Original Research Article

Anti-leukemic activity of phosphoproteins from Sesamin *via* induction of nuclear antigen H731and CLIP-associating protein 2 isoform X25 mediated apoptosis

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A b s t r a c t

Leukemia is an uncontrolled proliferation hematopoietic cancer cells that commonly treats with conventional therapies such as chemotherapy. However, many side effects were reported. Alternative medicines have been developed by using natural or herb compounds as therapeutic drug. Sesamin, a class of phytoestrogen isolated from sesame seed displaying potent anticancer activity in *vitro* and in *vivo*. However, the mechanism by which sesamin mediates anticancer effects on leukemic cells are not fully understood. In this study, the effects of sesamin on cell viability, cell apoptosis and expression of phosphoproteinsin Molt-4 and NB4 leukemic cell lines were investigated using MTT assay, flow cytometry and immobilized metal affinity chromatography (IMAC) phosphoprotein enrichment and LC-MS, respectively. The results showed that sesamin reduced viability and induced apoptosis in leukemic cells. In addition, 79 phosphoproteins were identified from LC-MS within three main clusters including biological regulation, cellular process and metabolic process. Interestingly, nuclear antigen H731 (PDCD4) and CLIP-associating protein 2 isoform X25 (CLASP2)showed increased in sesamin treated cells and associated protein-ligand interaction network with allicin, capsaicin, cucurbitacin B, and rapamycin which are known to activate apoptosis in cancer cells. Then, sesamin could be developed as candidate of alternative leukemia treatment in the future.

Keywords: Leukemia, Sesamin, Apoptosis, Phosphoprotein, Mass spectrometry

Introduction

Leukemia is a cancer that caused by uncontrolled proliferation of the hematopoietic cells in bone marrow, especially in leukocyte cells [1], [2]. According to French American British (FAB) and the World Health Organization (WHO) criteria, leukemia can be classified into four subtypes including acute myeloid leukemia, acute lymphoid leukemia, chronic myeloid leukemia, and chronic lymphoid leukemia based on its lineage and disease progression [3]. Leukemia is a treatable disease which has chemotherapy as medical treatment. However, chemotherapy is still a non-selective treatment that kills both cancer and normal cells, and has many side effects including hair loss, skin rashes, nausea, vomiting, infection, and weakness. Therefore, alternative treatment in term of using natural compounds and herbs have been studied [4].
Sesamin is one of the natural compounds that has been suggested to have various pharmaceutical activities such as antioxidant, anti-hypertension, anti-inflammation, and anti-cancer. Sesamin is a lignan extracted from sesame seeds named as *Sesamumindicum* [5], [6].There are many studies reported about the effect of sesamin

on inhibition of cell proliferation and induction of apoptosis in various types of cancer. For example, sesamin can inhibit cell proliferation of MCF-7 human breast cancer cell line by induction growth arrest at the G1 phase in cell cycle progression [7]. It was also reported to promote cell cycle arrest at G2/M phase and also induced apoptosis of HepG2 human hepatocellular carcinoma cell line through STAT3 signaling pathway [8].
Phosphoproteomictechnology has been developed to characterize protein phosphorylation, including enrichment of low-abundance phosphoproteins or phosphopeptides with immobilized metal affinity chromatography (IMAC), strong cation exchange chromatography (SCX), or the two in combination [9]. It is an alternative method to analyze phosphoproteins which exists on more than 30% of the proteome and regulates a series of biological processes including cell growth, differentiation, proliferation, apoptosis, and even intercellular communication [10], [11].Mass spectrometry-based methods for phosphoproteome analysis facilitates the studying ofsignal transduction pathways involved in diabetes, neurodegenerative diseases, autoimmune diseases and several forms of cancers [9]. The aim of the present investigation was to determine the signaling pathway involved in mediating the anti-cancer effects of sesamin by enrichment of intact

phosphoproteins using immobilized metal-ion affinity chromatography (IMAC) combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Materials and Methods

Compound reagent

Sesamin was purchased from Sigma-Aldrich (St. Louis,MO, USA). DMSO was used to dissolvedsesamin.

Cell culture

Molt-4 (acute lymphocytic leukemia) and NB4 (acute promyelocytic leukemia) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin. Cells were cultured in humidified incubators at 37∘C and 5% CO_2. Medium of cultured cell was changed every 3 days. Molt-4 and NB4 (5 x 10^5µg/ml) were treated with 100 µg/ml of sesamin, respectively,for 15, 30, 60 min, 12, 24, and 48 h. Untreated cell were used as control.

Cell viability assay

The leukemic cell lines ($1x10^4$cells/ml) were plated in 96-well plate and treated with sesamin at concentrations of 0, 50, 100, and 200 µg/ml and incubated for 24 and 48 hours at 37 °C with 5 % CO_2. Untreated cells (medium without sesamin) were used as a control. The viability of cells was determined by MTT assay which is a colorimetric assay for measuring the activity of mitochondrial dehydrogenase in viable cells that reduce yellow tetrazolium salt, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)) to its blue soluble formazan dyes. The absorbance of each well was measured by spectrophotometer at 570 nm single wavelength and then calculated for the percentage of cell viability. The inhibition concentration (IC_{50}) was calculated by linear regression analysis. Cell apoptosis assay by Annexin V– FITC and propidium iodide staining. The leukemic cell lines (1 x 10^5 cells/ml) were treated with sesamin at IC_{50} values of 100 and 120 µg/ml in Molt-4 and NB4, respectively, in 24-well plate and incubated for 48 h. Untreated cells (medium without sesamin) were used as a control. After incubation, cells were transferred into microcentrifuge tube and centrifuged at 12,000 rpm for 5 minutes. The supernatant was removed and resuspended pellet with PBS 1 ml and then centrifuged again at 12,000 rpm for 5 minutes for cell washing.Accordingly, the cells were added with Annexin V binding buffer 200 µl and transferred into flow cytometry tubes. Then, the cells were incubated with Annexin V- FITC (2 µl) and PropidiumIodide (PI) (5 µl) at 4 °C in the dark place for another 15

minutes. The percentage of apoptotic cell was analyzed by FACSCantoII flow cytometry (BD Biosciences, USA).

Phosphoprotein analysis

Phosphoproteomic profiling of Molt-4 (acute lymphocytic leukemia) and NB4 (acute promyelocytic leukemia) before and after sesamin treatment were performed.Following protein isolation and phosphopeptide enrichment, global phosphoproteomic analysis was done by mass spectrometry.Briefly, cells were collected before treatment and at 6 time-points following 100 and 120 µg/ml of sesamin. Cells were washed twice with HEPES andresuspended with 0.5% Sodium Dodecyl Sulphatefollowed by centrifugation at 10,000 g for 20 minutes at 4°C, the supernatant was collected. Lowry protein assay was used for determination of protein concentration in supernatant, bovine serum albumin used as a protein standard[12]. The phosphoproteins were enriched by immobilized metal affinity column (IMAC) from Pierce Phosphoprotein Enrichment Kit (Thermo Scientific). Then, Thermo Scientific Protein Desalting Spin Column Kit (Thermo Scientific) was used to remove salt and small molecules out of phosphoprotein samples. Protein concentration of desalted samples was determined according to Lowry assay[12]. Phosphoproteins were reduced with 10 mMDithiotheitol (DTT), alkylated with 55m MIodoacetamide (IAA) and digested with trypsin (Promega, Germany) for overnight at 37°C. The phosphorpeptidesamples were injected into a NanoAcquity system (Waters Corp., Milford, MA) equipped with a Symmetry C_{18} 5 µm, 180-µm x 20-mm Trap column and a BEH130 C_{18} 1.7 µm, 100-µm x 100-mm analytical reversed phase column (Waters Corp., Milford, MA) and analyzed by using a SYNAPT™ HDMS mass spectrometer (Waters Corp., Manchester, UK) which operated in V mode at 10,000 resolution with positive nanoelectrospray ion mode.

Protein quantitation and identification

DeCyderMS Differential Analysis software (DeCyderMS, GE Healthcare) was used for protein quantitation based on MS signal intensities of each LC-MS analyses. The analyzed MS/MS data from DeCyderMS was searched against UniprotKB database for protein identification using the Mascot software (Matrix Science, London, UK). Database interrogation was: taxonomy (*Homo sapiens*); enzyme (trypsin); fixed modifications (carbamidomethyl); variable modifications (oxidation of methionine residues, phospho ST and phospho Y); mass values (monoisotopic); protein mass (unrestricted); peptide mass tolerance (2 Da); fragment mass tolerance (± 2 Da), peptide charge state (1+, 2+ and 3+) and three missed cleavages. The relative quantitation ratios were displayed as log2 and processed with median normalization for each sample. Different levels of phosphoproteins were analyzed using the significant t-test at p-value 0.05.

Bioinformatic analysis

Protein categorization was analyzed using PANTHER database together with UniprotKB database for biological process, molecular function and classification[13]. Venn diagram was using for show all the possible relations between different set of phosphoprotein sample. The heatmap visualization of all differentially expressed phosphoproteinswas conducted usingMulti-Experiment Viewer (MeV) software[14]. In addition, potential interaction of the identified phosphoproteins was analyzed using public-domain software, the search tool for interacting chemicals known as STITCH 4.0[15].

Results

Cell viability of leukemic cells by MTT assay

The percentage of cell viability after sesamin treatment was measured by MTT assay at 24 h and 48 h which significantly showed the recession with dose- and time-dependent manner for both leukemic cell lines. The IC_{50} values at 48 h for Molt-4 and NB4 cells were 104.84 and 121.00 µg/ml, respectively (Figure 1).However, sesamin had no affect on normal PBMC.

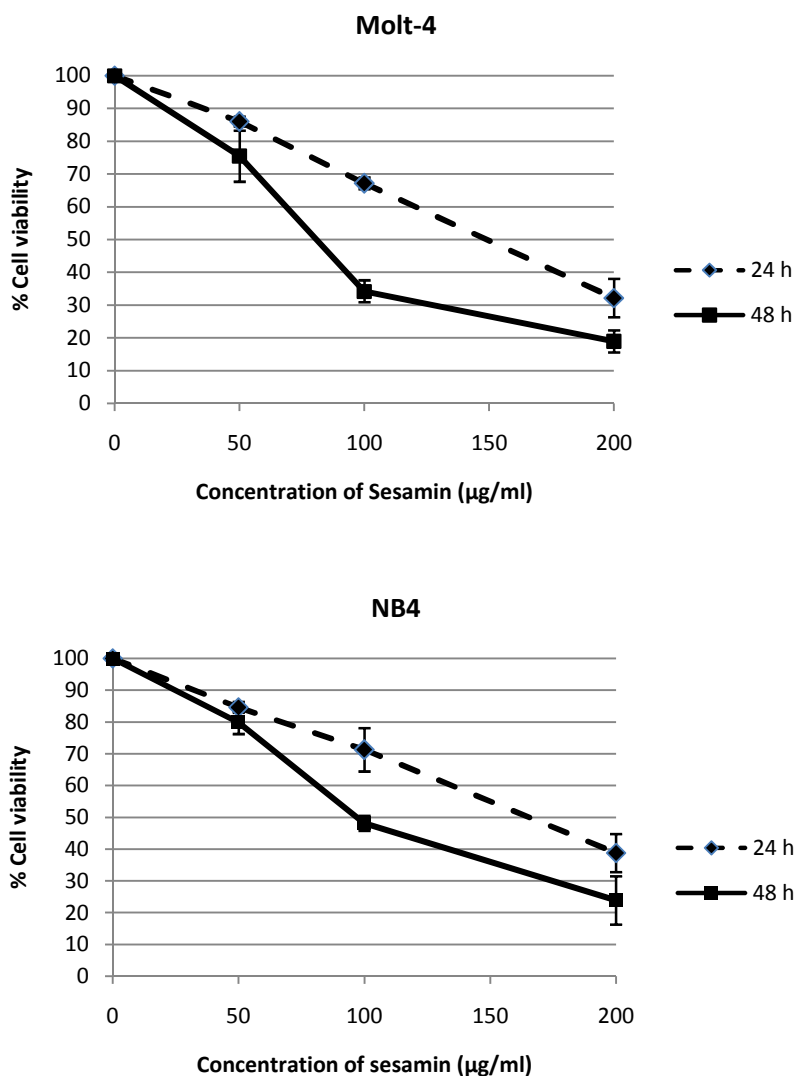**Figure 1** The percentage of cell viability of NB 4 and Molt4 leukemic cells after sesamin treatment for 24 h and 48 h were measured by MTT assay.

Cell apoptosis of leukemic cells by flow cytometry

The apoptotic cells percentageofMolt-4 and NB4 treated by sesamin at IC_{50} concentration value for 48 h were measured by Annexin V-FITC/PI staining and analyzed by flow cytometry. The proportions of Annexin V-positive cells and the percentage of apoptotic cells after 100µg/mland 120 µg/ml sesamin treatments in Molt-4 and NB4 cells, respectively, were significantly increased when compared to the control (Figure 2).
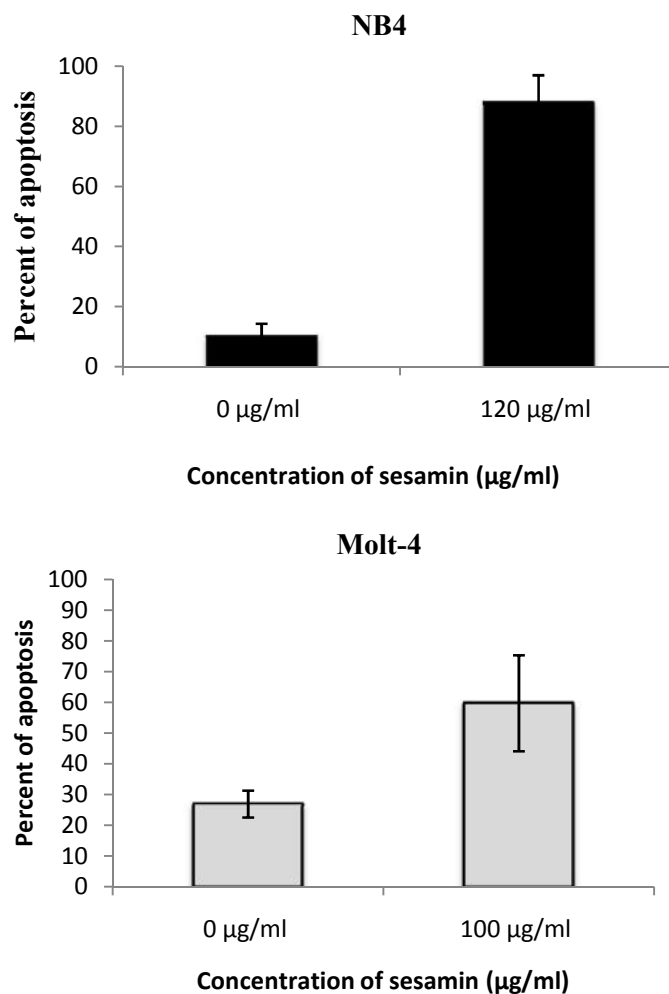
NB4

Molt-4

Figure 2. Percent of apoptosis from Molt-4 and NB4 leukemic cells treatedwith sesamin at IC_{50} concentration for 48 h were measured by Annexin V-FITC/PI staining and analyzed by flow cytometry.

Phosphoprotein profile in Molt-4 and NB4 leukemic cells analyzed by LC-MS

Molt-4 and NB4 leukemic cell lines were treated with sesaminor 15, 30, 60 min, 12, 24, and 48 h while untreated cells were used as control. Then, phosphoproteins were enriched by Pierce phosphoprotein enrichment kit and in-solution digested with trypsin. The phosphopeptides were analyzed by LC-MS/MS. The DecyderMSand Mascot software were used for quantitation and identification of phosphoproteins based on MS signal intensities of each LC-MS analyses.

A total of 79 differential expressed phosphoproteins from sesamin treated Molt-4 and NB4 cells were identified.A two-way hierarchical clustering analysis shown in Figure 3A indicated these proteins were differentially expressed in two cell lines grown in the presence or absence of sesamin.Biological processof 79 identified phosphoproteinswas categorized by PANTHER database together

with UniprotKB database. They were involved in various biological processes biological regulation (26%), cellular process (25%), metabolic process (18%), developmental process (6%), localization (6%), response to stimulus (6%), apoptotic process (4%), cellular component organization or biogenesis (2%), and others (3%)(Figure 3B).
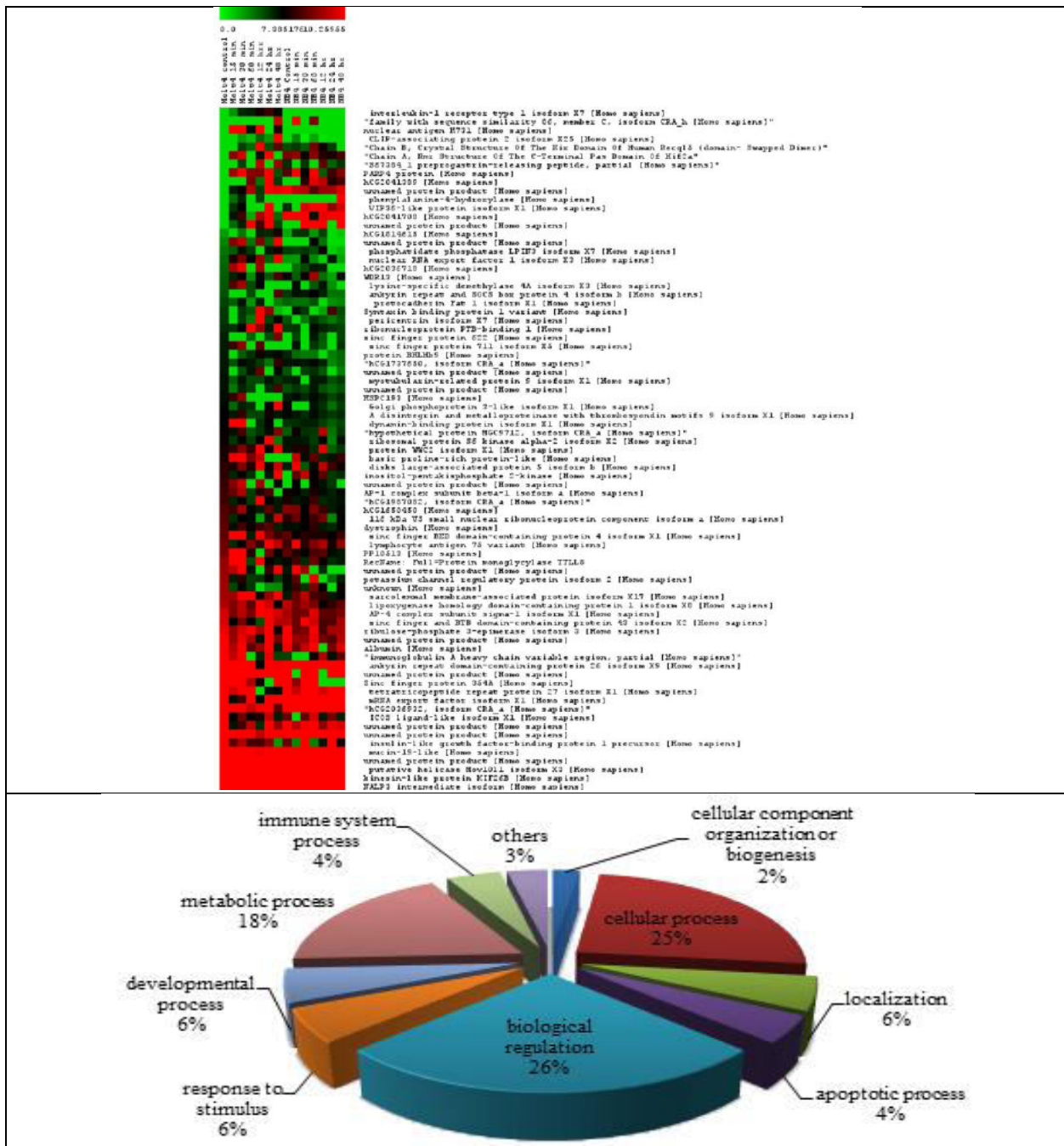

Figure 3. Heatmap and functional annotation of 79 phosphoproteins detected in Molt-4 and NB4 cells after sesamin treatment. (A) represents phosphoprotein level in Molt-4 and NB4 cells treated with sesamin for 15, 30, 60 min, 12, 24, and 48 h compared to control. Green, black and red colors represent proteins with low, average and high levels of expression, respectively. (B) represents function of phosphoproteins in leukemic cells observed after sesamin treatment.

Identification of sesamin responsive phosphoproteins

A full list of 7 phosphoproteins which are detected only in the sesamin treated Molt-4 and NB4 cells is shown in Table 1 and Figure 4. Of particular, interleukin-1 receptor type 1 isoform X7 (IL1R1), family with sequence similarity 86, member C, isoform

CRAh (FAM86A) and nuclear antigen H731 (PDCD4) were detected only in sesamin treated Molt-4 cells. However, CLIP-associating protein 2 isoform X25 (CLASP2), hCG2041389, phenylalanine-4-hydroxylase (PAH), and TRABD protein (TRABD) remained expressed in both treated cells.

Table 1. List of 7 phosphoproteinsdetected only in Molt-4 and NB4 after treatment with sesamin when compare to the untreated cells.

| Protein name	NCBI Accession number	STITCH accession number	Peptide sequence	Function
Interleukin-1 receptor type 1 isoform X7	gil530368626	IL1R1	RSPSSKHQLLSPATK	Signal transduction
Family with sequence similarity 86, member C, isoform CRA_h	gil119595208	FAM86A	AMLDWDQMGSGSSS	Transcription
Nuclear antigen H731	gil1825562	PDCD4	DSGRGDSVSESGSDALRSGLT VPTSPK	Apoptosis
CLIP-associating protein 2 isoform X25	gil578805860	CLASP2	KPGSAGGPK	Signal transduction
hCG2041389	gil119604326	-	TGPEMISNTQSLL	Unknown
Phenylalanine-4-hydroxylase	gil4557819	PAH	RSLPALTNIIK	Amino acid metabolism
TRABD protein	gil15214638	TRABD	AIAAXS	Unknown
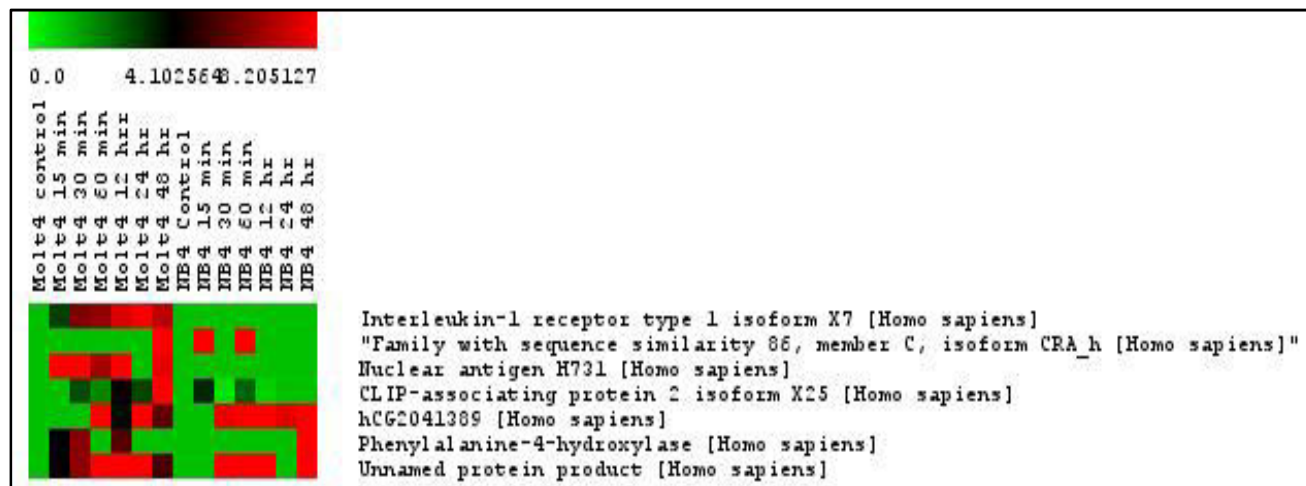
Figure 4.Heatmap of 7 phosphoproteinsdetected only in Molt-4 and NB4 cells after exposure to sesamin for 15, 30, 60 min, 12, 24, and 48 h were subjected to LC-MS.

Interaction between selected phosphoproteins and sesamin

To speculate the potential role of these 7 phosphoproteins in sesamin treated Molt-4 and NB4 cells, possible interaction between them and related proteins or chemical agents were predicted by STITCH version 4.0.The analysis was based on the following parameters: species, *Homo sapiens*; confidence score, medium;

active prediction methods, all and using a list of multiple protein names as input. As shown in Figure 5, thesephosphoproteins show interaction network with an apoptotic protein Caspase 3 (CASP3) and rapamycin. They were also associated withcucurbitacin B, capsaicin, allicinand sesamin.The STITCH analysis suggests that the increase of interleukin-1 receptor type 1 isoform X7 (IL1R1), family with sequence similarity 86, member C,

isoform CRAh (FAM86A), nuclear antigen H731 (PDCD4), CLIP-associating protein 2 isoform X25 (CLASP2), hCG2041389, phenylalanine-4-hydroxylase (PAH), and TRABD protein (TRABD) potentially play roles in theregulation of apoptotic signaling pathways in response tosesamin similar toallicin, capsaicin,cucurbitacin B or rapamycin.
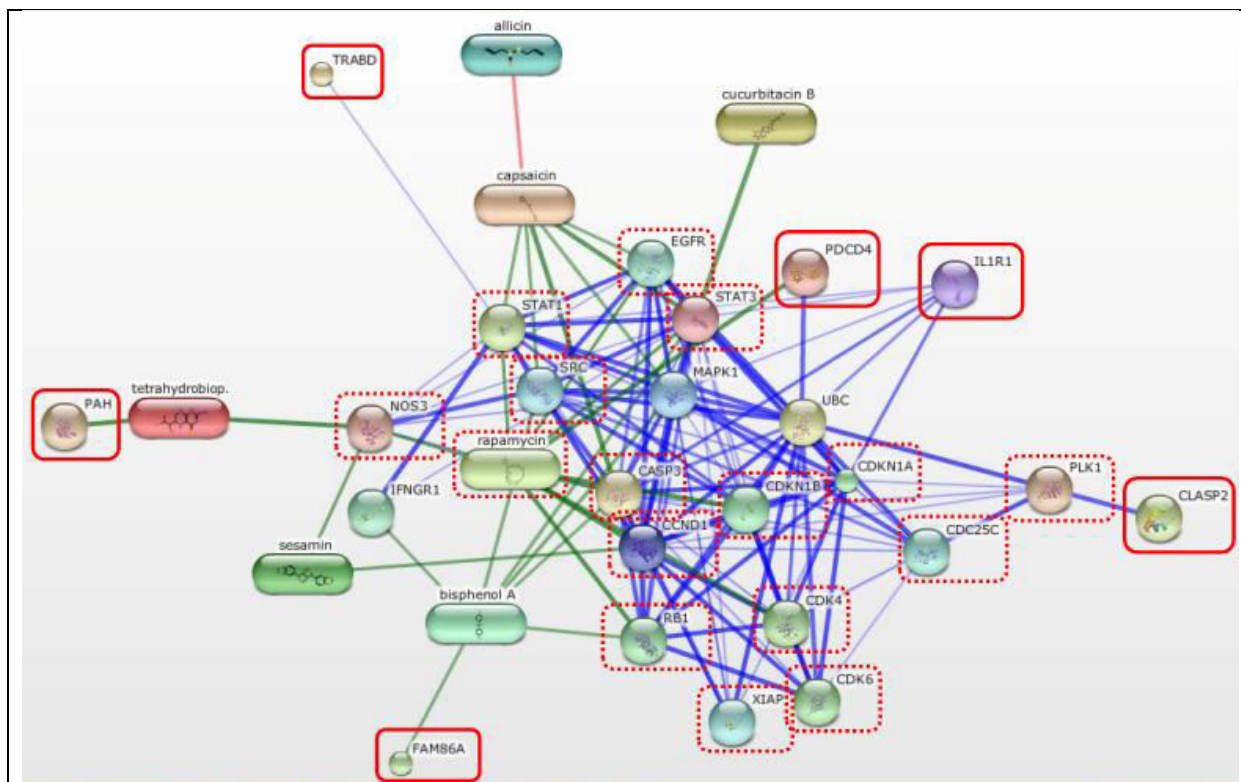

Figure 5.Protein-chemical interaction network of the identified 7phosphoproteins(in bold box) and apoptosis related factor (in bold dotted box) predicted by STITCH Version 4.0 (http://stitch.embl.de).Interleukin-1 receptor type 1 isoform X7 (IL1R1), family with sequence similarity 86, member C, isoform CRAh (FAM86A), nuclear antigen H731 (PDCD4), CLIP-associating protein 2 isoform X25 (CLASP2), hCG2041389, phenylalanine-4-hydroxylase (PAH), and TRABD protein (TRABD) were found to be associated with apoptosis related factors. Stronger associations are represented by the thicker lines. Weak associations are represented by thin lines. Protein-protein interactions are shown in solid lines, chemical-protein interactions in dashed lines.

Discussion

Leukemia is a cancer of hematopoietic cells; the patients mainly receive the treatment with chemotherapy which still caused many side effects. Natural compounds have been approached in alternative treatment and developed for new cancer therapeutics.Sesaminwas found to have various pharmaceutical activities including antioxidant, anti-hypertension, anti-inflammation, and anti-cancer. However, the mechanism of sesamin treated leukemic cells still unclear.Phosphoprotein analysis was then used to investigate the underlying mechanism and its signaling pathway.

A total of 79 phosphoproteins were expressed differentially inboth Molt-4 and NB4 cells after exposure to sesamin. Of particular, 7 out of 79phosphoproteins were found only in the both cells in response tosesamin treatment including interleukin-1 receptor type 1 isoform X7 (IL1R1), family with sequence similarity 86, member C, isoform CRAh (FAM86A), nuclear antigen H731 (PDCD4), CLIP-associating protein 2 isoform X25 (CLASP2), hCG2041389, phenylalanine-4-hydroxylase (PAH), and TRABD protein (TRABD).These phosphoproteins were identified to be associated with various functions including amino acid metabolism, apoptosis,

signal transduction, transcription, all of which potentiallysupport ability ofsesamin to lowersurvival ability of leukemic cells.

Nuclear antigen H731 or Programmed Cell Death 4 (PDCD4) was observed only in sesamin treated Molt-4 cells. Previous study reported that PDCD4might have a potential role in tumor suppressor[16], [17]and plays critical role as a prognostic marker in ovarian cancer[18]. High level of PDCD4 was found in Molt-4 leukemic cells after drug treatment[19]. It is indicated that Molt-4 cancer cells may employ the apoptoticpathways in response to sesamin and lead to cell death.

Rapamycin, an immune suppressant drug, was found to exhibit inhibitory activity on a number of tumor cells[20], [21].In addition, rapamycin shows anticancer activity in primary chronic lymphocytic leukemia cells in vitro[22]. The inhibitory mechanism induced by rapamycin in leukemic cells is modulated by apoptosis mediated signaling pathway[23], [24].Rapamycin was reported to associate with apoptosis via alteration in abundance ofcaspase 3 (CASP3), cyclin D1 (CCND1), cyclin-dependent kinase 4 (CDK4), epidermal growth factor receptor or EGFR, nuclear antigen H731 (PDCD4), retinoblastoma 1 or RB1[25]. Induction of NOS3 could lead to the production of nitric oxide that toxic to the cell and promote cell death through apoptosis process[26]. STAT3 also plays as a target for apoptosis induction in many types of cancer cells since abrogates STAT3 could suppress cancer cells growth and trigger the apoptosis pathway[27], [28].The association between identified phosphoproteins and caspase 3 predicted by STITCH 4.0 demonstrates interaction of 7 phosphoproteins to the apoptotic signaling network in Molt-4 and NB4 cells in response to sesamin treatment. These finding were consistent with these previous findings.

In addition, the predicted results based on network analysis revealed that allicin, capsaicin and cucurbitacin B have interactions with the identified 7 phosphoproteins. Allicin and capsaicin are related to caspase 3 (CASP3), epidermal growth factor receptor (EGFR), v-src sarcoma (SRC), STAT1 and STAT3. Interaction between cucurbitacin B and STAT3 was predicted. Cucurbitacin B is a biochemical compound found in Cucurbitaceae with anti-cancer activity. It inhibited cell proliferation by induced cell cycle arrest and apoptosis [29] through STAT3 and Raf/MEK/ERK pathway in leukemic cells [30]. Capsaicin, a compound from red peppers which belongs to the genus Capsicum, has shown an activity of anti-proliferation in cancer cells. It induces cell cycle arrest and apoptosis via caspase signaling pathway in KB cancer cells[31]. It is also reported to promote apoptosis through the suppression of STAT3 in multiple myeloma cells [32] and NF-kB in prostate cancer cells [33].Moreover, allicin which is a derived-compound from garlic reported to play roles as anti-cancer to inhibit cell proliferation and activate both extrinsic and intrinsic apoptotic pathway in gastric cancer cells [34]. It also investigated to prevent cancer by induce apoptosis via nrf2and NOS [35].

The protein-chemical interaction network to evaluate the anti-cancer mechanisms of sesaminsuggests thatCLIP-associating protein 2 isoform X25 (CLASP2) may be a novel candidate. CLASP2 shows direct interactionwith polo-like kinase 1(PLK1).A network neighborhood of proteins around these 2 proteins showed overlap with proteins involved in apoptosis pathway including cell division cycle 25 homolog C (CDC25C), cyclin D1 (CCND1), cyclin-dependent kinase inhibitor 1A (CDKN1A) and cyclin-dependent kinase inhibitor 1B(CDKN1B).CLASP2 protein is closely related to apoptosis mediated by sesamin treatment.

Conclusion

Correlating similar biological activities between apoptosis induced anticancer drugs and sesamin suggested that sesamin induced apoptosis through 2 phosphoproteins including nuclear antigen H731 (PDCD4) and CLIP-associating protein 2 isoform X25 (CLASP2)as allicin, capsaicin, cucurbitacin B and rapamycin.This finding would be useful for the development of sesamin as an alternative treatment for leukemia in the future. However, further studies are required to be more understanding on itsmechanism.

Acknowledgements

Conflict of interest

Authors declare that they have no conflict of interest.

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