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RESEARCH ARTICLE

Relationship between lunasin's sequence and its inhibitory activity of histones H3 and H4 acetylation

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Scope: Dysfunction of histone acetyltransferases (HATs) or histone deacetylases (HDACs) involved in histones acetylation has been associated with cancer. Inhibitors of these enzymes are becoming potential targets for new therapies.
Methods and Results: This study reports by Western-Blot analysis, that peptide lunasin is mainly an *in vitro* inhibitor of histone H4 acetylation by P300/cAMP-response element-binding protein (CBP)-associated factor (PCAF), with IC_{50} values dependent on the lysine position sensitive to be acetylated (0.83 μM (H4-Lys 8), 1.27 μM (H4-Lys 12) and 0.40 μM (H4-Lys 5, 8, 12, 16)). Lunasin is also capable of inhibiting H3 acetylation (IC50 of 5.91 μM (H3-Lys 9) and 7.81 μM (H3-Lys 9, 14)). Studies on structure-activity relationship establish that lunasin's sequence are essential for inhibiting H4 acetylation whereas poly-D sequence is the main active sequence responsible for H3 acetylation inhibition. Lunasin also inhibits H3 and H4 acetylation and cell proliferation (IC_{50} of 181μM) in breast cancer MDA-MB-231 cells. Moreover, this peptide decreases expression of cyclins and cyclin dependent kinases-4 and -6, implicated in cell cycle pathways.
Conclusion: Results from this study demonstrates lunasin's role as modulator of histone acetylation and protein expression that might contribute on its chemopreventive properties against breast cancer.

Keywords:
Breast cancer cells / Cell proliferation / Histone acetylation / Lunasin / Protein biomarkers

1 Introduction

Epigenetics is defined as changes in gene function, which occur without a change in DNA sequence and are mitotically, and sometimes meiotically, heritable. Numerous studies have demonstrated that both genetic and epigenetic alterations collaborate in cancer development [1]. Epigenetic changes in gene expression may occur via changes in the folding of DNA to form chromatin and the architecture of that chromatin within the nucleus [2]. The nucleosome is the fundamental unit of eukaryotic chromatin and consists of DNA wrapped around an octamer of core histone proteins. Histones H2A, H2B, H3 and H4 form the nucleosome core complex whereas histone H1 links the two nucleosomes [3]. Specific amino acids within the histone tails are the sites of a variety of post-translational modifications responsible for the dynamic changes in the chromatin structure and recognized to play a pivotal role in the regulation of gene expression [4]. These modifications mainly

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Abbreviations: CDK, cyclin-dependent kinase; **ER,** estrogen receptor; **HATs,** histone acetyltransferases; **HDACs,** histone deacetylases; **INHAT,** HAT inhibitor; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; **PCAF,** P300/CBP-associated factor; **RB,** retinoblastoma protein; **TBS-1T,** Tris-buffered saline 1% Tween 20

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include acetylation, methylation, phosphorylation, ADP-ribosylation and ubiquitination.

Acetylation has been considered one of the most important epigenetic modifications determining the structure, function and intracellular localization in cells playing important roles in signal transduction pathways [5]. This process, carried out by histone acetyltransferases (HATs) (e.g. p300, cAMP-response element-binding proteina (CBP) and P300/CBP-associated factor (PCAF) in mammalian cells), which transfer acetyl group to lysine residues in N-terminal tails of histones, is counteracted by the activity of histone deacetylases (HDACs). Acetylation of specific lysine residues in histones is generally linked to chromatin disruption and the transcriptional activation of genes [6]. Generally, histone acetylation catalyzed by HATs is associated with an open chromatin environment that permits access of transcription factors to DNA, whereas histone deacetylation catalyzed by HDACs results in chromatin condensation and transcriptional repression [7]. Mistargeted and deregulated HAT activities in cellular signaling can lead to human disease, including breast, colorectal and pancreatic cancers [8–10]. Thus, small chemical inhibitors of these enzymes represent novel candidates for drug development. To date, a limited number of HAT inhibitors have been described. Some of them, such as anacardic acid, garcinol, curcumin and gallic acid, are derived from natural sources and, interestingly, have also been shown to prevent growth of cancer cells [11–14]. Therefore, the development of specific HAT inhibitors would help to elucidate molecular mechanisms of the action and to design new agents with chemotherapeutic potential.

Lunasin is a 43 amino acid peptide, whose sequence is SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDD [15]. Initially identified in soybean in our laboratory, lunasin has also been isolated and characterized by other research groups in barley, wheat, amaranth and other plants [16]. Lunasin's cancer preventive properties have been demonstrated in mammalian cells against chemical carcinogens and viral oncogenes [17, 18]. First animal experiments have shown that this peptide reduces tumor incidence and multiplicity in a skin cancer mouse model [17], and also exerts preventive properties against breast cancer mouse models [19, 20]. Lunasin also acts as a potent anti-oxidant and exerts anti-inflammatory properties [21]. Recently, our previous studies have shown that lunasin inhibits MDA-MB-231 cell proliferation and induces apoptosis, affecting expression of different genes involved in cell cycle, apoptosis and signal transduction [22]. Moreover, studies carried out by Jeong et al. [23, 24] have demonstrated the potential of lunasin to inhibit HAT enzyme activity, histones H3 and H4 acetylation and retinoblastoma protein (RB) phosphorylation. However, the precise mechanism of lunasin as a HAT inhibitor (INHAT) in breast cancer cells is poorly understood.

The main objective of this work is to study lunasin's capacity to inhibit acetylation on different lysine residues of histones H3 and H4 sensitive to acetylation, carrying out analysis of structure–activity relationship with different lunasin fragments. Moreover, lunasin's effect on histone acetylation and protein biomarker levels implicated in carcinogenesis process of breast cancer cells was also evaluated in this study.

2 Materials and methods

2.1 Materials

Lunasin: SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDD, and lunasin fragments: P1: SKWQHQQDSCRKQLQGVNLTPC; P2: EKHIMEKIQGRG; P3: EKHIMEKIQGRGDDDDDDDDD and P4: DDDDDDDDD were synthesized by Chengdu KaiJie Bio-Pharmaceutical (Chengdu, P. R. China). The peptide pp32 corresponding to fragment f(150–180) of the INHAT complex [25] was synthesized by American Peptide American (Sunnyvale, CA, USA). The purity of these peptides was higher than 95%.

Histones H3 and H4 were purchased from Roche Applied Science (Indianapolis, IN, USA). HAT enzymes PCAF, p300 (HAT domain), HAT assay buffer and Acetyl-CoA were obtained from Millipore (Billerica, MA, USA).

2.2 HAT inhibitory activity assay by Western blot analysis

Analysis of HAT inhibitory activity was carried out following the method described by Kuninger et al. [26] with some modifications. Acetylation reactions were composed of histones H3 or H4 (1 μg) in 150 μL HAT assay buffer, containing 20 μM acetyl-CoA, HAT enzymes PCAF or p300 (1 μg) and synthetic peptides (pp32, lunasin or lunasin-peptides P1–P4 dissolved in sterile water) at concentrations ranged from 0.5 to 80 μM. Reactions were incubated at 30°C for 1 h, followed by addition of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA, USA, ratio 1:1 v/v) and boiling for 5 min. Proteins were loaded onto 18% Tris-HCl ready gels (Bio-Rad) and run in Mini Protean-2 Cells (Bio-Rad) using Tris-glycine as the running buffer and 200 V constant for 40 min as running conditions. The proteins on SDS-PAGE gel were transblotted to the Immun-Blot PVDF membrane (Bio-Rad) for 60 min at 100 V and 4°C. Upon completion of transfer, the nonspecific binding sites were blocked by 5% nonfat dry milk dissolved in Tris-buffered saline 1% Tween 20 (TBS-1T) for 1 h. The membrane was washed with TBS-1T and then incubated with specific primary antibodies, including anti-acetyl-H3(Lys 9) (1:250, Millipore), anti-acetyl-H3(Lys 14) (1:1000, Millipore), anti-acetyl-H3(Lys 9, 14) (1:3000, Millipore), anti-acetyl-H4(Lys 8) (1:1000, Millipore), anti-acetyl-H4(Lys 12) (1:500, Millipore) and anti-acetyl-H4(Lys 5, 8, 12, 16) (1:500, Millipore) for 1 h

at room temperature. After washing, the membrane was incubated for 1 h with an anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:2000 dilution in 3% nonfat dry milk in TBS-1T. The intensity of proteins was detected using the detection agent (Amersham Biosciences, Piscataway, NJ, USA) and immediately developed using 667 Polaroid films. The intensities of the bands were quantified using the software Un-SCAN-IT gel version 5.1 (Silk Scientific, Orem, UT, USA). Concentration of acetylated histone in a specific position was expressed as percentage of control, considered as 100%. The results were expressed as IC_{50} value or peptide concentration needed to inhibit 50% histone acetylation. Acetylation reactions were carried out by duplicate and two analyses of each reaction were performed. All data were analyzed from these two independent experiments. The results were expressed as the mean $\pm$ SD.

2.3 Cell culture

Human estrogen-independent MDA-MB-231 breast cancer cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). MDA-MB-231 cell were grown in Leibovitz's L-15 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA), in a humidified atmosphere at 37°C.

2.4 Cell proliferation assay

MDA-MB-231 cell cytotoxicity was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma Chemical, Saint Louis, MI, USA) assay. Cells were plated at a density of 2×10^4 cells/well in 96-well plate overnight, and then treated with various concentrations of lunasin or lunasin-related peptides for 48 h. At the end of treatment, 50 μL/well of MTT solution (0.5 mg/mL) was added and the plate was incubated for 3 h at 37°C. The supernatant was aspirated and formazan crystals formed were solubilized in 100 μL DMSO (Sigma) for 30 min. The absorbance at 570 nm was measured using a Multiskan Plus microplate reader (Thermo Electron Corporation, Waltham, MA, USA). The results were expressed as percentage of the control, considered as 100%. All data were analyzed from three independent experiments. The results were expressed as the mean $\pm$ SD.

2.5 Analysis of histone acetylation in human breast cancer MDA-MB-231 cells

Cells were plated at a density of 2×10^6 cells/flask overnight and then, treated with lunasin or lunasin-related peptides at concentrations of 3, 15 and 75 μM for 48 h. During the last 24 h peptide treatment, cells were treated without or with HDACs inhibitor sodium (Na)-butyrate (5 mM). At the end of experiment, cells were collected by trypsinization and washed with PBS. Histones extraction was carried out resuspending cells in Triton extraction buffer (TEB:PBS containing 0.5% Triton X 100 v/v, 2 mM PMSF, 0.02% w/v NaN_3) for 30 min on ice with gentle stirring. After centrifuging at $2000 \times g$ for 10 min, the supernatant was discarded and the cell pellet was resuspended in 0.2 N HCl to extract the histones overnight at 4°C. Samples were centrifuged and supernatant was collected. Protein content was analyzed by Bradford assay using BSA as the standard protein. Proteins were prepared and analyzed by SDS-PAGE and Western blot, using the protocol and specific primary antibodies against acetylated histones described above. Data were analyzed from two independent experiments.

2.6 Preparation of whole cell lysates and analysis by Western blot

Cells were plated at a density of 2×10^6 cells/flask overnight and then, treated with lunasin or related peptides at concentrations of 3, 15, and 75 μM for 48 h. After treatment, cells were harvested by trypsinization and washed with PBS. Total proteins were prepared using PRO-PREP™ protein extract solution (iNtRON, Seongnam, Korea) according to the manufacturer's instructions. Proteins were quantified by Bradford assay using BSA as the standard protein, and then, added to Laemmli sample buffer (Bio-Rad) at a ratio 1:1, and boiled for 5 min. Aliquots of 100 μg of proteins were loaded onto 15% Tris-HCl gels (Bio-Rad), run and transblotted following the same conditions described above. Upon completion of transfer, the nonspecific binding sites were blocked by immersing the membrane in the Odissey Blocking buffer (Li-Cor Biosciences, Lincoln, NE, USA) for 1 h. The membrane was washed with fresh changes of PBS-Tween 20 and incubated with specific primary antibodies, including actin (1:500, Cell Signaling Technology, Danvers, MA, USA), p15 (1:1000, Cell Signaling), p16 (1:1000, Cell Signaling), p21 (1:500, Cell Signaling), p27 Kip 1 (1:1000, Cell Signaling), cyclin D1 (1:2000, Cell Signaling), cyclin D3 (1:1000, Cell Signaling), cyclin-dependent kinase (CDK)4 (1:400, Cell Signaling) and CDK6 (1:2000, Cell Signaling) overnight at 4°C. After washing with PBS-Tween 20, the membrane was incubated with an anti-mouse or anti-rabbit IRDyE® secondary antibody (Li-Cor Biosciences) at 1:5000 dilution in Odissey blocking buffer-Tween 20 for 1 h. After washing the membrane four times with PBS-1T, and once with PBS, it was developed using the Odyssey® Infrared Imaging System (Li-Cor Biosciences). Data were analyzed from two independent experiments.

3 Results

3.1 Lunasin inhibits in vitro histones H3 and H4 acetylation

The effect of lunasin and lunasin-peptides on histones H3 and H4 acetylation was evaluated using antibodies specific for acetylated histones at different lysine positions. This method permits analysis of far smaller amounts of material, also facilitating investigation of the functional significance of the different sites. Peptides were added to acetylation reaction containing PCAF enzyme to acetylate histone H4 or p300 to acetylate histone H3. As an example, Fig. 1 shows Western blot results for lunasin, lunasin fragments and pp32, using PCAF as a HAT enzyme, and anti-acetyl-H4-Lys 5, 8, 12 and 16 as specific antibody. Lunasin, lunasin peptides and pp32 concentrations were added at concentrations ranged from 0.5 to 80 μM, and the IC_{50} value or peptide concentration needed to inhibit 50% HAT activity was calculated (Table 1). HAT inhibitory activity of peptide pp32, corresponding to fragment f(150–180) of the cellular INHAT complex, has been demonstrated by Seo et al. [25]. Thus, we used pp32 peptide as a positive control to compare the results obtained with lunasin and its derived fragments. As shown in Table 1, pp32 inhibited histone H4 acetylation by PCAF at all lysine positions studied. The IC_{50} values were 1.83 μM (H4-Lys 8), 1.34 μM (H4-Lys 12) and 1.23 μM (H4-Lys 5, 8, 12, 16). To date, only the p300 inhibitory activity of pp32 had been studied, and IC_{50} values ranged from 0.6 to 1.5 μM had been reported for histones H2A, H2B, H3 and H4 acetylation [25, 27]. In our study, lunasin has been also found to inhibit PCAF enzyme, with IC_{50} values of 0.83 μM (H4-Lys 8), 1.27 μM (H4-Lys 12) and 0.40 μM (H4-Lys 5, 8, 12, 16). No significant differences were observed when the effect was compared with that observed for pp32. The IC_{50} values corresponding to H4 acetylated in four Lys positions (5, 8, 12 and 16) were two and three times lower than those corresponding to H4 acetylated in Lys 8 and 12, respectively. These differences clearly demonstrated that lunasin acts preferentially on some Lys residues of this protein leading to their acetylation.

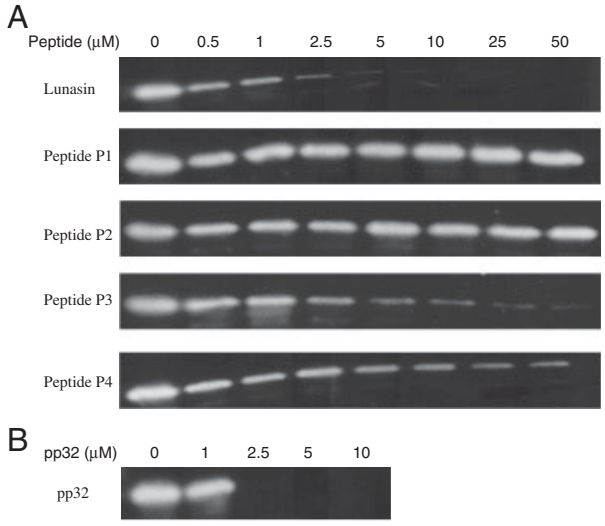

Figure 1. Inhibitory effect of lunasin and lunasin fragments on histone H4 acetylation by PCAF. (A) Histone H4 (1 μg) was incubated with PCAF (1 μg), acetyl-CoA (20 μM) and synthetic lunasin, lunasin fragments (concentrations ranged from 0 to 50 μM) or (B) pp32 (concentrations ranged from 0 to 10 μM) for 1 h. Proteins were separated by 18% SDS-PAGE and then subjected to Western blot analysis using a specific primary antibody against histone H4 acetylated on lysine positions 5, 8, 12 and 16. Acetylation reaction was carried out by duplicate and two analysis of each reaction were performed. All data were analyzed from these two independent experiments.

In order to elucidate structure–activity relationship, four fragments contained in lunasin's sequence and named as P1–P4 were synthesized and analyzed for their function as PCAF inhibitors. Low activity was found to be exerted by peptides P1 and P2. Peptides P3 and P4 mainly inhibited H4 acetylation at Lys-12 (IC_{50} values of 3.65 and 1.63 μM for P3 and P4, respectively), and Lys-5, 8, 12, 16 (IC_{50} values of 3.14 and 0.91 μM for P3 and P4, respectively), indicating their contribution to lunasin's effect. However, lunasin's inhibitory effect on histone H4 at Lys-8 was significantly higher than that shown by peptides P3 and P4. These peptides correspond to 21-amino acid sequence (P3) and poly-D sequence (P4) localized at N-terminus of lunasin. Among all peptides analyzed, lunasin demonstrated to be the most potent, indicating the both amino acid sequence and length of this peptide were determinants for its activity preventing H4 acetylation by PCAF.

The activity of lunasin, lunasin fragments and pp32 as inhibitors of histone H3 acetylation by p300 at Lys-9, Lys-14 and Lys-9, 14 positions were further analyzed. As shown in Table 1, no effect was observed for Lys-14. Lunasin inhibited H3 acetylation at Lys-9 (IC_{50} of 5.91 μM) and Lys-9, 14 (IC_{50} of 7.81 μM). This activity was lower than that observed for pp32, whose IC_{50} values were 0.67 μM (H3-Lys 9) and 0.78 μM (H3-Lys 9, 14), although no statistical differences were observed. Peptide P4, corresponding to poly-D sequence of lunasin, showed lower IC_{50} values (2.94 μM for H3-Lys 9 and 0.40 μM for H3-Lys 9, 14) than those calculated for lunasin. This indicates that a reduction of lunasin's length might contribute positively on its capacity to bind H3 histones and inhibit its acetylation.

3.2 Lunasin inhibits histones H3 and H4 acetylation in MDA-MB-231 cells

Lunasin inhibits both PACF and p300 enzyme activity; therefore, we analyzed the effect of this peptide on histone acetylation in human breast cancer MDA-MB-231 cells. These results would allow us to evaluate whether lunasin acts as an epigenetic modulator of MDA-MB-231 cell growth. Figure 2 shows the inhibition of histones H3 and

Table 1. HATs enzymes (PCAF or p300) inhibitory activity, expressed as IC_{50} (mean ± SD, $n = 4$), of lunasin, lunasin-peptides P1–P4 and pp32

Peptide	IC_{50} value (μM)					
	Acetyl-H4 (acetylated by PCAF)			Acetyl-H3 (acetylated by p300)		
	Lys(8)	Lys(12)	Lys(5, 8, 12, 16)	Lys(9)	Lys(14)	Lys(9, 14)
Lunasin[a)]	0.83 ± 0.06	1.27 ± 0.08	0.40 ± 0.15	5.91 ± 1.48	n.d.[b)]	7.81 ± 0.40
Peptide P1[c)]	n.d.[b)]	77.28 ± 8.63[g)]	74.57 ± 0.61[g)]	n.d.[b)]	n.d.[b)]	n.d.[b)]
Peptide P2[d)]	n.d.[b)]	45.65 ± 0.81[g)]	n.d.[b)]	n.d.[b)]	n.d.[b)]	n.d.[b)]
Peptide P3[e)]	52.79 ± 0.86[g)]	3.65 ± 0.69	3.14 ± 0.18	n.d.[b)]	n.d.[b)]	2.82 ± 0.27[g)]
Peptide P4[f)]	4.55 ± 0.42[g)]	1.63 ± 0.21	0.91 ± 0.25	2.94 ± 0.48	n.d.[b)]	0.40 ± 0.13[g)]
pp32	1.83 ± 0.16	1.34 ± 0.08	1.23 ± 0.01	0.67 ± 0.12	n.d.[b)]	0.78 ± 0.18[g)]

a) Lunasin: SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDD.
b) n.d. histone acetylation inhibitory activity not detected at higher concentration used in the experiment (80 μM).
c) Peptide 1: SKWQHQQDSCRKQLQGVNLTPC.
d) Peptide 2: EKHIMEKIQ.
e) Peptide 3: EKHIMEKIQGRGDDDDDDDD.
f) Peptide 4: DDDDDDDDD.
g) Differences among peptides were analyzed by Student's *t*-test, ($^*p < 0.05$ versus Lunasin treatment).

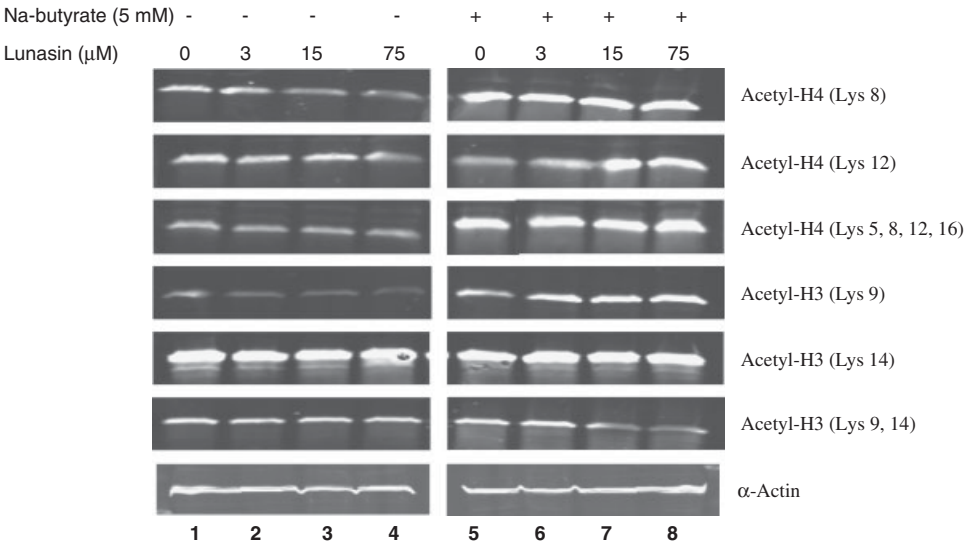

Figure 2. Lunasin's inhibitory effect on HAT activity in human breast cancer MDA-MB-231 cells. Cells were treated with various concentrations of lunasin for 48 h, histones were acid extracted and subjected to Western blot analysis using the indicated antibodies against histones H3 and H4 acetylated on different lysine positions. Electrophoresis pattern of histones extracted from untreated cells without (lane 1) and with sodium butyrate (lane 5); treated cells with 3 μM lunasin without (lane 2) and with (lane 6) sodium butyrate; treated cells with 15 μM lunasin without (lane 3) and with (lane 7) sodium butyrate; treated cells with 75 μM lunasin without (lane 4) and with (lane 8) sodium butyrate. Na-butyrate was added at a final concentration of 5 mM. Each well contains 100 μg of protein. Data were analyzed from two independent experiments.

H4 acetylation at different lysine positions in breast cancer cells after 48 h treatment with lunasin, with and without treatment with Na-butyrate, a well-known HDAC inhibitor. First, we observed that even without the addition of Na-butyrate and lunasin (negative control), acetylated H3 and H4 at all lysine positions analyzed were present in the nuclei of breast cancer cells. This result indicates that histone acetylation is involved in breast carcinogenesis and its inhibition might be involved in cell growth suppression. A dose-dependent inhibitory effect on H4 acetylation at positions H4-Lys 8 and H4-Lys 12 was observed after addition of lunasin to the culture medium. This inhibition reached 17 and 19% for both positions, respectively, when lunasin was treated at 75 μM. Treatment with lunasin at all tested concentrations produced 10% inhibition of H4 acetylation at positions Lys 5, 8, 12, 16 and 12 and 8% inhibition of H3 acetylation at positions Lys 9 and Lys 14, respectively. Moreover, no effect was observed for histone H3 acetylated at Lys 9, 14 (Fig. 2).

Second, when treated with Na-butyrate alone (positive control), a notable increase in acetylation level was detected for positions Lys-8, and Lys-5, 8, 12, 16 of histone H4, and positions Lys-9 and Lys-9, 14 of histone H3. This increase ranged from 16.6 to 59.2%. However, no stimulation of acetylation as H4-Lys 12 and H3-Lys 14 was observed after treating breast cancer cells with Na-butyrate, indicating that a maximum level of acetylation was reached in non-treated cells. Treatment with lunasin only inhibited H3 acetylation in positions Lys-9, 14 (30.2% inhibition at 75 μM lunasin). A slight increase in levels of acetylated histones in positions H4-Lys 12 and H3-Lys 9 is observed (Fig. 2). No effects were visible for the rest of positions evaluated, indicating that the effects on histones are dependent on lysine positions sensitive to epigenetic modification.

3.3 Lunasin and lunasin peptides inhibit breast cancer MDA-MB-231 cell proliferation

The present study shows that lunasin inhibits MDA-MB-231 cells H4 and H3 acetylation. To investigate whether this effect may contribute to the cell proliferation inhibitory properties, the cytotoxic effect of lunasin and lunasin peptides on breast cancer MDA-MB-231 cells was analyzed. Previous published studies had not observed any cytotoxic effect of this peptide on normal mammalian cells [17]. As shown in Fig. 3, lunasin and peptides P1, P3 and P4 reduced the number of viable cells in a dose-dependent manner, being statistically significant at the lowest concentration (10 μM) used in our study ($p<0.05$ for lunasin, and peptides P1 and P4; $p<0.005$ for peptide P3). The IC_{50} value, expressed as the peptide concentration needed to inhibit 50% cell number, was calculated. The IC_{50} value of lunasin was 181 μM. Higher values were obtained for P1 (323 μM) and P4 (218 μM). However, peptides P2 and P3 were more potent than lunasin, with IC_{50} values of 175 and 138 μM, respectively. These results suggest that mainly the 21-amino acid sequence localized at the N-terminus of lunasin is responsible for lunasin's inhibitory effect on breast cancer cell growth. Lunasin and peptide P3 showed inhibitory activity of histone acetylation that might be responsible of their inhibitory activity of breast cancer cells proliferation. However, peptide P2 did not show effect on histone acetylation, indicating that other molecular mechanism of action might be involved in cell proliferation inhibitory activity of lunasin and lunasin-related fragments.

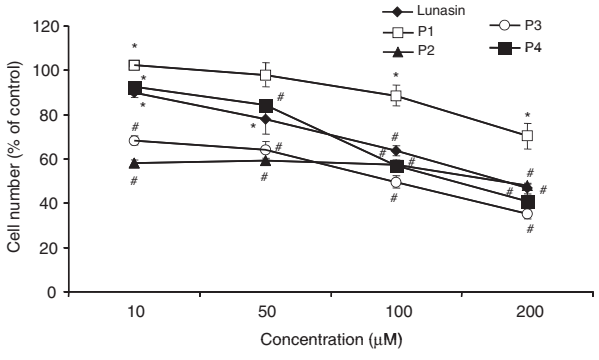

Figure 3. Lunasin and lunasin fragments inhibit human breast cancer MDA-MB-231 cells proliferation. Cells were treated with lunasin or lunasin peptides (P1– P4) (10–200 μM), for 48 h and cell proliferation was determined by MTT assay. Cell number in treated cells was expressed as percentage of that in control cells. (*$p<0.05$, ♯ $p<0.005$ versus vehicle-treated cells).

3.4 Effect of lunasin on cell cycle biomarkers

It is known that cell cycle progression in eukaryotic cells is governed by interactions between cyclins, CDKs and CDK inhibitors [28]. To determine whether lunasin-induced breast cancer cell growth inhibition was produced through cell cycle regulation, effect of this peptide on the expression of different cell cycle regulators was examined. As shown in Fig. 4, after 48 h treatment with lunasin, the levels of cyclin D1, cyclin D3, CDK4 and CDK6 expression were dose dependently down-regulated in MDA-MB-231 cells. However, no effects were observed for CDK inhibitors p15, p16, $p21^{Waf1}$ and $p27^{Kip1}$.

4 Discussion

Epigenetic modifications of chromatin and DNA have been recognized as important factors in controlling the expressed genome. Much of today's epigenetic research is converging on the study of covalent or noncovalent modifications of DNA and histone proteins [2]. Acetylation is considered one of the most important epigenetic modifications acting on signal transduction pathways including those involved in cancer development [5, 29]. HATs play crucial roles in the regulation of gene expression and have been found to be essential for normal cell proliferation, growth and differentiation [30]. Mistargeted and deregulated HAT activities of GCN5/PCAF and p300 as well as their over-expression have been reported to play an important role in genetic diseases and in human colorectal, breast and pancreatic cancer [31, 32]. In the recent years, considerable efforts have been put to develop HAT modulators either for mechanistic studies or for anti-cancer values.

The results of the present study demonstrate that lunasin is a potent in vitro inhibitor of PCAF and p300 HATs' activity. Lunasin's activity is comparable to peptide pp32 that is included in a complex playing an important function in regulation of chromatin remodeling, transcription, transformation and apoptosis [25, 27]. Despite the efforts on searching naturally occurring INHATs, only few of them has been characterized [33]. Anacardic acid derived from cashew nut shell liquid showed IC_{50} values of 5 and 8.5 μM

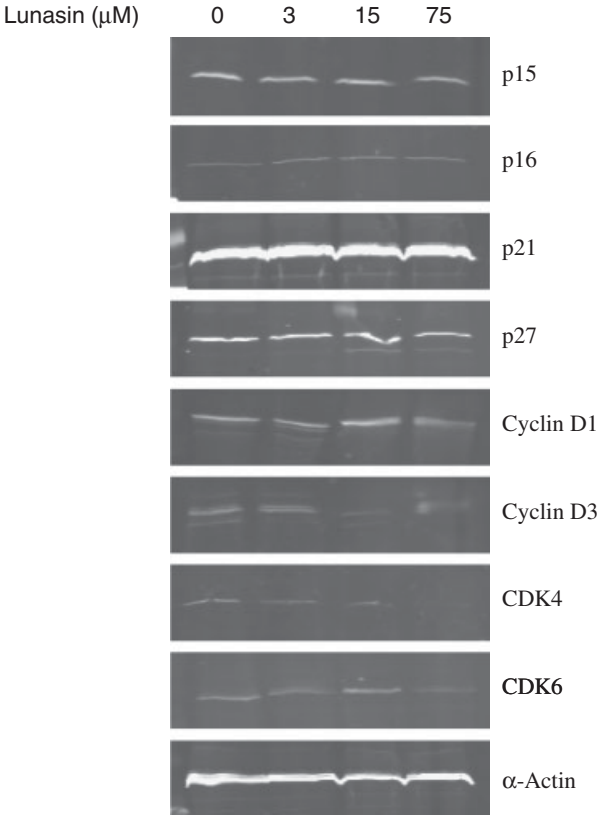

Figure 4. Effects of lunasin on the expression level of $p15^{Ink4B}$, $p16^{Ink4A}$, $p21^{Waf1/Cip1}$, $p27^{Kip1}$, cyclin D3, CDK4 and CDK6 in human breast cancer MDA-MB-231 cells. Cells were treated with indicated concentrations of lunasin for 48 h. Cell extracts were separated by 18% SDS-PAGE and immunoblotted with specific primary antibodies. The immunoblots are representative of two independent experiments with similar results.

when PCAF and p300 were used as HAT enzymes, respectively, to acetylate histones [11]. More recently, these authors discovered that garcinol, derived from *Garcinia indica* fruit, was a potent inhibitor of PCAF (IC_{50} of 5 μM) and p300 (IC_{50} of 7 μM) [12]. Curcumin also has been reported to inhibit HAT activities with an IC_{50} of 25 μM [13]. The HAT inhibitory activity of these compounds has been described as crucial for their anti-cancer properties. Our results demonstrate that lunasin is more potent than these compounds, suggesting a promising role of this peptide as a cancer-preventive agent.

Previous studies have reported a 75% reduction of H4 acetylation by PCAF when 1 μM lunasin was added to reaction medium [23]. However, these authors did not analyze lunasin's inhibitory effect on different lysine positions sensitive to acetylation. The results derived from the structure–activity relationship analysis show that both sequence and length of lunasin is essential for inhibiting histone H4 acetylation whereas poly-D sequence is active by itself to inhibit histone H3 acetylation. Galvez et al. [17] had observed that lunasin likely binds to deacetylated histone by ionic interaction with its negatively charged poly-D end. These authors also had described that the N-terminus of lunasin including a helical region might play a role in targeting lunasin to deacetylated histones. Our results suggest that lunasin tends mainly to inhibit PCAF activity, due to the fact that the reaction mechanisms for PCAF and p300 to acetylate the lysine residues are contrastingly different [34]. These authors have reported that PCAF employs ternary complex mechanisms that involve the ordered binding and release of substrates and products, whereas p300/CBP family follows the double displacement (ping-pong) mechanisms.

A plethora of chromatin alterations appears to be responsible for the development and progression of various types of cancers, including breast cancer. Although the ''epigenetic'' code is not fully understood, specific marks such as lysine acetylation has been found to be associated with regulation of gene expression, transcription, replication and repair. A global histone modification analysis revealed that in the majority of breast cancers, histone H4 acetylation at position Lys 16 was reduced or absent, suggesting that this alteration may represent an early sign of breast cancer [35]. In addition, moderate to low levels of histone H3 acetylation at positions Lys 9 and Lys 18, and histone H4 acetylation at Lys 12 were observed in breast carcinomas, and they were associated with poor prognosis and clinical outcome [36]. Such changes of global histone modification patterns can be predictive of clinical outcome as recently shown for prostate, lung and gastric cancers [37–39]. Therefore, evaluation of inhibitory effect on specific lysine residues of histones seems to be very promising for searching new therapies against breast cancer. The present study shows that lunasin inhibits MDA-MB-231 cells growth mainly mediated via inhibition of H4 acetylation at positions Lys 8 and Lys 12, and H3 acetylation at position Lys 9. This effect could contribute to the cell proliferation inhibitory properties shown by lunasin. Thus, these findings provide data about lunasin's molecular mechanism of action on epigenetic alterations involved in breast cancers that would be very useful to define new prognostic markers and therapeutic targets.

Up to one-third of breast cancers that are initially estrogen receptor (ER)-positive lose ER expression making tumors resistant to endocrine therapy during tumor progression [40]. Due to this emergence of hormone resistance, it is necessary to search for alternative therapies. Recently, it has been demonstrated that lunasin reduces breast tumor incidence in a MDA-MB-231 xenograft mouse model [19]. These authors have demonstrated that lunasin arrests cell cycle progression in the S-phase, induces cell apoptosis and modulate expression of different genes involved in breast carcinogenesis process [22]. Inhibition of deregulated cell cycle progression in cancer cells is an effective strategy to halt tumor growth. It is well established that cyclins play a positive role in promoting cell cycle

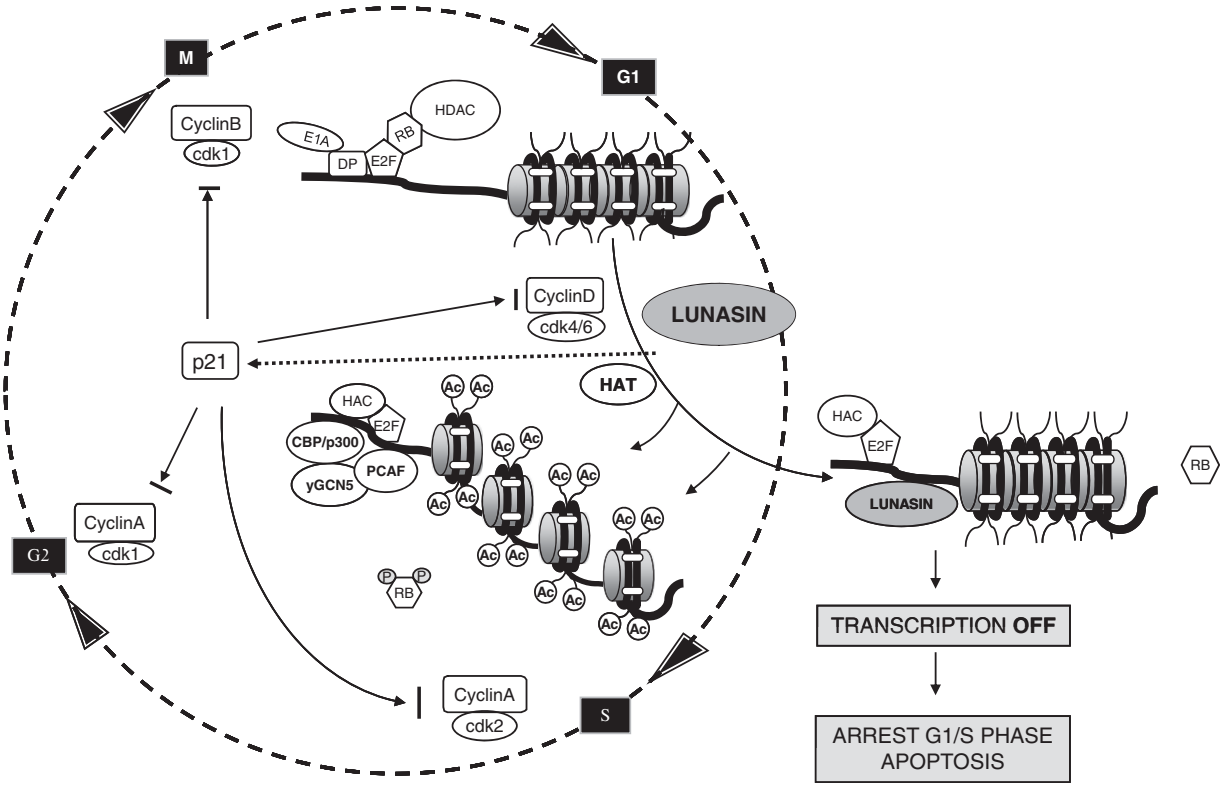

Figure 5. Model proposed to explain the mechanism of action of lunasin as cancer-preventive agent. RB and RB-P represent the unphosphorylated and the phosphorylated forms of RB, respectively. In normal cells, during early G1 phase, RB–E2F complex recruits HDAC keeping the core histones in the deacetylated (repressed) state. E1A inactivates RB by phosphorylation, dissociates the RB–E2F complex, exposing the deacetylated histones to the HATs (PCAF, CBP/p300 and yGCN5). Histone acetylation allows the expression of genes that encodes products needed for S-phase, activating the cell cycle progression. Lunasin competes with the HATs in binding to the deacetylated histones, turning off the transcription that is perceived as abnormal by cells, resulting in arrest G1/S phase and apoptosis. Lunasin also has been demonstrated to up-regulate RB gene and protein expression, and to down-regulate protein expression of cyclins and CDKs involved in G1/S phase transition, thus contributing to cell cycle arrest and apoptosis induction.

transitions via their ability to associate with their cognate CDKs and to activate them [41]. Over-expression of cyclins D1 and D3 is one of the most frequent alterations present in breast tumors [42]. Cyclins D interacts with CDK4 or CDK6 to form a catalytically active complex, which phosphorylates RB to free active E2F [43].

Mechanistic investigation carried out in the present study shows the potential molecular mechanisms of action of lunasin in ER-independent breast cancer MDA-MB-231 cells (Fig. 5). This peptide inhibits histone acetylation through HAT inhibition, thus provoking cell cycle arrest in G1/S phases and apoptosis induction, as previously reported [22]. Up-regulation of RB gene and protein expression [22] as well as inhibition of RB phosphorylation [23] might also contribute to previous effects on breast cancer cell cycle and programmed cell death. Our present study has also demonstrated lunasin's capacity to down-regulate protein expression of several cyclins and CDKs in MDA-MB-231 cells. This action might also affect cell cycle control pathway, especially G1/S phase arrest, inhibiting abnormal cell growth.

Importantly, our finding is the first report on lunasin's global histone acetylation inhibitory properties and its structure–activity relationship in ER-independent human breast cancer MDA-MB-231 cells. This peptide shows to be a potential strategy against breast cancer, although studies focused on determining its activity in humans through its oral administration would be needed. Moreover, further research should be relevant to complete the elucidation of lunasin's mechanism of action in this type of cancer.

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