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RESEARCH ARTICLE

Lunasin, with an arginine–glycine–aspartic acid motif, causes apoptosis to L1210 leukemia cells by activation of caspase-3

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Lunasin is a novel chemopreventive peptide featuring a cell adhesion motif composed of arginine–glycine–aspartate (RGD) which has been associated to cytotoxicity to established cell lines. The objectives of this study were to determine the effect of lunasin on the viability of L1210 leukemia cells and to understand the underlying mechanisms involved. Pure lunasin and lunasin enriched soy flour (LES) caused cytotoxicity to L1210 leukemia cells with IC_{50} of 14 and 16 μM (lunasin equivalent), respectively. Simulated gastrointestinal digestion showed that 25% of the original amount of lunasin survived 3 h of pepsin digestion and 3% of lunasin remained after sequential pepsin–pancreatin digestion for a total of 6 h. Cell cycle analysis showed that lunasin caused a dose-dependent G2 cell cycle arrest and apoptosis. Treatment of L1210 leukemia cells with 1 mg/mL of LES for 18 h led to an increase in the amount of apoptotic cells from 2 to 40%. Compared to untreated cells, treatment with 1 mg/mL LES showed a 6-fold increase on the expressions of caspases-8 and -9, and and a 12-fold increase on the expression of caspase-3. These results showed for the first time that lunasin, a naturally occurring peptide containing an RGD motif, caused apoptosis to L1210 leukemia cells through caspase-3 activation.

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Apoptosis / Leukemia / Lunasin / RGD motif / Soybean

1 Introduction

Soybean is not only an important source of nutrients but also a source of different biologically active compounds including peptides. These biologically active peptides are either naturally present or derived by hydrolysis via fermentation or enzymatic degradation [1]. Lunasin is a novel chemopreventive peptide isolated from soybean, barley, wheat and other plant sources [2–6]. It is composed of 43 amino acid residues with nine aspartic acid residues at its carboxyl end and a predicted helix with a structural homology to a conserved region of chromatin-binding proteins [2, 3]. Also, its amino acid sequence features a cell adhesion motif composed of arginine, glycine and aspartic acid (RGD) residue [2]. Lunasin sequence was discovered in 1987 by Odani *et al.* [7] but the biological properties of this peptide were discovered in 1999 by de Lumen and co-workers. Galvez and de Lumen [8] reported that the small subunit peptide of Gm2S-1 (lunasin) is capable of arresting mitosis resulting in cell death when lunasin gene is transfected and expressed inside mammalian cells. This capability of arresting mitosis was attributed to the binding of the polyaspartic residue found in the carboxyl end of the lunasin molecule to regions of hypoacetylated chromatin. The binding of the polyaspartic residue resulted in the inability of kinetochore binding complex to form properly, leading to mitotic arrest and eventually cell death [8]. Another report by Galvez *et al.* [2] showed that exogenous application of the lunasin peptide inhibits chemical carcinogen-induced transformation of murine fibroblast cells to

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Abbreviations: GI, gastrointestinal; **LES,** lunasin enriched soy flour; **MEM,** minimum essential medium; **PI,** propidium iodide; **RGD,** arginine–glycine–aspartic acid

cancerous foci. The mechanisms involved include the internalization of lunasin in the cell through the RGD motif, colocalization with hypoacetylated chromatin, preferential binding to deacetylated histone H4 *in vitro* and inhibition of histone H3 and H4 acetylation *in vivo* in the presence of a histone deacetylase inhibitor. Jeong *et al.* [4] reported that lunasin from lunasin enriched soy flour (LES) was protected from *in vitro* digestion by pepsin, and lunasin extracted from blood and liver of rats fed with LES was intact and also inhibited core histone acetylation.

Peptides containing the RGD motif have been used extensively in the study of cell adhesion, migration, growth and differentiation [9–11]. The RGD tripeptide is the cell attachment site, recognized by integrins, present in many extracellular matrix and cell surface proteins [11]. Also, several studies have shown the capability of peptides containing RGD motif to induce apoptosis on different cell lines [12–15]. Very recently, RGD peptide has been shown to induce apoptosis in cultured chondrocytes as well as in cells in cartilage explants in synovial cells through direct activation of caspase-3 [16].

Leukemia is considered to be the most common type of cancer in children [17]. Leukemia disrupts the normal reproduction and repair processes of white blood cells causing them to divide too quickly before they mature and resulting in the arrest on the proper production of all blood cells. According to the American Cancer Institute, in 2008 there was an estimated 44 270 new cases of leukemia and approximately 21 710 number of deaths. The annual treatment cost of leukemia in the United States alone is approximately $ 2.6B [18].

In this study, we hypothesized that lunasin, a naturally occurring peptide from soybean containing the RGD motif, will exert cytotoxicity to leukemia cells via apoptosis and will influence the cell cycle of leukemia cells. The objectives were to determine the effect of lunasin on the viability of L1210 leukemia cells and to understand the underlying mechanisms involved.

2 Materials and methods

2.1 Materials

Leukemia cell line L1210 was purchased from American Type Culture Collection (Manassas, VA, USA). Minimum essential medium (MEM), fetal bovine serum (FBS), apoptosis assay and caspase expression kits were purchased from Invitrogen (Carlsbad, CA, USA). Pepsin and pancreatin were purchased from Sigma (St. Louis, MO, USA). Purified lunasin and rabbit lunasin polyclonal antibody against the lunasin epitope – EKHIMEKIQGRGDDDDD were purchased from the University of California at Berkeley. LES containing 27% w/w lunasin was prepared by extracting soybean flour with distilled water in a 1:6 w/v at 4°C for 48 h. After extraction, the mixture was filtered and allowed to settle for 48 h at 4°C. The supernatant of the solution was then freeze dried to obtain LES as indicated by Jeong *et al.* [4]. Mouse monoclonal antibodies for actin, p21 and p27 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and antimouse IgG horseradish peroxidase conjugate secondary antibody and antirabbit IgG alkaline phosphatase conjugate secondary antibody were purchased from GE Healthcare (Buckinghamshire, UK). All other reagents were purchased from Sigma unless otherwise specified.

2.2 Lunasin digestion stability assay

2.2.1 Simulated gastrointestinal digestion of lunasin

In order to determine the stability of lunasin upon gastrointestinal (GI) digestion, a simulated GI digestion was carried out following a previous protocol [19]. Briefly, 5% w/v LES in deionized distilled water was prepared and brought to 37°C. Pepsin (EC 3.4.23.1, 662 units/mg) was added at 1:20 w/w enzyme/flour ratio and the pH was adjusted to 2.0 using 0.1 N HCl. Pepsin digestion was performed for a total of 3 h and samples were taken at 0, 10, 30, 60, 90 and 180 min of digestion. After pepsin digestion, pancreatin (8 × USP, from porcine pancreas) was added at 1:20 w/w enzyme/flour ratio and the pH was adjusted to 7.5. Pancreatin digestion was carried out for a total of 3 h and samples were taken at 0, 10, 30, 60, 90 and 180 min of digestion. Simulated GI digestion was stopped by heating at 75°C for 20 min. Hydrolyzed samples were neutralized, centrifuged at 14 000 × *g* for 30 min and filtered using a 0.45 μm membrane and lyophilized in FreeZone freeze dried system (Kansas City, MO, USA) and stored at −20°C until analysis.

2.2.2 Quantification of lunasin in hydrolysates using ELISA

Approximately 0.05 g of lyophilized samples from the simulated GI digestion were extracted with 1 mL of 0.05 M Tris-HCl buffer, pH 8.2 with sonication at 40°C for 70 min with vortexing every 10 min to avoid settling down of samples. The mixture was then centrifuged at 20 000 × *g* for 30 min at 4°C and the supernatant tested for lunasin using ELISA as described previously [20]. Briefly, 100 μL of diluted samples (1:6000) were plated in 96-well plate and stored at 4°C for at least 14 h. The plate was then washed with 0.01 M PBS with 0.05% Tween-20, pH 7.4 using ELX 50 Auto Strip Washer from Biotek Instruments (Winooski, VT, USA). Immediately after that, the plate was blocked by incubating with 300 μL of 5% BSA in TBS-1% Tween-20 for 1 h. The plate was washed and incubated with 50 μL of rabbit lunasin polyclonal antibody (1:4000 dilution) for 1 h at room

temperature. The plate was then incubated with 50 μL of antirabbit IgG alkaline phosphatase conjugate secondary antibody (1:7000 dilution) for 1 h at room temperature. After washing, the color was developed by adding 100 μL of color reagent *p*-nitrophenyl phosphate to each well. The reaction was stopped by adding 25 μL of 3 N NaOH at 30 min and the absorbance was read after 35 min at 405 nm using an ELISA plate reader ELX 808 IU from Biotek. Lunasin concentration was quantified using a standard curve from different concentrations of synthetic lunasin. Measurements were done in triplicate and results represent mean value ± SD.

2.2.3 Analysis of lunasin in hydrolysates using Western blot

A 5 mg/mL sample of lyophilized hydrolysates was prepared using deionized distilled water. A 1:1 dilution of the sample with Tris-Tricine buffer (Biorad Laboratories, Hercules, CA, USA) containing 5% β-mercaptoethanol was boiled for 5 min and loaded into a 16.5% polyacrylamide gel. Protein separation by electrophoresis was carried out at 125 V for 60 min. After separation, the gel was transferred to a polyvinylidene fluoride membrane at 40 V for 90 min. The membrane was then blocked with ECL advance Western blot blocking reagent for 1 h and washed with Tris-buffered saline with 1% Tween 20 (TBST) three times for 5 min. The membrane was then incubated with rabbit lunasin polyclonal antibody (1:4000) at 4°C overnight. After incubation, the membrane was washed with TBST and incubated with antirabbit IgG alkaline phosphatase conjugate for 1 h at room temperature. After washing, lunasin band was detected using an Immun-Star Chemiluminescent Substrate solution (Bio-Rad Laboratories) following the manufacturer's recommendations and visualized using Kodak Image station 440 CF (Eastman Kodak, New Haven, CT, USA).

2.3 Cell viability of L1210 leukemia cells

Murine leukemia cell line L1210 was cultured in MEM (Invitrogen) supplemented with 10% FBS at 37°C in 5% CO_2/95% air. Cell viability assay was performed using a Cell Counting Kit-8 (CCK-8) reagent (Dojindo, Gaithersburg, MD, USA). Briefly, 2×10^3 cells (approximately 90 μL of cell suspension at the logarithmic phase) were seeded in a 96-well flat bottom cell culture plate. After incubation for 24 h the L1210 leukemia cells were treated with 10 μL of samples with different concentrations (0.01–5 mg dry material/mL). The plate was then incubated at 37°C in 5% CO_2/95% air for 48 h after which 10 μL of CCK-8 reagent was added to determine the number of viable cells. The plate was incubated another 4 h and the absorbance read at 450 nm using an ELX 808 plate reader from Biotek. The results were expressed as percent viability of treated cells compared with the untreated control. Data are presented as mean values ± SD of three independent experiments.

2.4 Cell cycle distribution of L1210 leukemia cells

Analysis of the cell cycle was performed by flow cytometry following previously reported protocol [21]. L1210 leukemia cells were seeded in a 24-well plate and treated with lunasin at concentrations ranging from 0.1 to 5 mg/mL for 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) (Molecular Probes, Eugene, OR, USA) solution (PBS containing 0.1% v/v Triton X-100, 0.2 mg/mL DNase-free RNase A and 0.02 mg/mL of PI). Cell cycle distribution analysis was performed using a LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) at excitation wavelength of 488 nm. Fluorescence emission was measured using a 695/40 nm band pass filter. A total of 20 000 events were collected for each sample. Data are presented as mean values ± SD of three independent experiments.

2.5 Apoptosis and caspase expression analysis

Apoptosis analysis was performed using a Vybrant® FAM (a carboxyfluorescein group) polycaspases assay kit and expressions of caspases 3, 8 and 9 were determined by Vybrant® FAM caspase 3, 8 and 9 assay kit, respectively, following manufacturer's instructions. Briefly, L1210 leukemia cells at logarithmic phase were seeded in 24-well plate and treated with LES at 0.3 and 1 mg/mL concentrations for 4–32 h at 37°C in 5% CO_2/95% air. After treatment, cells were stained with FAM-VAD-FMK polycaspases reagent (for caspase-1, -3, -4, -5, -6, -7, -8 and -9), FAM-DEVD-FMK (for caspase 3), FAM-LETD-FMK (for caspase 8), FAM-LEHD-FMK (for caspase 9) and PI. Cell fluorescence was measured using LSR II flow cytometer at excitation wavelength of 488 nm. Flourescence emission was measured at 530/30 nm for FAM and 695/40 nm for PI. A Hoescht staining procedure was performed using microscopic analysis to determine chromatin condensation and caspase induction on L1210 leukemia cells treated with LES.

2.6 Analysis of p21 and p27 expression

2.6.1 Cell treatment and preparation of cell lysate

L1210 leukemia cells, at a density of 2.5×10^5 cells/mL, were seeded in a 24-well plate in MEM supplemented with 10% FBS and treated with LES at 0.3 and 1 mg/mL concentrations for 24 h at 37°C in 5% CO_2/95% air. After treatment, cells were washed with PBS and resuspended in radioimmunoprecipitation buffer (Boston Bioproduct) containing

protease inhibitor (Sigma, 20 μL/mL radioimmunoprecipitation buffer). The suspension was allowed to sit on ice for 10 min and sonicated 20 times (2 s per time, 12 s interval between sonication) on ice. After sitting on ice for another 10 min, the cell lysate was centrifuged at 12 000 × *g* at 4°C for 15 min. The supernatant was collected and the protein concentration was determined using protein DC assay kit (Biorad Hercules) following manufacturer's instructions.

2.6.2 Western blot analysis of p21 and p27

Equal amount of protein (20 μg) were loaded in each well of 15% polyacrylamide gel. Protein separation by electrophoresis was done at 125 V for 60 min. After separation, the gel was transferred to a polyvinylidene fluoride membrane at 40 V for 90 min. The membrane was then blocked with ECL advance Western blot blocking reagent for 1 h and washed with TBST three times for 5 min. The membrane was then incubated with mouse monoclonal p21 antibody (1:200, sc-6246) or mouse monoclonal p27 antibody (1:200, sc-1641) or mouse monoclonal actin antibody (1:200, sc-8432), at 4°C overnight. The membrane was washed with TBST and incubated with antimouse horseradish peroxidase conjugate secondary antibody (1:15000) for 1 h at room temperature. The signal was detected by adding HRP substrate from ECL Advance Western Blotting Detection Kit (Amersham Biosciences) and visualized using Kodak Image station 440 CF (Eastman Kodak).

2.7 Statistical analysis

The concentration needed to inhibit 50% of L1210 leukemia cell growth (IC_{50}) was determined by non-linear regression (curve fit) using the GraphPad Prism® software. Data were analyzed by one-way ANOVA and significant difference was reported when *p*-value was less than 0.05.

3 Results and discussion

3.1 Stability of lunasin after simulated GI digestion

Stability towards GI digestion is an important characteristic of any bioactive compound for it to be absorbed by the GI system thereby exerting its effect on the target organ or tissues. Figure 1 presents the effect of pepsin and pancreatin *in vitro* digestion on the amount of lunasin in LES. Quantification of lunasin using ELISA showed that the amount of lunasin decreased rapidly in the first 60 min of pepsin digestion and reached an equilibrium concentration at 90 min. It can also be seen that approximately 25% of the original concentration of lunasin remained after 180 min of pepsin digestion. Further digestion of LES with pancreatin resulted in a dramatic decrease in lunasin concentration after 30 min. Additional pancreatin digestion for 150 min resulted in an equilibrium concentration in the amount of lunasin in LES. Quantification of the amount of lunasin remaining after sequential pepsin–pancreatin digestion showed that only 3% of the original lunasin concentration was left. The results of the Western blot analysis confirmed the trend on the amount of lunasin remaining after pepsin–pancreatin digestion (Fig. 1 inset). Lunasin must first survive GI digestion before it can be internalized into the cells. The result of this study showed that lunasin, an important biologically active peptide can survive sequential pepsin–pancreatin digestion, a very important characteristic to keep its integrity and activity. Previous result showed that 30% of lunasin remained after 120 min of pepsin digestion as determined by Western blot [4]. This previous result is very much in agreement with our current findings showing that 25% of lunasin was still present after 180 min of pepsin digestion as quantified by ELISA. The US Food and Drug Administration approved a claim for soybean protein indicating that consumption of 25 g of soybean protein per day as part of a diet low in saturated fat and cholesterol may lead to reduction of risk of heart disease. de Mejia *et al.* [20] showed that soy protein isolate contains 3.8% lunasin, thus, consumption of 25 g soy protein per day can lead to a total intake of 0.94 g of lunasin. Of this total lunasin intake, 3% will survive sequential pepsin–pancreatin digestion resulting in 28 mg of biologically active lunasin. This amount of lunasin, when completely absorbed, will lead to a plasma concentration of approximately 9 μg/mL for a 70 kg subject with a plasma volume of 3 L. Previous study on experimental animals has shown that lunasin can be found in the liver and blood of rats fed with lunasin-enriched soybean protein [4]. Very recently, our laboratory demonstrated that lunasin can be found in plasma of men after five days of soy protein consumption [22]. These findings support our

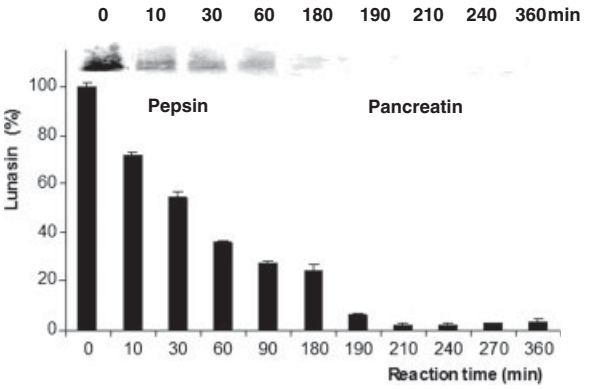

Figure 1. *In vitro* stability of lunasin during simulated GI enzyme digestion. Lunasin enriched flour was subjected to sequential pepsin–pancreatin digestion for 3 h. Lunasin concentrations at different time points were measured by ELISA (bar graph). Measurements were done in triplicate and results represent mean value ± SD. Western blot results are shown in inset.

results on the stability of lunasin, a very important requirement for lunasin to exert its biological activity.

3.2 Effect of lunasin on viability of L1210 leukemia cells

Figure 2 shows the effect of purified lunasin and LES on the viability of L1210 leukemia cells. As shown in Fig. 2A, purified lunasin caused a dose-dependent cytotoxicity on L1210 leukemia cells. Lunasin inhibited proliferation of L1210 leukemia cells by 19% at 10 μg/mL (2 μM) with an IC_{50} of 78 μg/mL (14 μM). LES containing 27% w/w lunasin showed the same capability of inhibiting the proliferation of L1210 leukemia cells. At a concentration of 0.34 mg/mL equivalent to 16 μM lunasin, LES caused 50% cytotoxicity to L1210 leukemia cells. Also, a dose-dependent anti-proliferation effect of LES was observed in L1210 leukemia cells as shown in Fig. 2B. These results demonstrated for the first time the capability of soybean lunasin to inhibit proliferation of an established cancer cell line. This has not been the case for other cell types. A previous study has shown that lunasin at a concentration of up to 10 μM did not cause any cytotoxicity on human breast cancer cell line MCF-7 and human lung cancer cell line NCI-H460 [23]. Results from our laboratory also showed that LES at concentrations of 1.5 and 3 mg/mL did not cause any cytotoxicity to HL60 leukemia cell line and HepG2 hepatoma cell line, respectively (data not shown). These observations indicated that the anti-proliferative effect of lunasin from soybean depends on the type of cell line tested. This might be attributed to the specificity of lunasin on exerting its anti-proliferative effect [23]. This specificity is also supported by Matsuki *et al.* [16]. More studies are needed to further clarify this effect, probably by increasing the concentrations used. Also, studies of LES on apoptosis of human leukemia cells are warranted. Soybean lunasin features a cell adhesion motif composed of RGD residues. Previous studies have shown the capability of peptides with RGD motif to cause cytotoxicity in established cell lines. Anuhadra *et al.* [15] showed that RGD peptide resulted in a time-dependent death of HL60 cells when exposed to RGD at a concentration of 1 mM from 24 to 48 h causing 50% death after 36 h of exposure. Also, the results showed the importance of the RGD motif in inducing cell cytotoxicity since RAD (arginine–alanine–aspartate) peptide showed no inhibition of HL60 growth at the same concentrations used for RGD. The same results were observed when lymphocytes [14] and chondrocytes and synovial cells [16] were treated with peptides containing RGD motif.

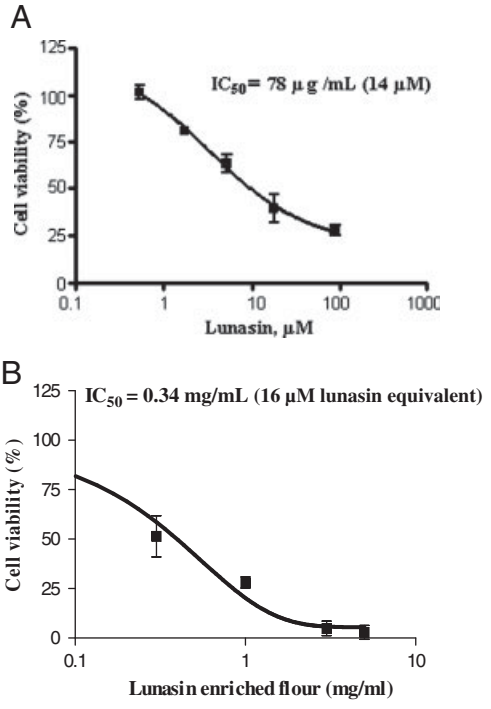

Figure 2. Effect of purified lunasin (A, 98%) and lunasin enriched flour (B, 27%) on the viability of L1210 leukemia cells. Data are presented as mean values ± SD for three independent experiments.

3.3 Effect of lunasin-enriched soy flour on L1210 leukemia cells cell cycle distribution

Figure 3 presents the effect of LES on cell cycle distribution of L1210 leukemia cells. Treatment of L1210 leukemia cells with LES for 24 h led to an increase in the amount of cells in the sub-G1 fraction in a dose-dependent manner. LES at concentrations of 0.3 and 1.0 mg/mL resulted in 43.7 and 73.2% of the cells in the sub-G1 fraction, respectively, as compared to 1.0% of the untreated cells. Cells at the sub-G1 fraction already lost its integrity and contained less amount of DNA than G1 cells. A large amount of cells in the sub-G1 fraction is an indicator of DNA fragmentation and apoptotic body formation [24]. Previously, it has been shown that treatment with a RGD peptide at a concentration of 1 mM for 24 and 48 h resulted in a time-dependent internucleosomal DNA fragmentation in HL60 leukemia cells [15]. Figure 4 shows the effect of LES in the cell cycle distribution of L1210 leukemia cell line without considering the sub-G1 fraction. It can be seen that treatment of L1210 leukemia cells with LES resulted in a dose-dependent induction of G2 cell cycle arrest. The amount of cells in the G2 phase increased from 24.5 (untreated cells) to 28.2, 29.6 and 35.5% for L1210 leukemia cells treated with 0.3, 1.0 and 5.0 mg LES/mL, respectively. G2 cell cycle arrest can be triggered by various stress stimuli through down regulating the expression of Cdk1 kinase, topoisomerase IIα, and/or upregulating the cyclin-dependent kinase inhibitor p21 and p27 [25]. However, treatment of L1210 leukemia cells with LES up to 1 mg/mL showed no effect on the expression of p21 and p27 (data not shown). This indicates that other

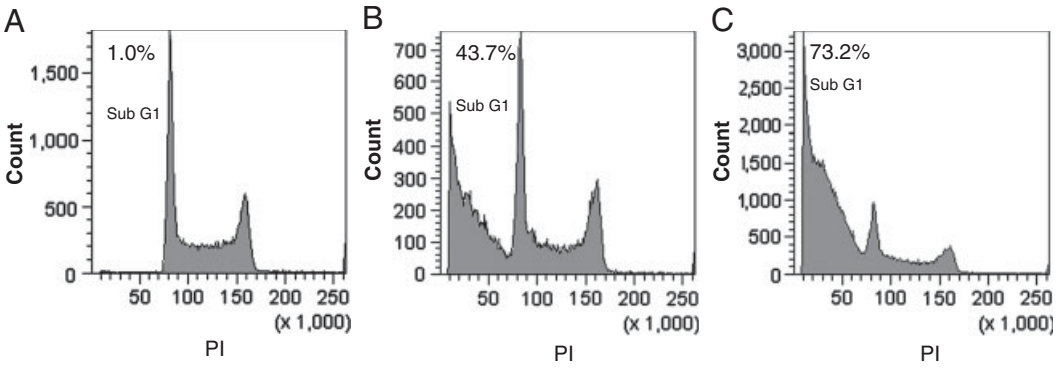

Figure 3. Lunasin enriched flour treatment lead to dose-dependent increase in the percentage of sub-G1 fraction of L1210 leukemia cells. (A) Untreated L1210 leukemia cells; (B) L1210 leukemia cells treated for 24 h with 0.3 mg/mL LES and (C) L1210 leukemia cells treated for 24 h with 1.0 mg/mL LES.

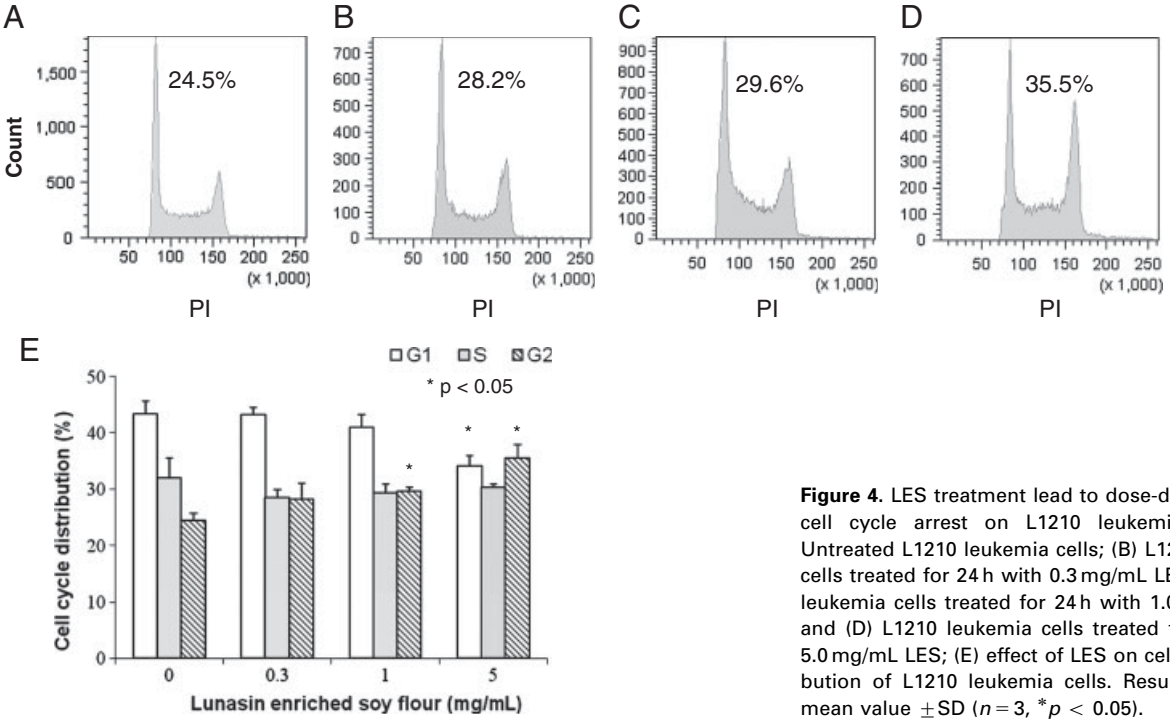

Figure 4. LES treatment lead to dose-dependent G2 cell cycle arrest on L1210 leukemia cells. (A) Untreated L1210 leukemia cells; (B) L1210 leukemia cells treated for 24 h with 0.3 mg/mL LES; (C) L1210 leukemia cells treated for 24 h with 1.0 mg/mL LES and (D) L1210 leukemia cells treated for 24 h with 5.0 mg/mL LES; (E) effect of LES on cell cycle distribution of L1210 leukemia cells. Results represent mean value $\pm$SD ($n = 3$, $^{*}p < 0.05$).

mechanisms might be involved by which lunasin induced G2 cell cycle arrest. The presence of polyaspartic residue on the carboxyl end of the lunasin molecule led to its binding to the regions of hypoacetylated chromatin [8]. This binding resulted in the mitosis arrest [8] which might explain the ability of lunasin to induce G2 cell cycle arrest.

3.4 Apoptosis and caspase activation

Figure 5 shows that LES caused a time- and dose-dependent induction of apoptosis in L1210 leukemia cells. Higher concentration (1 mg/mL) of LES and longer treatment times (13 and 18 h) increased the percentage of apoptotic cells. This event was accompanied with an increase in the number of post-apoptotic cells and a decrease in the amount of viable cells. The apoptosis inducing effect of LES on L1210 leukemia cells was observed at a concentration as low as 0.3 mg/mL when exposed for at least 13 h. Treatment of LES at a concentration of 1 mg/mL for 18 h led to a significant increase in the amount of apoptotic cells from 1.9 to 39.7 %. This apoptotic inducing effect of LES was confirmed by microscopic analysis of the cells treated with 1 mg/mL for 24 h and stained with Hoechst reagent. Figure 6 shows that LES led to chromatin condensation and caspase induction on L1210 leukemia cells supporting the induction of apoptosis previously observed.

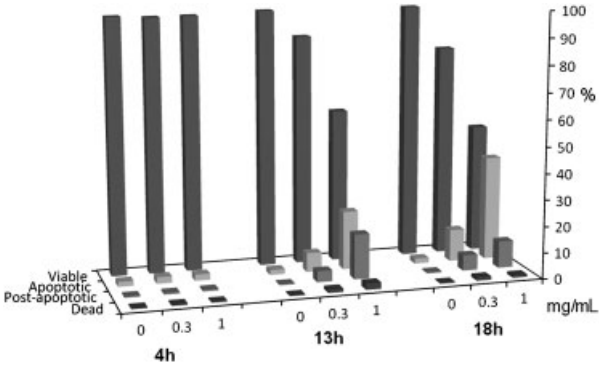

Figure 5. LES induced apoptosis in L1210 leukemia cells in a time and dose-dependent manner. Cell fluorescence was measured using LSR II flow cytometer at excitation wavelength of 488 nm; flourescence emission was measured at 695/40 nm for PI.

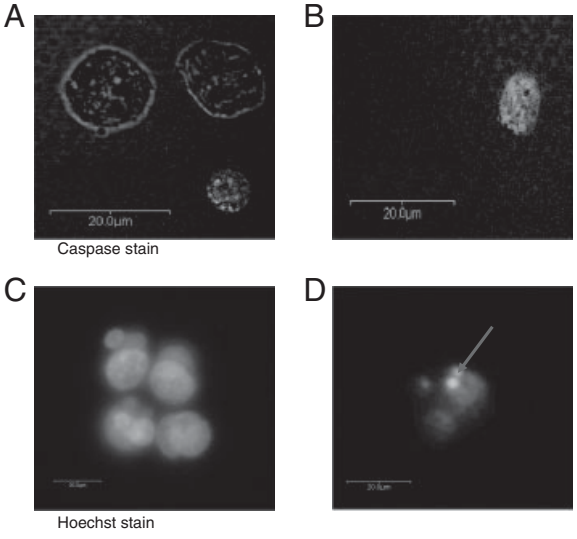

Figure 6. LES induced apoptosis on L1210 leukemia cells. (A) L1210 leukemia cells untreated with LES and stained with caspase stain; (B) L1210 leukemia cells treated with LES (1 mg/mL) for 24 h and stained with caspase stain; (C) L1210 leukemia cells untreated with LES and stained with Hoechst stain and (D) L1210 leukemia cells treated with LES (1 mg/mL) for 24 h and stained with Hoechst stain. The slides were viewed with a phase-contrast microscope under UV light at 100 ×.

Caspases are enzymes that belong to the cysteine family that cleave their substrates at aspartic acid residues and play a key role in apoptosis [26]. Caspases exist as inactive zymogens in the form of procaspases [27] and, once activated, process themselves and can activate other inactive zymogens [16]. This cascade of events can lead to activation of apoptotic initiators caspases-8 and -9 and apoptotic executioners caspases-3 and -7 [28]. Figure 7 shows the effect of LES treatment on the expression levels of caspases-3, -8 and -9. LES treatment at a concentration of 1 mg/mL resulted in the 5–6-fold and 12-fold increase in the expressions of caspases-8 and -9 and -3, respectively, when compared to untreated L1210 leukemia cells. For caspases 3, 8, and 9 an average of 1.9 ± 0.2, 3.2 ± 0.5, and 3.6 ± 0.5% of cells, in the absence of lunasin, were positive to the caspase reagent, respectively. These values obtained only with water and no lunasin (control), were used as baseline to calculate the caspases fold increases. Each data point in Fig. 7 represents mean values generated by the flow cytometer system measuring 25 000 different events. This figure shows a tendency on the fold increase of each caspase. More studies are needed to better understand the variability due to different concentrations of lunasin. Further analysis on caspase expression will reveal the specific pathways, internal or external signal cascade, through which lunasin affects L1210 leukemia cells. Caspase-8 reached a maximum level after 18 h of LES treatment while caspase-9 is at its highest expression after 24 h of treatment. On the other hand, caspase-3 reached its highest expression level after 24 h treatment indicating that it is a later event after caspases-8 and -9 activation. These results showed that LES activated caspases-8 and -9 expression which triggered the activation of caspase-3, an executioner of apoptosis. LES also induced caspase-3 expression even in the presence of inhibitors for caspases-8 and -9. This result suggests that LES caused an induction of caspase-3 expression independently of caspase-8 and -9 pathways (data not shown). This demonstrated that the mechanism by which LES induced apoptosis is through a caspase dependent pathway. Buckley *et al.* [14] showed that RGD-containing peptides were able to directly induce apoptosis and demonstrated that these peptides can enter cells and directly induce autoprocessing and enzymatic activity of procaspase-3, a pro-apoptotic protein. They also showed that caspase-3 gene is an important requirement for RGD-mediated cell death. Similar result was reported by Anuhadra *et al.* [15] which showed that RGD-treated HL60 leukemia cells resulted in caspase-3 activation thereby inducing apoptosis in HL60 cells. They showed that caspase-3 might have a critical role in the induction of apoptosis by RGD-containing peptides. A study on RGD-peptides treated chondrocytes and synovial cells showed that these peptides

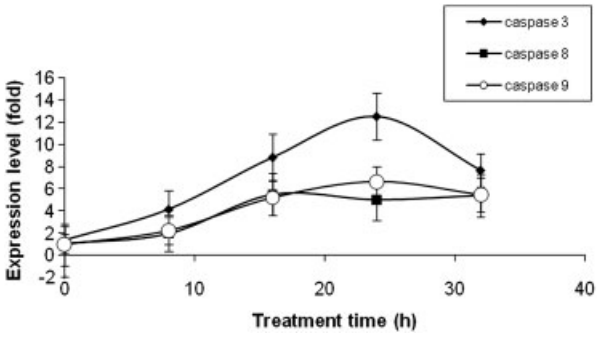

Figure 7. Effect of lunasin (50 μM) on the activation of caspase-8, -9 and -3 in L1210 leukemia cells. Each point represents the mean value (±SD) generated by the flow cytometer system measuring 25 000 different events.

induced apoptosis through direct activation of caspase-3 [16]. The results suggest that LES caused increased expression of different caspases through an RGD-mediated action.

It has been shown previously that peptides containing RGD sequence can target $\alpha_v\beta_3$ on angiogenic epithelia cells [29] presenting a possible role of RGD peptides in cancer therapy. Also, RGD peptide analogs have been used recently in tumor imaging with fluorophores, or radionucleotides in angiogenesis studies and in tumor therapy with chemotherapeutic drugs [30–36]. Chen *et al.* [37] showed that paclitaxel, an antitumor agent, conjugated with a dimeric RGD peptide inhibited cell proliferation *via* an arrest of G2/M phase of the cell cycle followed by apoptosis in a metastatic breast cancer cell line MDA-MB-435. Recently, it has been shown that paclitaxel in conjugation with a dimeric RGD peptide had higher initial tumor exposure dose and prolonged tumor retention than the unconjugated paclitaxel [38]. Also, Zhao *et al.* [39] have shown that RGD peptide modified liposomes containing paclitaxel (RGD-SSL-PTX) showed 3.5 times lower than IC_{50} in causing significant cytotoxicity in SKOV-3 human ovarian cancer cells than unmodified liposomes. They also showed that RGD-SSL-PTX had the strongest tumor growth inhibition in BALB/c mice xenografted with SKOV-3 solid tumor without causing any significant change in the body weight of the animals treated at a concentration of 12.5 mg/kg administered *via* intravenous injection. These studies show the importance of the presence of the RGD motif in increasing the efficacy of several antitumor drugs. Lunasin, a novel peptide from soybean bearing the RGD motif, might be considered a natural source of RGD peptide needed for increasing the efficacy of these drugs.

4 Concluding remarks

Purified lunasin from soybean and LES cause cytotoxicity to L1210 leukemia cells through induction of G2 cell cycle arrest and apoptosis. It has been demonstrated for the first time that lunasin, a naturally occurring peptide containing an RGD-motif, causes apoptosis through increased activation of caspases-8 and -9 and -3. Furthermore, lunasin can survive a simulated GI digestion, an important property for a compound to exert its biological activity. These findings may have important implications on the search of compounds that can alleviate diseases like leukemia.

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