaction study between lunasin (10 and 25 μM) and oxaliplatin (0.5, 1.0 and 2.0 μM) was performed for KM12L4 colon cancer cells following the same cell proliferation protocol described above. For OxR cells, 2 μM oxaliplatin (final concentration) was added throughout the treatment. Each test was conducted for at least two independent trials with three replicates for each trial.

2.3 Cell-cycle distribution of KM12L4 colon cancer cells

Analysis of cell-cycle distribution was performed using flow cytometry as we have previously reported [9]. Briefly, KM12L4 cells were seeded at a density of 2×10^5 cells *per* well in a six-well plate and allowed to grow for 48 h at 37°C in 5% CO_2/95% air. Cells were then treated with different concentrations of lunasin (0–10 μM) for another 24 h at 37°C in 5% CO_2/95% air. After treatment, cells were fixed overnight with 70% ethanol at 4°C and stained with propidium iodide (PI) solution. Cell-cycle distribution analysis was performed using a LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) at an excitation wavelength of 488 nm. Fluorescence emission was measured using a 695/40 nm band pass filter. The analysis was performed in triplicates.

2.4 Apoptosis of KM12L4 colon cancer cells

Apoptosis of KM12L4 colon cancer cells was evaluated by flow cytometry using an Annexin V-FITC apoptosis detection kit (Sigma-Aldrich, St Louis, MO, USA). Briefly, 2×10^5 cells *per* well were seeded in a six-well plate and allowed to grow for 48 h at 37°C in 5% CO_2/95% air. The cells were then treated with different concentrations of lunasin

(0–25 μM) for 24 h at 37°C in 5% CO_2/95% air. After treatment, cells were washed with PBS twice, trypsinized and suspended in binding buffer at a concentration of 1×10^6/mL. Five hundred microliters of lunasin-treated and untreated cells was transferred into a plastic test tube and stained with 5 μL Annexin V-FITC and 10 μL PI solution for 10 min. PI staining was performed concomitantly with Annexin V-FITC staining to determine whether any DNA/nuclei were present in the colon cancer cells. The cells were analyzed immediately by LSR II flow cytometer (BD Biosciences). The analysis was performed in triplicate.

2.5 Fluorescence microscopy of KM12L4 colon cancer cells

Fluorescence microscopic analysis of cells was performed at the Institute of Genomic Biology Microscopy Facility, University of Illinois at Urbana-Champaign. Briefly, 1×10^3 cells suspended in 300 μL medium were seeded in an eight-well ibiTreat plate (ibidi Integrated BioDiagnostics, Martinsried, Germany) allowed to grow to 60–70% confluency at 37°C in 5% CO_2/95% air. Cells were treated with 10 and 25 μM lunasin for 24 h. After treatment, cells were washed and fixed with 4% paraformaldehyde in PBS for 30 min. The cells were washed with PBS three times and permeabilized with 0.1% Triton-X for 30 min and washed again with PBS. The cells were stained with Hoechst 33342 stain (Invitrogen, CA) and mounted with prolong gold (Invitrogen) for 24 h in the dark at room temperature. The plate was kept at 4°C in the dark until analysis. The cells were imaged using Zeiss Axiovert 200M with the Apotome Structured Illumination Optical Sectioning System fluorescence microscope (Zeiss Obercohen, Germany).

2.6 Protein expression in KM12L4 and KM12L4OxR cells by Western blot

KM12L4 and KM12L4OxR cells were seeded at a density of 2×10^5 and 1×10^5 cells *per* well, respectively, in a six-well plate for 48 h at 37°C in 5% CO_2/95% air. In case of KM12L4OxR cells, 2 μM oxaliplatin (final concentration) was added throughout treatment. Cells were then treated with different concentrations of lunasin (0–25 μM) for 24 h. Cells were washed with PBS twice, trypsinized, suspended in lysis buffer composed of 62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol and protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). The cell suspension was sonicated and boiled for 5 min. Equal amount of protein (approximately 15 μg) was loaded in 4–20% Tris-HCl ready gels (Biorad, Hercules, CA, USA). The separated proteins were transferred to PVDF membrane and blocked with 5% non-fat dry milk in 0.1% Tris-buffered saline Tween-20 for 1 h at 4°C. After blocking, the membrane was washed with 0.1% Tris-buffered saline Tween-20 (five times, 5 min each) and incubated with actin, Bax, Bcl-2, clusterin, cytochrome *c*, integrins α_5 and β_1, p27, or p21 primary antibodies (1:200) (Santa Cruz Biotechnology, CA, USA) at 4°C overnight. The membrane was washed again and incubated with antimouse IgG horseradish peroxidase conjugate secondary antibody for actin, Bax, Bcl-2, cytochrome *c*, caspase-3, integrins α_5 and β_1, p27 and p21 (GE Healthcare, Buckinghamshire, UK) and antirabbit IgG horseradish peroxidase conjugate secondary antibody for clusterin (GE Healthcare) for 3–4 h at room temperature. After incubation and repeated washings, the expression of proteins was visualized using chemiluminescent reagent (GE Healthcare). Analyses were conducted in triplicate.

2.7 Caspase activity

The activity of caspases-2, -3, -6, -8 and -9 was determined using the caspase colorimetric assay kit (Invitrogen) following manufacturer's protocol. Briefly, cells were seeded at a density of 2×10^5 *per* well in a six-well plate and allowed to grow for 48 h at 37°C in 5% CO_2/95% air. Cells were treated with different concentrations of lunasin (0–10 μM) for 24 h. After treatment, cells were pelleted and resuspended in 50 μL chilled cell lysis buffer and incubated on ice for 10 min. The cells were then centrifuged at $10\,000 \times g$ for 1 min. Approximately 50 μg of protein was assayed for caspase activity analysis by adding 50 μL reaction buffer containing 10 mM dithiothreitol and 5 μL of the 4 mM corresponding caspase substrates in a 96-well plate. The plate was incubated at 37°C in the dark for 2 h. The absorbance was read at 405 nm using ELx808 microplate reader (Biotek, Winooski, VT, USA). Analyses were performed in four replicates.

2.8 ECM gene expression profile using real-time polymerase chain reaction

KM12L4 cells were plated at a density of 2×10^6 cells in a 75-cm^2 flask, cultured overnight and treated with 10 μM lunasin or PBS for 24 h. Cells were harvested by trypsinization and total RNA was harvested using RNeasy Mini Kit with an on-column DNase treatment step (Qiagen, CA). Four hundred nanograms of RNA from each sample was used to carry out reverse transcription using a RT^2 First Strand Kit (SA Biosciences, MD). The cDNA samples were incubated with RT^2 SYBR Green/ROX qPCR Master Mix (SA Biosciences), and the expression of genes was determined using RT^2 Profiler for human ECM and adhesion molecules array on the 7900HT ABI 384-well block. Cycling conditions used were 10 min at 95°C (1 cycle) and 15 s at 95°C, 1 min at 60°C (40 cycles). Gene expression was quantified following manufacturer's instructions using the $\Delta\Delta C_t$ method in which C_t refers to the fractional cycle number where the fluorescent signal reaches a detection threshold. The ΔC_t was normalized

using a total of five endogenous housekeeping genes (B2M, HPRT1, RPL13A, GADPH and ACTN). Fold change values were reported as $2^{-(\Delta\Delta C_t)}$ and when the value was <1 it was converted to its negative inverse to report downregulated genes. When the C_t value was >35, the gene was considered non-detectable as *per* manufacturer's instructions.

2.9 Statistical analysis

Data were analyzed using ANOVA. Means were generated and adjusted with least significant difference using Statistical Analysis System software version 9.1. Significant differences were reported at *p*-values <0.05. All analyses with regard to apoptosis-inducing properties were performed in three independent experiments; the caspase activity measurements were performed in four replicates while the PCR array was done in duplicate.

3 Results

3.1 Lunasin caused cytotoxicity to different human CRC cells and their oxaliplatin-resistant variants

Lunasin caused dose-dependent cytotoxicity to KM12L4, HT-29, HCT-116, RKO colon cancer cells and their OxR variants except for HT-29OxR wherein no significant effect was observed when treated with up to 50 μM lunasin (Fig. 1A and B). Among the parental cell lines used, lunasin most potently inhibited the growth of the metastatic KM12L4 colon cancer cells with an IC_{50} of 13.0 μM. A concentration as low as 1 μM, lunasin caused 19.8% inhibition of KM12L4 metastatic colon cancer growth ($p<0.05$). At 50 μM, lunasin caused at least 90% reduction in the viability of KM12L4 cells. Lunasin also caused a significant reduction in the viability of RKO ($IC_{50}=21.6$ μM), HCT-116 ($IC_{50}=26.3$ μM) and HT-29 ($IC_{50}=61.7$ μM) CRC cells. It was also cytotoxic for KM12L4OxR ($IC_{50}=34.7$ μM), RKOOxR ($IC_{50}=38.9$ μM) and HCT116OxR ($IC_{50}=31.6$ μM). On the other hand, lunasin up to 100 μM showed no cytotoxicity to normal human colon fibroblast CCD-33Co (Fig. 1A). The cytotoxic effect of lunasin correlated with the expression of $\alpha_5\beta_1$ integrin (Fig. 1C) in different colon cancer cell lines. KM12L4 colon cancer cells highly expressed this integrin while HT-29 did not express α_5 integrin. This is the first report on lunasin anti-cancer potential attributed to integrin signaling. Lunasin synergistically interacted with oxaliplatin causing cytotoxicity in KM12L4 cells (Fig. 1D).

3.2 Lunasin caused G2/M cell-cycle arrest on KM12L4 colon cancer cells

Lunasin increased, in a dose-dependent manner, the amount of cells at G2 phase of the cell cycle (Fig. 2A). It also

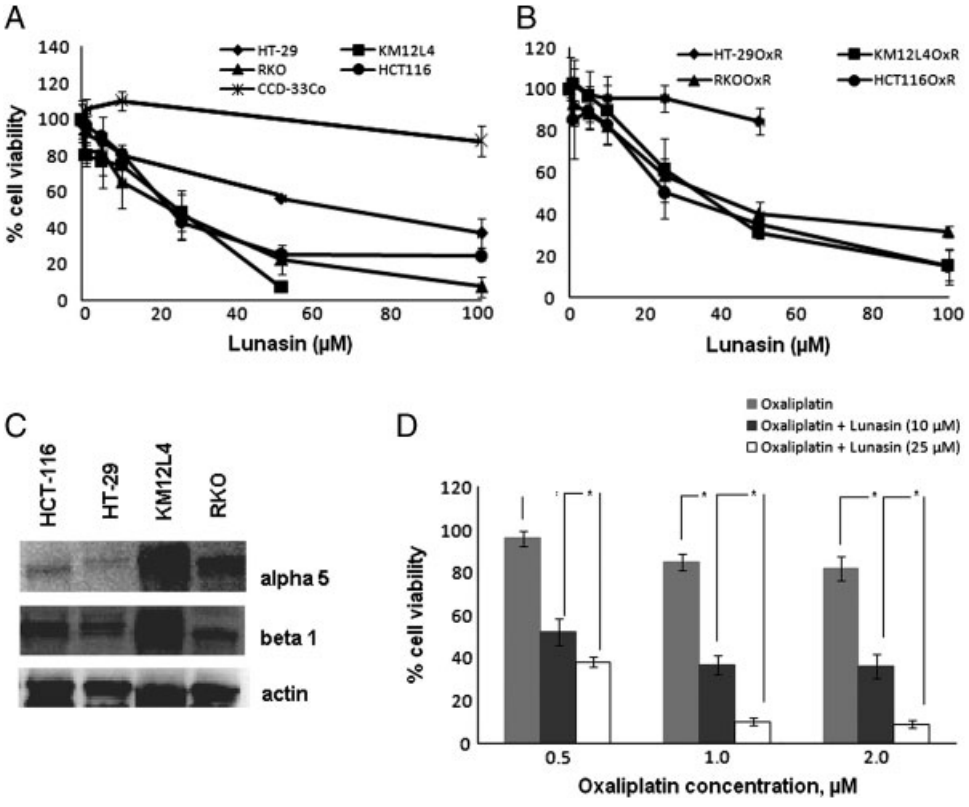

Figure 1. Lunasin cytotoxicity to different colon cancer cells correlated with the expression of $\alpha_5\beta_1$ integrin of KM12L4 colon cancer cells. (A) Parental colon cancer cells. (B) OxR colon cancer cells. (C) Expression of α_5 and β_1 integrins by different colon cancer cells. (D) Interaction study between lunasin and oxaliplatin in causing cytotoxicity to KM12L4 human colon cancer cells. Error bars indicate the standard deviation; * indicate significant differences, $p<0.05$, $n=3$.

increased the expression of cyclin-dependent kinase inhibitors p21 and p27 (Fig. 2B). Lunasin at 10 μM resulted in a 2.2-fold increase in the expression of p21 and 2.3-fold increase in the expression of p27. The same trends were observed in the case of KM12L4OxR cells but at a higher concentration of lunasin (25 μM; Fig. 2C).

3.3 Lunasin promoted apoptosis on KM12L4 colon cancer cells

Figure 3A shows the percentage of KM12L4 colon cancer cells undergoing apoptosis in the presence or absence of lunasin after treatment for 24 h. Lunasin increased the amount of cells undergoing apoptosis from 13.6% (untreated) to 21.7, 24.7 and 27.7% for cells treated with 5, 10 and 25 μM lunasin, respectively. Figure 3B shows the representative pictures from cells treated with 10 and 25 μM lunasin for 24 h as viewed by fluorescence microscopy. Untreated control cells showed normal morphology with intact cell structures, whereas cells treated with lunasin showed characteristics of apoptosis such as nuclear condensation and DNA fragmentation. Furthermore, the population of cells became significantly reduced as seen in Fig. 3B, confirming the cytotoxic effect of lunasin toward KM12L4 cells.

3.4 Lunasin activated the mitochondrial pathway of apoptosis

To further understand the mechanism involved in lunasin-mediated apoptosis, we measured the expression of proteins associated with the mitochondrial pathway of apoptosis (Fig. 4A and B). The expression of the pro-apoptotic Bax was increased by lunasin (10 μM) treatment by 2.2- and 2.3-fold in KM12L4 and KM12L4OxR cells, respectively. Treatment of KM12L4 with 10 μM lunasin resulted in the reduced expression of anti-apoptotic Bcl-2 by two-fold, whereas the same concentration of lunasin resulted in 1.5-fold decrease in KM12L4OxR. In addition, the expression of the pro-apoptotic form of clusterin, nuclear clusterin (nCLU), was also increased. Lunasin (10 μM) increased the release of cytosolic cytochrome c, a major product of mitochondrial permeabilization as a result of Bax mitochondrial translocation, by 2.1- and 1.8-fold in KM12L4 and KM12L4OxR cells, respectively. The expression of the effector of apoptosis caspase-3 increased by 1.8- and 1.7-fold in KM12L4 and KM12L4OxR colon cancer cells, respectively, after treatment with lunasin (10 μM; Fig. 5A). As shown in Fig. 5B, lunasin (5 and 10 μM) increased the activity of initiator caspases 2 and 9 but not caspase-8 indicating that lunasin activated the mitochondrial pathway of apoptosis but did not involve the activation of receptor-mediated apoptosis. An increase in

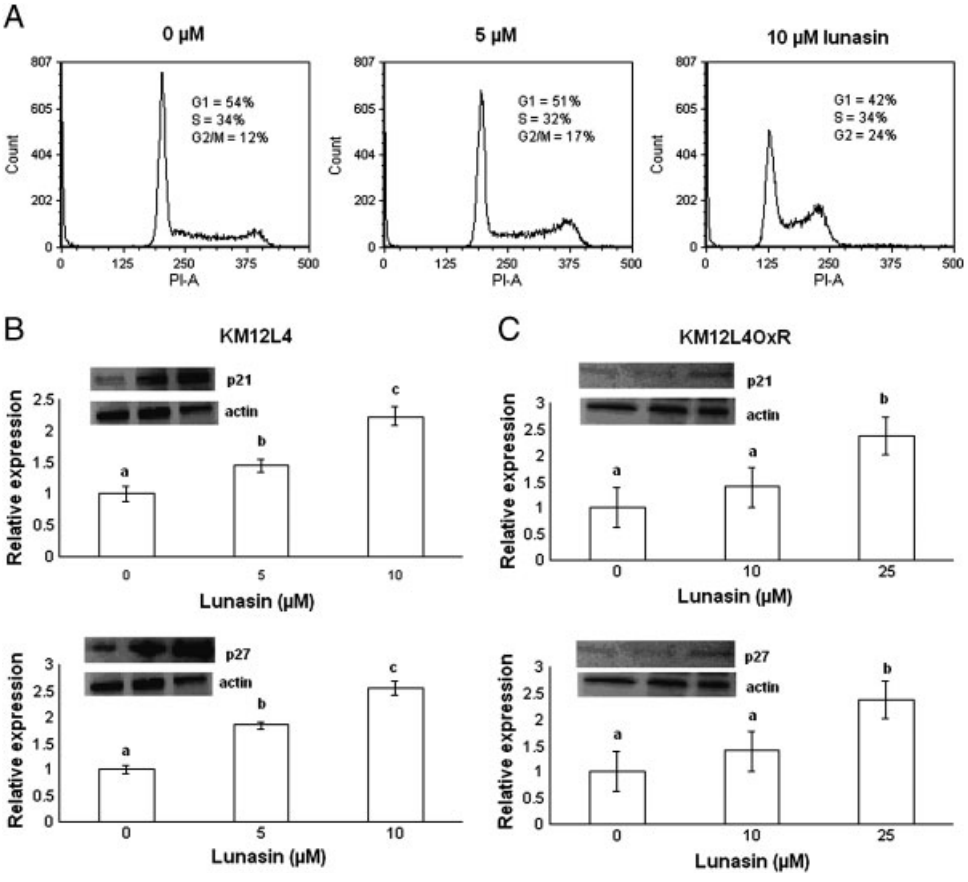

Figure 2. Lunasin arrested cell-cycle progression at G2/M phase of KM12L4 colon cancer cells and increased the expression of cyclin-dependent kinase inhibitors p21 and p27 in KM12L4 and KM12L4OxR colon cancer cells. (A) Cell-cycle progression analysis of cells as affected by lunasin treatment. (B) p21 and p27 expressions of KM12L4 colon cancer cells as affected by lunasin treatment. (C) p21 and p27 expressions of KM12L4OxR colon cancer cells as affected by lunasin treatment. Error bars indicate the standard deviation; different letters indicate significant differences, $p < 0.05$, $n = 3$.

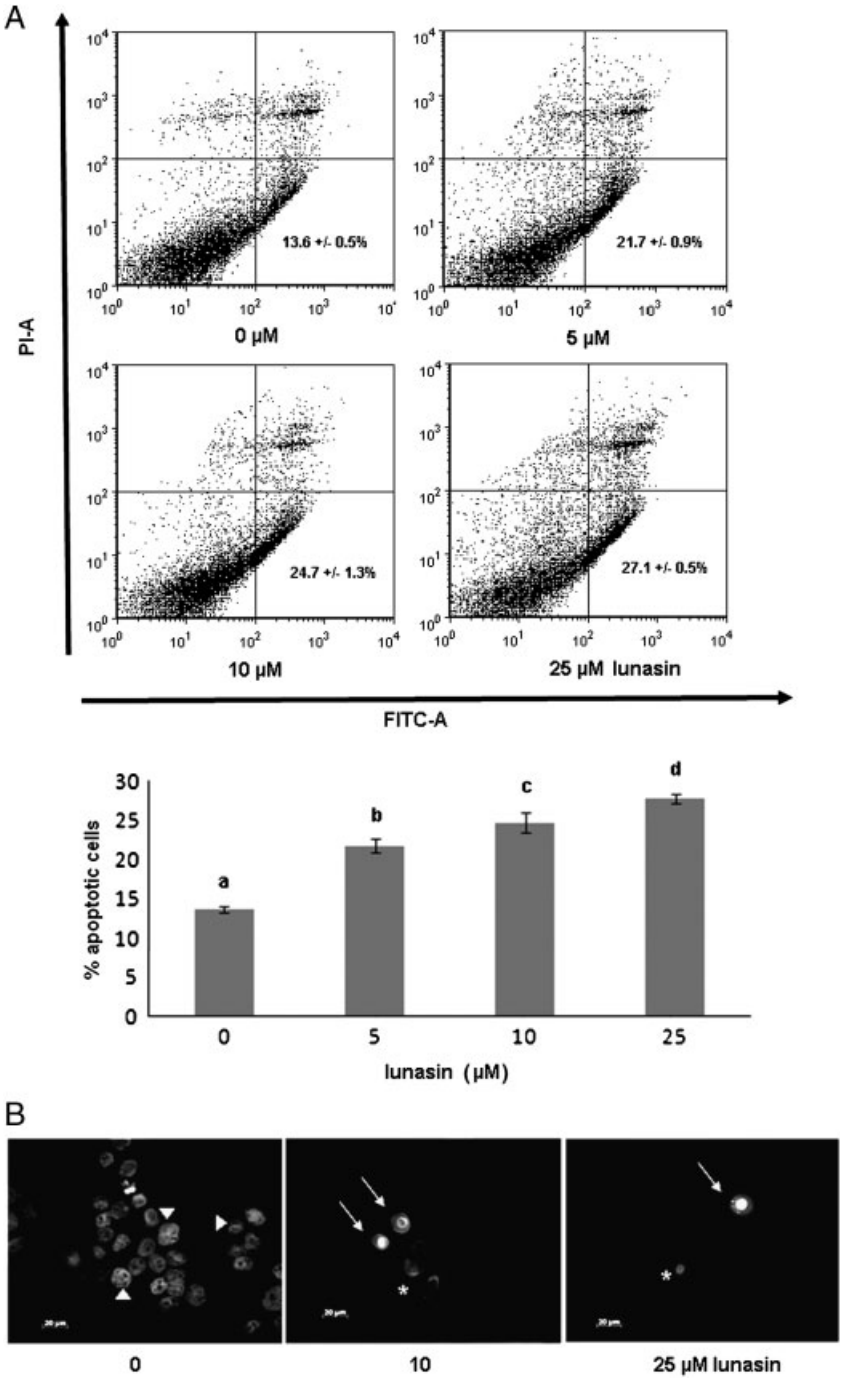

Figure 3. Lunasin induced apoptosis of KM12L4 colon cancer cells. (A) Apoptosis was measured after treatment of confluent cells for 24 h with different concentrations of lunasin. Error bars indicate the standard deviation; different letters indicate significant differences, $p < 0.05$, $n = 3$. (B) Fluorescence microscopic images of KM12L4 colon cancer cells treated with lunasin at 0, 10 and 25 μM for 24 h. Cells treated with lunasin showed the characteristics of apoptosis such as condensation (asterisks) and fragmentation (arrows) compared with untreated cells with normal nucleoli (triangle). All the images were taken at a scale of 20 μm.

the activity of caspase-9 resulted in an increased 2.3-fold activity of effector caspase-3 in KM12L4 cells treated with 10 μM lunasin when compared with untreated cells. Whether lunasin can directly activate caspase-3 independent of caspase-9 was also elucidated. When caspase-9 inhibitor was added into the cell culture of lunasin (10 μM) treatment, there was no upregulation of caspase-3 activity, indicating that lunasin activation of caspase-3 was dependent of caspase-9 activation, whereas caspase-9 was still active even in the presence of caspase-3 inhibitor (data not shown).

3.5 Lunasin modified the expression of human ECM and adhesion genes

Table 1 summarizes the effect of lunasin on the expression of human ECM and adhesion genes in KM12L4 cells. Out of

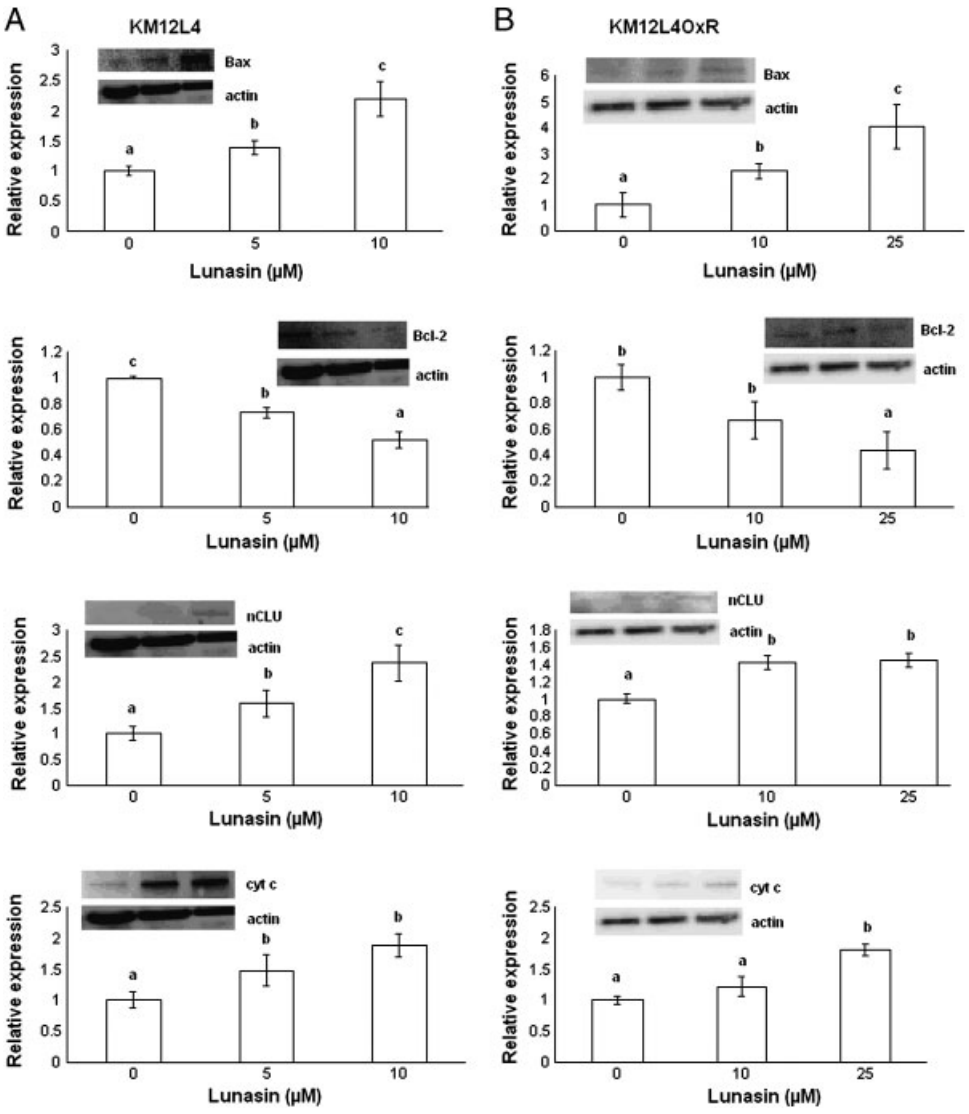

Figure 4. Lunasin modified the expression of proteins associated with mitochondrial pathway of apoptosis in colon cancer cells. (A) KM12L4 and (B) KM12L4OxR. Error bars indicate the standard deviation; different letters indicate significant differences, $p<0.05$, $n=3$.

62 genes that were detected by the array, lunasin caused upregulation of 48 genes and downregulation of 14 genes. The most downregulated genes included COL7A1 (10.10-fold), integrin β_2 (8.19-fold), MMP10 (7.71-fold), SELE (6.53-fold) and integrin α_5 (5.01-fold), while COL14A1 (11.62-fold) was the most upregulated gene. These results demonstrated the possible role of lunasin in mediating angiogenesis and metastasis of cancer cells by affecting genes associated with ECM and cell adhesion.

4 Discussion

Lunasin exhibited different cytotoxic effects against different CRC cell lines; being more effective against parental cell lines than against OxR forms. Lunasin most potently inhibited the growth of KM12L4 with an IC_{50} of 13 μM. This is five times more potent than against HT-29 colon cancer wherein lunasin exhibited an IC_{50} of 61.7 μM [10]. On the other hand, this IC_{50} was close to a previous study wherein lunasin exhibited an IC_{50} of 16 μM against L1210 leukemia cell lines [9].

We explored the possibility that the four colon cancer cells tested differentially expressed a certain type of integrin, a receptor for RGD as the recognizing sequence. We found that the cytotoxic effect of lunasin against colon cancer cells correlated with the expression of $\alpha_5\beta_1$ integrin ($R^2 = 0.78$). Lunasin had the most potent effect on KM12L4 which highly expressed this integrin receptor; while HT-29 was the least susceptible to lunasin's effect and did not express α_5 integrin. Since lunasin features a unique RGD motif known as a recognition sequence for integrins, we hypothesized that these colon cancer cells differentially expressed the $\alpha_5\beta_1$ integrin. The $\alpha_5\beta_1$ integrin is one of the most widely studied integrin in cancer research. It is important for cell adhesion [26] thereby controlling cell migration, growth, proliferation

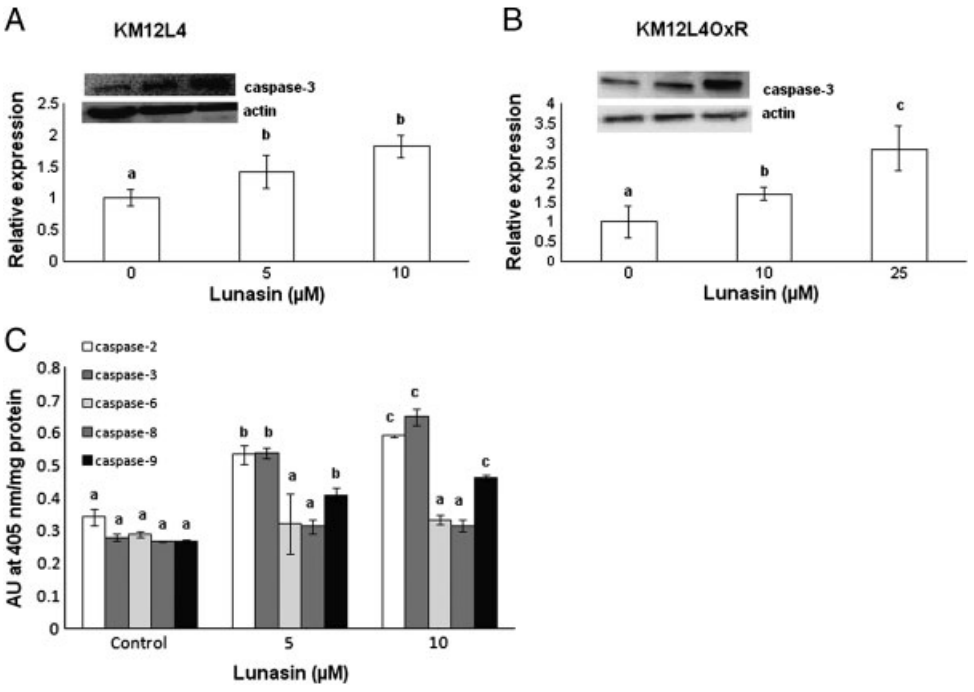

Figure 5. Lunasin increased the expression of caspase-3 in (A) KM12L4 and (B) KM12L4OxR and (C) induces caspase-9 and -3 activity in KM12L4 colon cancer cells. Error bars indicate the standard deviation; different letters indicate significant differences, $p<0.05$, $n=4$.

and apoptosis [27]. In colon cancer, inhibition of the $\alpha_5\beta_1$ function resulted in the reduction of CRC liver metastasis and improved survival in mice [28]. Also, targeting the $\alpha_5\beta_1$ integrin in colon cancer cells using a polyethylene glycolylated liposome RGD peptide PR_b resulted in its internalization *via* $\alpha_5\beta_1$-mediated mechanism and improved cytotoxic effect of 5-fluorouracil-encapsulated stealth liposomes against CT26.WT CRC cells [29]. Moreover, transfection of the α_5 integrin in HT-29 colon cancer cells resulted in the suppression of apoptosis triggered by serum deprivation [30]. The expression of $\alpha_5\beta_1$ integrins also correlated with the invasive capability of different CRC cell lines and contributed to malignant progression in colon carcinoma [31]. Our results suggest that lunasin can be used as a potential adjuvant therapeutic agent against cancer cells highly expressing the $\alpha_5\beta_1$ integrin receptors thereby avoiding the side effects and resistant development associated with the use of chemotherapeutic drugs alone.

Since lunasin most potently affected the proliferation of KM12L4 and KM12L4OxR, we determined its molecular basis. Lunasin affected cell-cycle progression of the metastatic KM12L4 CRC cells, arresting the cell cycle at G2/M phase. This result is in accord with our previous studies in L1210 and HT-29 cancer cells [9, 10]. On the other hand, our results differ with other studies showing the capability of RGD-containing peptides to arrest cell cycle at G1 phase [32, 33], which can be attributed to the cyclic nature of the RGD peptide used in these studies. Moreover, lunasin has a polyaspartic acid tail that caused mitotic arrest [1, 12]. The G2/M arrest caused by lunasin was accompanied by increased expressions of the cyclin-dependent kinase inhibitors p21 and p27. This observation was found in both parental and OxR variant forms of KM12L4 CRC cells, with higher expression found in non-resistant cells. Previous studies showed that the loss of p21 and p27 was linked to drug resistance. The loss of p21 function was associated with a tamoxifen growth-inducing phenotype in breast cancer cells [34] while increase in p27 restored tamoxifen sensitivity in tamoxifen-resistant breast cancer cell lines [35]. Our results showed that lunasin can increase the expressions of p21 and p27 in KM12L4OxR which suggest that lunasin might sensitize back OxR colon cancer cells to oxaliplatin.

Lunasin increased the amount of cells undergoing apoptosis and presented apoptotic-morphological changes on KM12L4 CRC cells such as DNA fragmentation and nuclear condensation. This increase in apoptotic cells was accompanied by the modification of expression of Bcl-2 family of proteins. Lunasin increased the expression of the pro-apoptotic Bax in both KM12L4 and KM12L4OxR cells, which might be attributed to blocked expression of Bcl-2 and increased expression of the pro-apoptotic nCLU. This increase in the expression of nCLU might be attributed to concomitant increase in the expression of p21 as a previous study in colon cancer cells showed that nCLU-mediated apoptosis was dependent on p21 [36]. Upon DNA damage, Ku70 is complexed with nCLU and goes to the nucleus leading to the release of Bax from Ku70–Bax complex and its translocation to the mitochondria [37]. The translocation of Bax into the mitochondria leads to the increased permeabilization of mitochondrial membrane, resulting in the release of death-promoting factor like cytochrome *c*. Lunasin treatment led to an increased amount of released cytochrome *c* further establishing the series of apoptotic events

Table 1. Effect of lunasin on gene expression of extracellular matrix and cell adhesion in human KM12L4 colon cancer

Functional gene groupings	Gene	Description	Fold change[a)]
Transmembrane molecules	CD44	CD44 molecule	1.32
	CHH1	Cadherin-1, type 1, E-cadherin (epithelial)	1.16
	HAS1	Hyaluronan synthase 1	2.19
	ICAM1	Intercellular adhesion molecule (CD54)	3.14
	ITGA1	Integrin, α1	−1.16
	ITGA2	Integrin, α2	1.79
	ITGA3	Integrin, α3	1.36
	ITGA4	Integrin, α4	−1.71
	ITGA5	Integrin, α5	−5.01
	ITGA6	Integrin, α6	1.28
	ITGAM	Integrin, αM	3.90
	ITGAV	Integrin, αV	1.70
	ITGB1	Integrin, β1	1.38
	ITGB2	Integrin, β2	−8.19
	ITGB3	Integrin, β3	−1.01
	ITBB4	Integrin, β4	1.07
	ITGB5	Integrin, β5	1.59
	NCAM1	Neural cell adhesion molecule 1	3.92
	SELE	Selectin E (endothelial adhesion molecule 1)	−6.53
	SELL	Selectin L (lymphocyte adhesion molecule 1)	1.77
	SGCE	Sarcoglycan, epsilon	1.50
	SPG7	Ostoenectin	1.38
	VCAM1	Vascular adhesion molecule	−1.41
Cell–cell adhesion	COL14A1	Collagen, type XIV, α1	11.62
	CTNND1	Cadherin-associated protein, δ1	1.25
Cell–matrix adhesion	ADAMTS13	ADAM metallopeptidase with thrombospondin type 1 motif, 13	1.58
	THBS3	Thrombospondin 3	2.00
Other adhesion molecules	COL12A1	Collagen, type XII, α1	2.00
	COL16A1	Collagen, type XVI, α1	2.85
	COL6A1	Collagen, type VI, α1	−10.10
	COL7A1	Collagen, type VII, α1	−1.05
	COL8A1	Collagen, type VIII, α1	−1.29
	CTGF	Connective tissue growth factor	1.46
	CTNNA1	Cadherin-associated protein, α1, 102 kDa	1.45
	CTNNB1	Cadherin-associated protein, β1, 88 kDa	1.58
	FN1	Fibronectin 1	1.06
	LAMA2	Laminin, α2	1.01
	LAMA3	Laminin, α3	1.62
	LAMB1	Laminin, β1	1.30
	LAMB3	Laminin, β3	2.09
	LAMC1	Laminin, γ1	1.35
	THBS1	Thrombospondin 1	−2.61
	THBS2	Thrombospondin 2	1.08
	CLEC3B	C-type lectin domain family 3, member B	2.78
	TNC	Tenascin C	1.55
	VTN	Vitronectin	1.07
Extracellular matrix proteins	COL4A2	Collagen, type IV α2	−1.43
	COL1A1	Collagen, type I, α1	3.38
	ECM1	Extracellular matrix protein 1	2.10
	TGFBI	Transforming growth factor, β-induced, 68 kDa	1.22
Extracellular matrix proteases	ADAMTS8	ADAM metallopeptidase with thrombospondin type 1 motif, 8	2.56
	MMP1	Matrix metallopeptidase 1	2.48
	MMP3	Matrix metallopeptidase 3	1.47
	MMP7	Matrix metallopeptidase 7	1.09
	MMP9	Matrix metallopeptidase 9	3.71
	MMP10	Matrix metallopeptidase 10	−7.71

Table 1. Continued

Functional gene groupings	Gene	Description	Fold change[a)]
	MMP11	Matrix metallopeptidase 11	2.33
	MMP12	Matrix metallopeptidase 12	1.43
	MMP14	Matrix metallopeptidase 14	1.85
	MMP15	Matrix metallopeptidase 15	−1.18
Extracellular matrix	TIMP1	TIMP metallopeptidase inhibitor 1	1.12
Protease inhibitors	TIMP2	TIMP2 metallopeptidase inhibitor 2	1.84

a) Fold change was calculated as $2^{-(\Delta\Delta C_t)}$ and when the value was <1 it was converted to its negative inverse to report downregulated genes, $n = 2$.

that happened when KM12L4 and KM12L4OxR were treated with lunasin. This is followed by the increase in the activity of caspase-9, leading to the activation of the effector of apoptosis caspase-3 in KM12L4 cells with concomitant increased expression of caspase-3. However, KM12L4OxR required a higher concentration of lunasin to undergo apoptosis, as it developed resistance to chemotherapy. Moreover, the OxR variant showed increased expression of nuclear β-catenin and transcription factor Snail which can explain why it needed higher concentration of lunasin for apoptosis [25].

Several studies showed the ability of RGD-containing peptides to induce apoptosis in a variety of cell lines. Recent investigations showed that RGDS inhibited the growth of melanoma cells with an adhesion-independent mechanism through internalization in melanoma cells and specific interaction with survivin [38] and induced caspase-8 and -9 activity in human endothelial cells [39]. They suggested a mechanism wherein the RGD motif can recognize intracellular target *via* cell internalization leading to procaspase autoprocessing and activation. This suggested mechanism is also supported by studies demonstrating the capability of RGD-containing peptides to promote apoptosis through direct caspase-3 activation [14, 40, 41]. Recent studies also showed the apoptosis-inducing effect of lunasin in breast cancer. Hsieh *et al.* [11] showed that lunasin inhibited cell proliferation and induced cell death in the breast tumor sections of a MDA-MB-231 xenograft breast cancer mouse model. The same group also reported that lunasin was able to sensitize human breast cancer MDA-MB-231 cells to aspirin-arrested cell cycle and induced apoptosis [42].

The PCR array study showed that lunasin was able to downregulate α5 integrin, which might explain the cytotoxic effect of lunasin on colon cancer cells expressing this type of integrin. Also, the results of this array serve as a ground point for future studies on the mechanism by which lunasin can prevent the outgrowth of metastasis of colon cancer cells. Lunasin, a peptide with an RGD motif, was able to downregulate the expression of α5 integrin, an adhesion molecule associated with the control of apoptosis. Lunasin might be able to block the interaction of the integrin receptor to its ligand, leading to the activation of apoptosis.

Lunasin modified the expression of human ECM and adhesion genes indicating the role of lunasin in angiogenesis and metastasis of cancer cells. Lunasin upregulated COL14A1 (11.62-fold), a molecule which when knockdown caused increased growth of renal cell carcinoma cell lines [43]. Also, COL14A1 methylation was associated with a poorer prognosis in renal cell carcinoma patients [43] and usually absent in the vicinity of invading tumors such as Kaposi sarcoma and oral squamous cell carcinoma [44]. On the other hand, lunasin downregulated integrins α_5 and β_2, indicating that lunasin can actually participate in integrin signaling that can be attributed to its RGD motif. Previous studies showed that upregulation of α_5 integrin is correlated with invasion and epithelial–mesenchymal transition of CRC cells [45] and its inhibition resulted in decreased activation of the PI3K pathway and cell adhesion [46]. The expression of MMP10, a matrix metalloproteinase associated with metastasis [47] and tumor growth acceleration [48], was downregulated by lunasin by 7.71-fold. These results suggest that lunasin can actually participate in the modification of genes associated with angiogenesis and metastasis. It is therefore important to study further the molecular basis of the anti-invasive and anti-metastatic potential of lunasin in CRC.

Our previous study [49] found that lunasin is bioavailable in men fed 50 g soy protein *per* day. However, the amount needed *in vivo* to cause the effects observed in colon cancer cells has not been determined. A previous study *in vivo* showed that lunasin, when given at a concentration found in 25 g soy protein, was able to induce apoptosis of breast cancer cells in a xenograft mouse model [11].

In summary, this is the first report on the apoptosis-inducing property of lunasin in human metastatic colon cancer cells and its OxR variant suggesting its potential as an agent to combat metastatic colon cancer particularly in cases where resistance to chemotherapy develops.

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