Autophagy and apoptosis induction by sesamin in MOLT-4 and NB4 leukemia cells

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Abstract. Sesamin, the major furofuran lignan found in the seeds of *Sesamum indicum* L., has been investigated for its various medicinal properties. In the present study, the anti-leukemic effects of sesamin and its underlying mechanisms were investigated in MOLT-4 and NB4 acute leukemic cells. Leukemic cells were treated with various concentrations of sesamin. Cell viability was determined using an MTT assay. Flow cytometry using Annexin V-FITC/PI staining and anti-LC3/FITC antibodies was applied to detect the level of apoptosis and autophagy, respectively. Reverse transcription-quantitative PCR was performed to examine the alterations in the mRNA expression of apoptotic and autophagic genes. In addition, bioinformatics tools were used to predict the possible interactions between sesamin and its targets. The results revealed that sesamin inhibited MOLT-4 and NB4 cell proliferation in a dose-dependent manner. In addition, sesamin induced both apoptosis and autophagy. In sesamin-treated cells, the gene expression levels of caspase 3 and unc-51 like autophagy activating kinase 1 (ULK1) were upregulated, while those of mTOR were downregulated compared with in the control. Notably, the protein-chemical interaction network indicated that caspase 3, mTOR and ULK1 were the essential factors involved in the effects of sesamin treatment, as with anticancer agents, such as rapamycin, AZD8055, Torin1 and 2. Overall, the findings of the present study suggested that sesamin inhibited MOLT-4 and NB4 cell proliferation, and induced apoptosis and autophagy through the regulation of caspase 3 and mTOR/ULK1 signaling, respectively.

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Introduction

Leukemia is a hematological malignancy characterized by an increase of abnormal leukocytes in the blood and bone marrow (1). In 2018, a total of 437,000 new cases and 309,000 deaths of leukemia were estimated worldwide (2). Chemotherapy is the main treatment for leukemia for decreasing the excessive number of leukocytes, but the efficacy is limited by its side effects, such as nausea, hair loss and changes in appetite (3). Therefore, complementary and alternative medicine using natural compounds has been used to enhance the efficacy of leukemia treatment (4). Sesamin is the major oil-soluble furofuran lignan abundantly found in sesame seeds and related products (5). Several studies have reported the medicinal properties of sesamin, including antioxidant (6), anti-inflammatory (7), anti-hypertensive (8) and anti-hypercholesterolemic activities (9). The anticancer activity of sesamin has been revealed in a number of types of cancer, such as lung cancer, breast cancer and leukemia (10). Sesamin is able to inhibit the proliferation and induce apoptosis of lung cancer cells via cyclooxygenase (COX)-2 (11). Additionally, it advocates cell cycle arrest by increasing the levels of p53 and checkpoint kinase 1 phosphorylation, and inducing apoptosis via Bax and caspase 3 in MCF-7 cells (12). Moreover, sesamin has been reported to potentiate autophagy; it triggers autophagy through the regulation of EPH receptor (Eph)A1 and EphB2 in colon cancer cells (13). Additionally, it activates autophagic cell death in the HeLa cervical cancer cell line (14). However, evidence of the anti-leukemic effects of sesamin is limited.

Sesamin possesses anti-leukemic activities by decreasing the proliferation and inducing apoptosis of HL-60 cells via the mitochondrial and endoplasmic reticulum stress pathways (15). It promotes TNF-induced apoptosis by suppressing the expression of survival genes (such as Bcl-2 and survivin), cell proliferative genes (such as cyclin D1 and COX-2) and genes involved in invasion and angiogenesis, including intracellular adhesion molecule 1 and matrix metalloproteinase 9, in KBM-5 cells (16).

Thus, the present study investigated the effects of sesamin on MOLT-4 and NB4 leukemic cells *in vitro* and examined

the underlying mechanisms associated with apoptosis and autophagy.

Materials and methods

Leukemic cell culture and peripheral blood mononuclear cell (PBMC) isolation. The human T-lymphocytic leukemic MOLT-4 cell line and human acute promyelocytic leukemic NB4 cell line were purchased from CLS Cell Lines Service GmbH. The cells were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator at 37°C with 5% CO_2. The isolation of PBMCs from healthy adult volunteers (1 female and 2 males; mean age, 27 years; age range, 26-27 years) was performed using Lymphoprep™ (Axis-Shield PoC AS) according to the manufacturer's protocol. Briefly, 10 ml of blood was collected from volunteers in August 2019 and diluted with an equal volume of PBS. The diluted blood was gently layered onto the Lymphoprep solution. The separation was done by centrifugation at 800 x g for 30 min at 25°C. The PBMC layer consisting of lymphocytes and monocytes was collected and washed twice with medium before being used for the cell viability assay. All healthy adult volunteers provided written informed consent for donating peripheral blood to the present study, which was in accordance with the guidelines of the Committee for Research of the Faculty of Medicine Ramathibodi Hospital, Mahidol University (Bangkok, Thailand), based on the Declaration of Helsinki.

Determination of cell viability by MTT assay. The effects of sesamin on cell viability were determined using an MTT assay. Sesamin (Sigma-Aldrich; Merck KGaA) was dissolved in DMSO at a stock concentration of 50 mg/ml and small aliquots were stored at -20°C. Immediately before use, individual aliquots were thawed and diluted in cell culture medium to the required concentrations. The leukemic cells and PBMCs were treated with various concentrations of sesamin (0, 100, 300 and 500 μg/ml). Following 24 and 48 h of incubation at 37°C, 5 mg/ml MTT (Thermo Fisher Scientific, Inc.) was added, followed by incubation for 4 h at 37°C. The formazan crystals were dissolved with solubilizing solution (10% SDS in 0.01 N HCl) and the optical density was measured at a wavelength of 570 nm using a spectrophotometer. The IC_{50} was calculated from linear regression for further experiments.

Analysis of apoptotic cells using flow cytometry. The leukemic cells were treated with sesamin at the IC_{50} concentration (400 μg/ml for MOLT-4 and 600 μg/ml for NB4). Following 48 h of incubation at 37°C, the cells were washed twice with cold PBS and resuspended in 1X binding buffer. Apoptosis was examined using the FITC Annexin V Apoptosis Detection kit (BD Biosciences) by staining the cells with 5 μl Annexin V and 5 μl PI for 15 min at room temperature in the dark. The apoptotic cells were detected using a FACS Canto II flow cytometer (BD Biosciences) within 1 h and analyzed using the BD FACSDiva software version 6.1.3 (BD Biosciences).

Analysis of autophagy induction using flow cytometry. The leukemic cells were treated with sesamin at the IC_{50} concentration (400 μg/ml for MOLT-4 and 600 μg/ml for NB4) at

37°C for 48 h. The LC3-II level was determined using the FlowCellect™ Autophagy LC3 Antibody-based Assay kit (Merck KGaA) following the manufacturer's protocol. At 30 min before the end of the time point, 10 μl diluted reagent A were added, followed by incubation at 37°C for 30 min. The medium was aspirated and 100 μl reagent B were added, followed by immediate spinning. The cells were resuspended in 1X assay buffer and stained with anti-LC3/FITC antibody for 30 min at room temperature in the dark, followed by flow cytometric detection using a FACS Canto II flow cytometer (BD Biosciences) within 1 h. The level of LC3-II was calculated from the mean fluorescence intensity analyzed using the BD FACSDiva software version 6.1.3 (BD Biosciences).

Reverse transcription-quantitative PCR (RT-qPCR). Leukemic cells were treated with sesamin at the IC_{50} concentration (400 μg/ml for MOLT-4 and 600 μg/ml for NB4) at 37°C for 48 h. Total RNA was extracted using GENEzol™ reagent (Geneaid Biotech Ltd.) and converted to 2 μg of cDNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was performed using the Bio-Rad CFX96 touch™ real-time PCR detection system (Bio-Rad Laboratories, Inc.) with 2 μl template, 0.5 μl of each primer, 10 μl Luna® Universal qPCR Master Mix (New England Biolabs, Inc.) and nuclease-free water to reach a final volume of 20 μl. The thermocycling conditions were as follows: Initial denaturation at 95°C for 15 sec, followed by 40 cycles of denaturation at 95°C for 15 sec and extension at 60°C for 30 sec. The following primers were used for detection: Caspase 3 (CASP3) forward, 5'-TTCAGAGGGGATCGTTGTAGAAGTC-3' and reverse, 5'-CAAGCTTGTCGGCATACTGTTTCAG-3'; mTOR forward, 5'-CGCTGTCATCCCTTTATCG-3' and reverse, 5'-ATGCTCAAACACCTCCACC-3'; unc-51 like autophagy activating kinase 1 (ULK1) forward, 5'-GGCAAG TTCGAGTTCTCCCG-3' and reverse, 5'-CGACCTCCAAAT CGTGCTTCT-3'; and GAPDH forward, 5'-GCACCGTCA AGGCTGAGAA-3' and reverse, 5'-AGGTCCACCACTGAC ACGTTG-3'. GAPDH was used as an endogenous control for normalization. The mRNA expression was calculated from the mean C_q value using the $2^{-\Delta\Delta Cq}$ method (17).

Prediction of protein-chemical interactions using bioinformatics tools. The interaction network between sesamin and possible targets was predicted using the Search Tool for Interactions of Chemicals (STITCH) version 5.0, which is a search tool used for predicting the interactions of chemicals and proteins that integrates information and databases, including metabolic pathways, crystal structures, binding experiments and drug-target associations to predict biological events (18). The parameters were determined as follows: List of names, sesamin, caspase3, mTOR and ULK1; species, Homo sapiens; confidence score, medium (0.400).

Statistical analysis. The experiments were performed in triplicate. All data are presented as the mean ± SEM. For comparisons between 2 groups, unpaired Student's t-test was used. For comparisons between >2 groups, data were analyzed using a one-way ANOVA followed by Dunnett's multiple comparisons test using GraphPad Prism 6
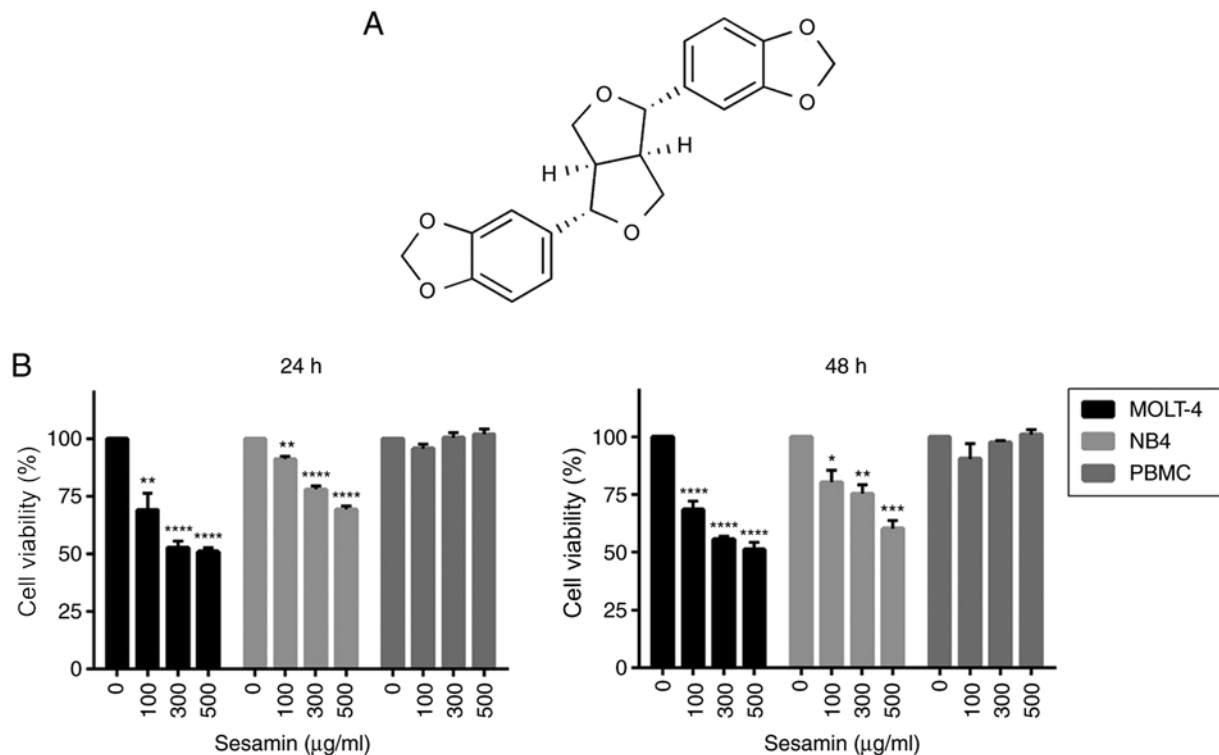
Figure 1. Sesamin suppresses the viability of leukemic cells. (A) Chemical structure of sesamin. (B) MOLT-4 and NB4 leukemic cells, and healthy PBMCs were treated with sesamin for 24 and 48 h. Cell viability was examined using an MTT assay. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$ vs. control (0 μg/ml). PBMC, peripheral blood mononuclear cell.

(GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Sesamin selectively exhibits cytotoxicity to leukemic cells. The chemical structure of sesamin is shown in Fig. 1A. To determine the anti-leukemic potential of sesamin, MOLT-4 and NB4 cells were treated with various concentrations of sesamin (0, 100, 300 and 500 μg/ml). Following 24 and 48 h of incubation, cell viability was examined using an MTT assay. Sesamin significantly decreased the viability of MOLT-4 and NB4 cells in a dose-dependent manner (Fig. 1B). The IC$_{50}$ value of sesamin at 48 h of incubation was 400 and 600 μg/ml in the MOLT-4 and NB4 cells, respectively. Notably, sesamin did not affect the viability of healthy PBMCs (Fig. 1B).

Sesamin induces the apoptosis of MOLT-4 and NB4 cells. To investigate the apoptosis induced by sesamin, MOLT-4 and NB4 leukemic cells were treated with sesamin at their IC$_{50}$ concentration for 48 h. The assessment of apoptosis was performed via Annexin V-FITC/PI staining and flow cytometry. The Annexin V-FITC$^+$ cells in Q2 (late apoptotic cells) and Q4 (early apoptotic cells) were defined as total apoptotic cells. The results revealed that sesamin significantly induced apoptosis of MOLT-4 and NB4 cells (Fig. 2A and B).

Sesamin induces autophagy by increasing the LC3-II level. To evaluate the induction of autophagy, leukemic cells were treated with sesamin at their IC$_{50}$ concentration for 48 h, and autophagy assay was performed using flow cytometry. The

detection was based on the level of LC3-II in the cells that accordingly indicated the level of autophagy. Compared with the control, sesamin significantly increased the LC3-II level by ~1.6-fold in both cell lines (Fig. 3A and B).

Sesamin contributes to the alteration of mRNA expression involved in apoptosis and autophagy. To examine the alteration of genes resulting from sesamin treatment, the expression levels of key genes associated with apoptosis, namely CASP3, and autophagy, namely mTOR and ULK1, were evaluated. The results of mRNA expression analysis in both cells demonstrated that sesamin induced apoptosis through the upregulation of CASP3 expression, and induced autophagy through the downregulation of mTOR and upregulation of ULK1 expression (Fig. 4).

Protein-chemical interaction of sesamin predicted by STITCH. STITCH was used to predict the association between sesamin and the proposed proteins (CASP3, mTOR and ULK1). The results from STITCH software analysis revealed that sesamin exhibited an association with CASP3 and mTOR via nitric oxide synthase 3. Moreover, ULK1, which is directly downstream of mTOR, exhibited high-score interactions with the target of rapamycin complex subunit LST8 (MLST8), regulatory-associated protein of mTOR (RPTOR) and proline-rich AKT1 substrate 1 (AKT1S1), as well as low-score interactions with ras homolog enriched in brain (RHEB), rapamycin-insensitive companion of mTOR (RICTOR), eukaryotic translation initiation factor 4E-binding protein 1 (EIF4EBP1) and eukaryotic translation initiation factor 4E (EIF4E). This interaction network indicated that CASP3, mTOR and ULK1 served a role
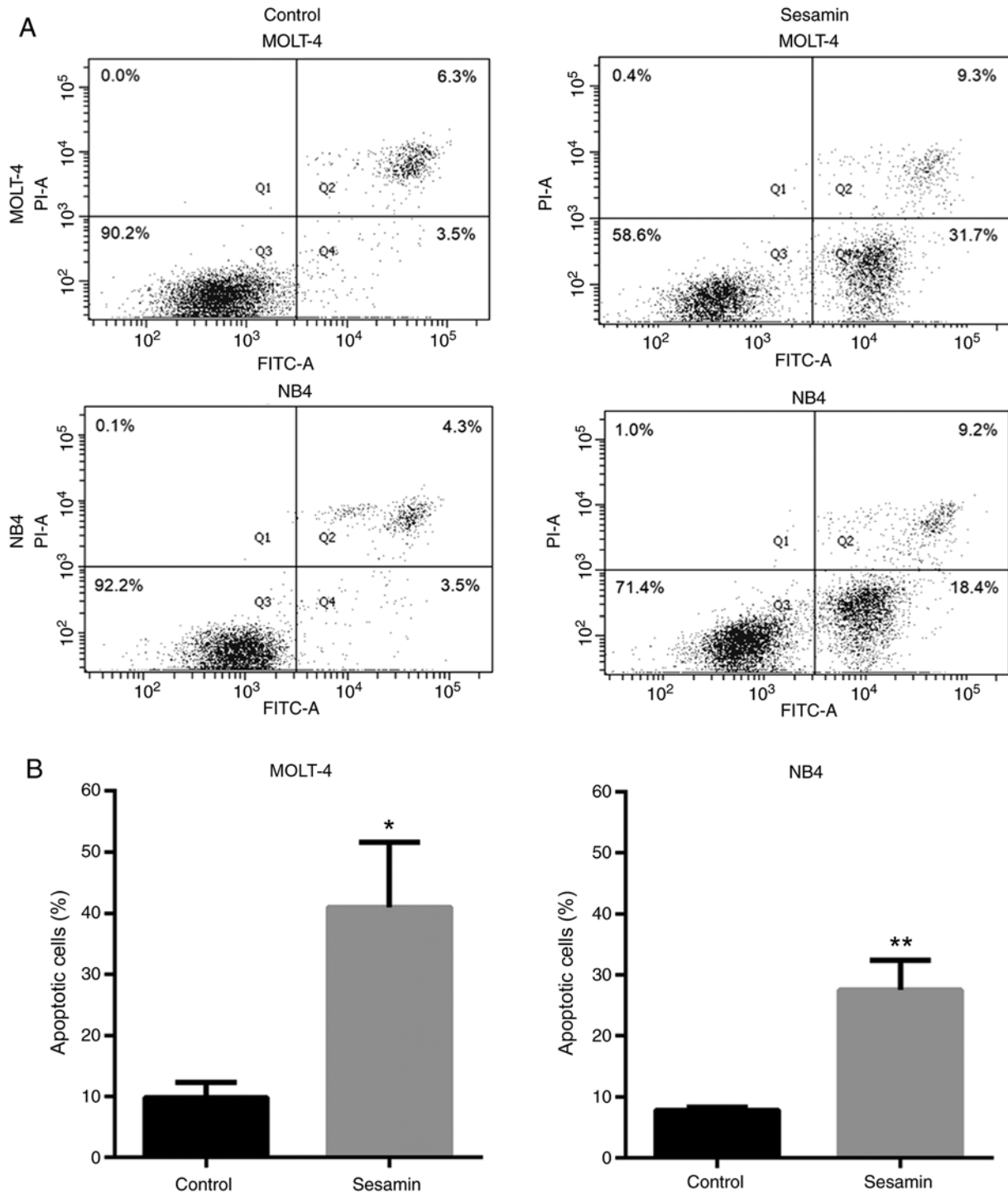
Figure 2. Sesamin induces the apoptosis of leukemic cells. (A) MOLT-4 and NB4 cells were incubated with sesamin at their IC_{50} value for 48 h, and apoptotic cells were analyzed via flow cytometry. Q1/upper left quadrant, necrotic cells; Q2/upper right quadrant, late apoptotic cells; Q3/lower left quadrant, viable cells; and Q4/lower right quadrant, early apoptotic cells. (B) Quantified results of apoptosis assay. *P<0.05 and **P<0.01 vs. control.

in apoptosis and autophagy in response to sesamin treatment, similar to rapamycin, AZD8055, Torin1 and Torin2 (Fig. 5).

Discussion

In the present study, the most substantial lignan in sesame seeds, known as sesamin, was investigated for its anti-leukemic effects on MOLT-4 and NB4 cell lines. It was revealed that sesamin exerted selective cytotoxic effects by inhibiting the viability of leukemic cells in a dose-dependent manner; however, it had a minimal effect on healthy PBMCs. Additionally, it increased apoptosis of both leukemic cell lines via the regulation of CASP3. When comparing the two cell lines, the MOLT-4 cells exhibited lower cell viability and a higher percentage of apoptosis compared with NB4 cells, suggesting that MOLT-4, an acute lymphoblastic leukemic cell line, was more sensitive to sesamin compared with NB4, an acute promyelocytic leukemic cell line. Previous studies
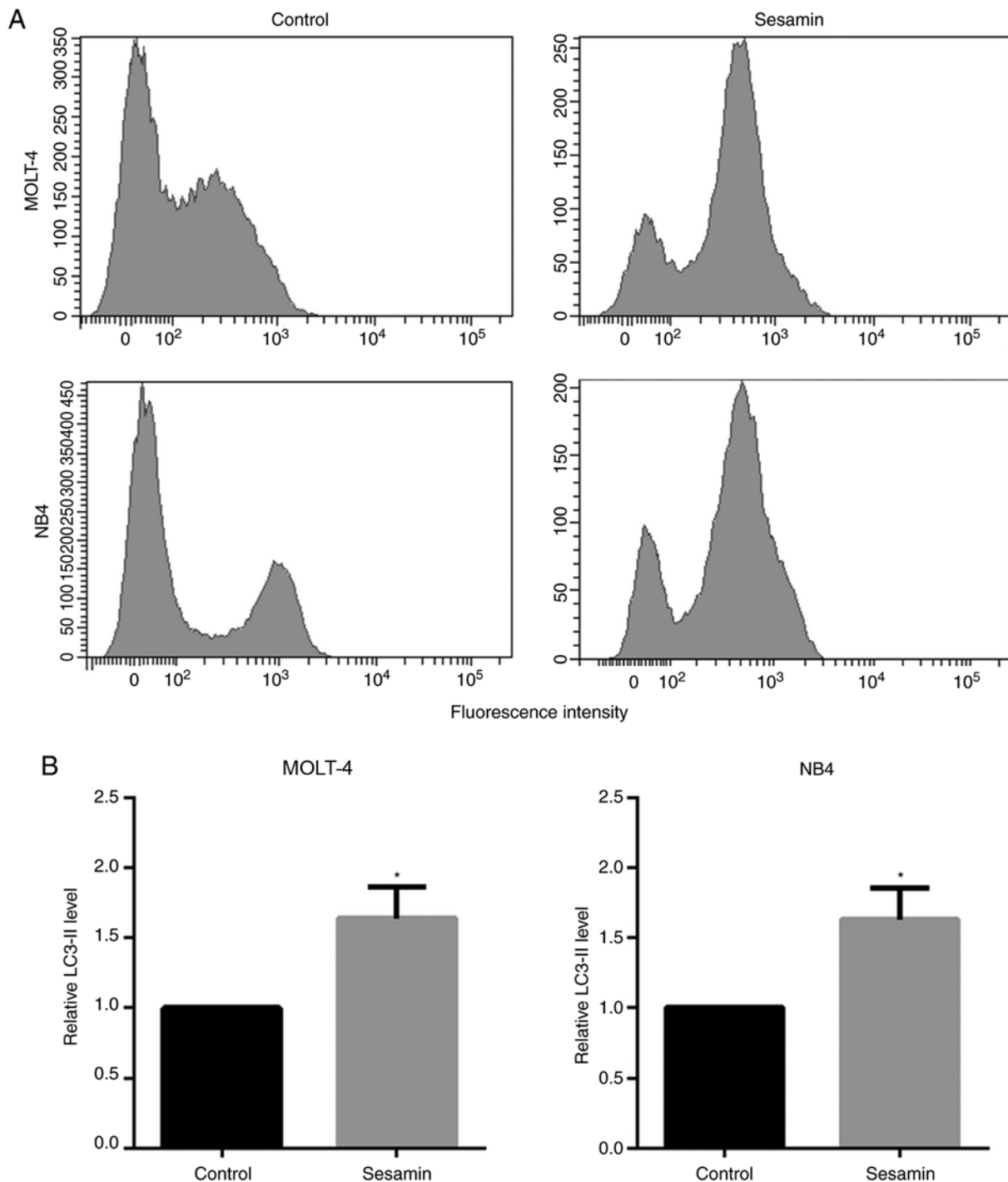
Figure 3. Sesamin induces autophagy in leukemic cells. (A) MOLT-4 and NB4 cells were treated with their IC_{50} concentration of sesamin for 48 h. Autophagy was quantitatively measured as the mean fluorescence intensity of LC3-II via flow cytometry. (B) Quantified results of autophagy assay. *P<0.05 vs. control.

have reported the induction of apoptosis by sesamin in several types of cancer. For example, the regulation of the apoptotic proteins Bax/Bcl-2 and endoplasmic reticulum stress proteins in the inositol-requiring enzyme 1α/JNK signaling pathway have been reported as key factors in cervical cancer cell apoptosis induced by sesamin (14). Additionally, sesamin is able to induce cell cycle arrest and apoptosis through the inhibition of STAT3 in hepatocellular carcinoma HepG2 cells (19).

In addition, previous studies have focused on the induction of autophagic cancer cell death by natural products. For example, ailanthone, the extract from *Ailanthus altissima*, induces autophagic cell death through the modulation of Beclin1, p62 and LC3 expression in HL-60 cells (20). Allicin and diallyl disulfide, the bioactive compounds in garlic extract, promote the mTOR-mediated autophagic death of human liver cancer and leukemic cells (21,22). mTOR is a protein kinase that serves a role in cell proliferation, survival, metabolism and autophagy; the activation of mTOR is frequently observed in cancer (23). Thereby, the inhibition of mTOR has been considered for cancer therapy (24). ULK1 is an autophagic marker, and its downstream signals are triggered by decreased mTOR levels; it has been reported as a
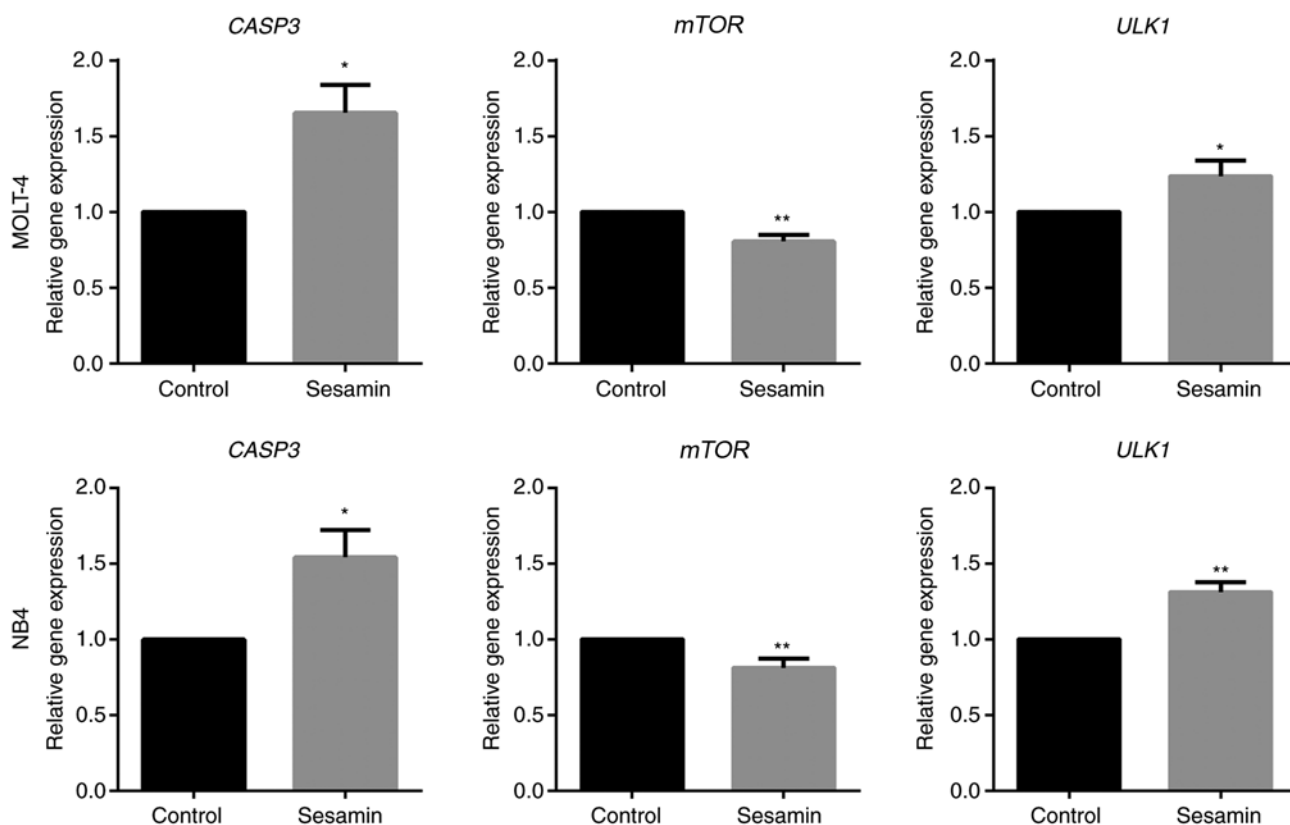
Figure 4. Sesamin induces leukemic cell death via the regulation of apoptotic and autophagic genes. Leukemic cells were incubated with sesamin at their IC_{50} value for 48 h. The mRNA expression levels of CASP3, mTOR and ULK1 were examined via reverse transcription-quantitative PCR. *$P<0.05$ and **$P<0.01$ vs. control. CASP3, caspase 3; ULK1, unc-51 like autophagy activating kinase 1.
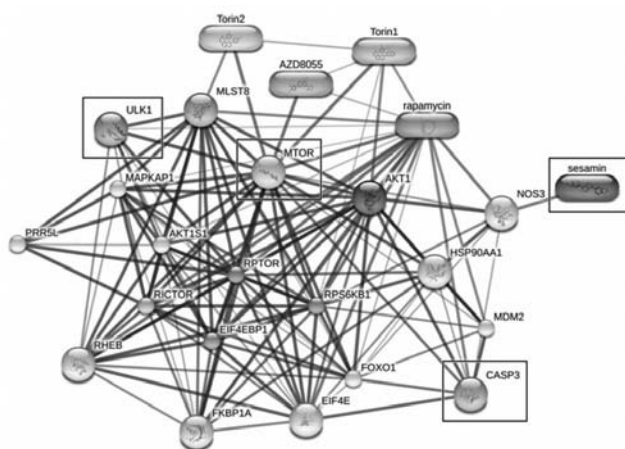

Figure 5. Prediction of protein-chemical interaction using the Search Tool for Interactions of Chemicals with the input of sesamin and associated factors (CASP3, mTOR and ULK1). Thick lines represent strong associations, while thin lines represent weak interactions. CASP3, caspase 3; ULK1, unc-51 like autophagy activating kinase 1; NOS3, nitric oxide synthase 3; MLST8, target of rapamycin complex subunit LST8; RPTOR, regulatory-associated protein of mTOR; AKT1S1, proline-rich AKT1 substrate 1; RHEB, ras homolog enriched in brain; RICTOR, rapamycin-insensitive companion of mTOR; EIF4EBP1, eukaryotic translation initiation factor 4E-binding protein 1; EIF4E, eukaryotic translation initiation factor 4E.

promising target for cancer therapy (25). Baicalein, a flavonoid from *Scutellaria baicalensis*, and LYN-1604, a candidate ULK1 agonist, exert antitumor effects through ULK1 activation in cancer cells (26,27). The results of the present study

demonstrated that sesamin treatment stimulated autophagy, represented by the increased levels of LC3-II in MOLT-4 and NB4 leukemic cells. This effect was accompanied by the downregulation of mTOR and the upregulation of ULK1 expression. This suggested that the induction of autophagy through mTOR/ULK1 signaling by sesamin may be an alternative treatment option for leukemia.

Bioinformatics tools have facilitated the comprehensive overview of available databases that are helpful to the understanding of pharmacology and biochemistry. The study of the interaction between proteins and small molecules is essential for the development of therapeutic drugs for diseases (28). The association between sesamin and possible associated molecules (CASP3, mTOR and ULK1) demonstrated that several targets participated in the response to sesamin. MLST8, RPTOR and AKT1S1, which exhibited strong interaction networks, were recognized as subunits of mTOR and their overexpression has been reported in a number of types of cancer cells, including colon, prostate, breast and lung cancer cells (29-31). It has been reported that the downregulation of MLST8, RPTOR and AKT1S1 expression inhibited tumor growth and induced cell death in colon cancer, lymphoma and sarcoma (29,30,32,33). RHEB and RICTOR, which exhibited an interaction in the current network, are also involved in the mTOR signaling pathway (24). The deletion of RHEB and RICTOR can enhance cell cycle arrest and apoptosis of acute leukemic cells (34,35). The other identified associated proteins, the translation factors EIF4E and EIF4EBP1, affect leukemogenesis by promoting growth and impairing the differentiation

of blood cell precursors (36). Additionally, STITCH predicted that other anticancer agents, including rapamycin, AZD8055, Torin1 and Torin2, exhibited similar mechanisms to sesamin. These chemicals are categorized as mTOR inhibitors with anti-leukemic properties. Rapamycin treatment enhances the anti-neoplastic effects of the chemotherapeutic drug arabinozide cytarabine in acute myeloid leukemia (AML) (37). AZD8055 exerts cytotoxic effects in clinical AML cells through cell cycle blocking, caspase-dependent apoptosis and autophagic cell death (38). The mTOR inhibitors Torin1 and 2 have been investigated for their anticancer effects, revealing that they can suppress cell proliferation and promote the death of colon cancer and hepatocellular carcinoma cells (39,40) and have thus progressed to clinical trials (41).

The present study suggested that sesamin may be a potent anti-leukemic agent, since it exerted anti-proliferative effects on MOLT-4 and NB4 leukemic cell lines. Further experiments indicated that these effects were mediated via the modulation of apoptosis and autophagy. The expression levels of the effective apoptotic factor CASP3 were increased in sesamin-treated cells. Autophagy occurred through the regulation of LC3-II triggered via the mTOR and ULK1 signaling pathway. The protein-chemical interaction analysis indicated that CASP3, mTOR and ULK1 were associated with several biomolecules in response to sesamin. The underlying mechanisms of sesamin were associated with the effects of the anti-neoplastic drug rapamycin and those of the well-known agents AZD8055, Torin1 and 2.

The findings of the present study demonstrated the anti-leukemic effects of sesamin on MOLT-4 and NB4 cell lines through apoptotic and autophagic signaling pathways. These findings may be beneficial for the improved understanding of the role of sesamin in human leukemia. However, the present study only performed *in vitro* experiments. Thus, further studies on the effects of sesamin *in vivo* are required in the future for the development of alternative therapeutic strategies for leukemia.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DT was involved in the conception of the study. KD and DT were involved in the experimental procedures. KD, CC, SR, UA and DT were involved in the interpretation and discussion of the results. KD was involved in the manuscript preparation. SR and DT were involved in the revision of the manuscript.

All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The experiments involving human blood samples were approved by the Committee for Research of the Faculty of Medicine Ramathibodi Hospital, Mahidol University (Bangkok, Thailand; approval no. MURA2019/679). All healthy adult volunteers provided written informed consent for donating peripheral blood.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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