Inhibitory effect of recombinant tyrosine-sulfated madanin-1, a thrombin inhibitor, on the behavior of MDA-MB-231 and SKOV3 cells *in vitro*

GUK HEUI JO[1], SUN AH JUNG[1], TAE HOON ROH[2], JIN SOOK YOON[3] and JOON H. LEE[1]

[1]Myung-Gok Eye Research Institute, Kim's Eye Hospital, Konyang University College of Medicine, Seoul 07301, Republic of Korea; [2]Department of Medicine, Yonsei University College of Medicine, Seoul 03722, Republic of Korea; [3]Department of Ophthalmology, Institute of Vision Research, Severance Hospital, Yonsei University College of Medicine, Seoul 03722, Republic of Korea

Received December 20, 2023; Accepted April 9, 2024

DOI: 10.3892/mmr.2024.13238

Abstract. Thrombin, which plays a crucial role in hemostasis, is also implicated in cancer progression. In the present study, the effects of the thrombin-targeting recombinant tyrosine-sulfated madanin-1 on cancer cell behavior and signaling pathways compared with madanin-1 wild-type (WT) were investigated. Recombinant madanin-1 2 sulfation (madanin-1 2S) and madanin-1 WT proteins were generated using *Escherichia coli*. SKOV3 and MDA-MB-231 cells were treated with purified recombinant proteins with or without thrombin stimulation. Migration and invasion of cells were analyzed by wound healing assay and Transwell assay, respectively. Thrombin markedly increased cell migration and invasion in both SKOV3 and MDA-MB-231 cells, which were significantly suppressed by madanin-1 2S ($P<0.05$). Madanin-1 2S also significantly suppressed thrombin-induced expression of phosphorylated (p)-Akt and p-extracellular signal-regulated kinase in both cell lines ($P<0.05$), whereas madanin-1 WT had no effect on the expression levels of these proteins in MDA-MB-231 cells. Furthermore, madanin-1 2S significantly reversed the effects of thrombin on E-cadherin, N-cadherin and vimentin expression in MDA-MB-231 cells ($P<0.05$), whereas madanin-1 WT did not show any effect. In conclusion, madanin-1 2S suppressed the migration and invasion of cancer cells more effectively than madanin-1 WT. It is hypothesized that inhibiting thrombin via the sulfated form of madanin-1 may be a potential candidate for enhanced cancer therapy; however, further *in vivo* validation is required.

Correspondence to: Professor Jin Sook Yoon, Department of Ophthalmology, Institute of Vision Research, Severance Hospital, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun, Seoul 03722, Republic of Korea
E-mail: yoonjs@yuhs.ac

Professor Joon H. Lee, Myung-Gok Eye Research Institute, Kim's Eye Hospital, Konyang University College of Medicine, 1 Yeongsin-ro 34-gil, Yeongdeungpo, Seoul 07301, Republic of Korea
E-mail: joonhlee@konyang.ac.kr

Key words: tyrosine sulfation, thrombin, madanin-1, cancer, migration, invasion, epithelial-mesenchymal transition

Introduction

Thrombin, a serine protease derived from the plasma protein prothrombin, is widely recognized for its role in hemostasis (1). Previous studies have reported that thrombin plays a diverse role as a growth-regulatory protein in addition to its role in hemostasis; thrombin has been shown to enhance mitogenesis (2), the proliferation of vascular smooth muscle cells (3) and fibroblasts (4), cellular adhesion (5,6) and angiogenesis (7). Moreover, accumulating evidence has suggested that hemostatic system components, such as thrombin, are involved in cancer progression through a variety of mechanisms (8,9). Protease-activated receptor-1 (PAR-1), which is activated by proteases including thrombin, is a G-protein coupled receptor (GPCR) (10); PAR-1 signaling is constitutively activated in cancer cells in contrast to normal cells, and is involved in carcinogenesis, metastasis and angiogenesis (11). PAR-1 has also been observed to be upregulated in numerous types of cancer, including breast and ovarian cancer, and may be utilized as a therapeutic target (10,11).

Several anticoagulant substances have been isolated and characterized to date from blood-feeding invertebrates (12-14). Madanin-1 (MEROPS inhibitor family I53) is a small cysteine-free protein isolated from the salivary glands of ticks (*Haemaphysalis longicornis*), which acts as a competitive inhibitor of thrombin (15). According to a previous study, madanin-1 also competes with physiological substrates for binding to the exosite I of α-thrombin, a secondary recognition site that interacts with fibrinogen and PARs (16). An assay using human plasma showed that madanin-1 can prolong the prothrombin time and activated partial thromboplastin time in a dose-dependent manner (16), and can double the thrombin time (TT) at a concentration of 5 μM (15). It has previously been shown that post-translational sulfation of tyrosine residues within acidic stretches substantially enhances the thrombin inhibitory and anticoagulant potency of madanin-1 by binding the molecule to the active site and exosite II (17).

Although anticoagulants are widely used for treating and preventing cancer-associated venous thromboembolism (18), they have not yet been used as a direct anticancer therapy in clinical settings. However, in the context of the comprehensive role of thrombin in cancer biology, several studies have reported the potential therapeutic effects of targeting thrombin and PAR-1 (19-21). Given that metastatic progression is the main cause of mortality in patients with cancer (22), and that thrombin plays a significant role in promoting the migration and invasion of various tumor cells (23-25), the present research focused on investigating the effect of recombinant tyrosine-sulfated madanin-1 on thrombin-induced migration and invasion of cancer cells, and the intracellular signaling pathways. An *in vitro* assay was performed using SKOV3, an ovarian cancer cell line, and MDA-MB-231, a highly aggressive breast cancer cell line. Well-established and widely used analytical techniques were employed, including the wound healing assay and Transwell cell invasion assay (26); these methods are not only commonly used in cancer research but are also relatively straightforward, making them essential tools for investigating migration and invasion (26).

Materials and methods

Expression and purification of madanin-1 wild-type (WT) and 2 sulfation (2S) proteins. Madanin-1 double-stranded oligonucleotides were purchased from CosmoGenetech Co., Ltd. Codon optimization was performed to improve recombinant protein expression, and the two TAC codons of the madanin-1 WT sequence were changed to two TAG codons to generate the madanin-1 2S protein (Table I). Nucleotides were added to make *Eco*RI and *Hin*dIII restriction sites in parenthesis (brackets in Table I). The synthesized madanin-1 WT and madanin-1 2S double-stranded oligonucleotides were inserted into the *Eco*RI and *Hin*dIII digested pET-41a vector (Novagen; Merck KGaA), which has an N-terminal glutathione S-transferase, polyhistidine (6xHis) tag and a C-terminal polyhistidine for purification.

Madanin-1 WT pET-41a (50 μg/ml kanamycin was used for the selection of transformants), madanin-1 2S pET-41a and pSUPAR6-L3-3SY (50 μg/ml kanamycin and 50 μg/ml chloramphenicol were used for the selection of transformants) were transformed into *Escherichia coli* BL21 (DE3; RBC Bioscience Corp.; Fig. 1A and C); the pSUPAR6-L3-3SY plasmid encodes the components required for the translational incorporation of sulfotyrosine in response to the TAG codons (27). Subsequently, the selected BL21 colony was cultured in 3 ml Luria-Bertani (LB) medium (BD Biosciences) overnight at 37˚C. The following day, cultured madanin-1 WT transformants were inoculated into 250 ml LB medium until OD_{600}=0.5, then 0.1 mM IPTG was added for 5 h at 37˚C to overexpress the fusion proteins. Cultured madanin-1 2S transformants were also inoculated into 250 ml LB medium with 10 mM sulfotyrosine (Bachem) and cultured until OD_{600}=1, then 1 mM IPTG was added for 20 h at 25˚C to overexpress the madanin-1 2S proteins.

After centrifuging the medium at 10,000 x g for 10 min at 25˚C, the bacterial pellets containing proteins suspended in 10 ml binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9) were sonicated. The sonication process was conducted as follows: The sonic probe was activated for 30 sec (65 Hz), followed by a pause during which the cell suspension was removed from the probe to chill for 2 min at 4˚C. This cycle was repeated 5 times to ensure lysis of the majority of the cells. Again, the disrupted cells were centrifuged at 14,000 x g for 30 min at 4˚C, and the supernatant was then used for further purification. The supernatant was applied to pre-equilibrated Ni-NTA resin (Novagen; Merck KGaA) with distilled water and binding buffer and allowed to flow by gravity. The column was washed with 10 volumes of binding buffer and elution buffer (100 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Subsequently, dialysis (20 mM Tris-HCl, 50 mM NaCl, 0.5 mM β-mercaptoethanol, pH 7.5) was accomplished to remove imidazole for properly storing purified proteins. Finally, madanin-1 WT and madanin-1 2S proteins were resolved by SDS-PAGE on 10% gels. After electrophoresis, the gels were stained with Coomassie Blue R-250 for 1 h at 25˚C and then destained (Fig. 1B).

Cell culture and treatment. SKOV3 and MDA-MB-231 cells were purchased from the Korean Cell Line Bank (Korean Cell Line Research Foundation). The cells were cultured in RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all Gibco; Thermo Fisher Scientific, Inc.), in a humidified 5% CO_2 incubator at 37˚C. For migration assays, invasion assays and western blot analyses, cells not treated with thrombin, madanin-1 WT or madanin-1 2S were defined as the control group. In cell viability and apoptosis assays, cells not treated with either madanin-1 WT or madanin-1 2S were defined as the control group.

Wound healing assay. SKOV3 ($2x10^5$ cells/well) and MDA-MB-231 cells ($3x10^5$ cells/well) were seeded into 24-well plates. After reaching 100% confluence, the cell monolayers were scratched with a 200-μl sterile pipette tip to create a vertical wound (26). To avoid any influence from the cell growth rate, the cell culture medium was changed from RPMI-1640 supplemented with 10% FBS to serum-free RPMI-1640. The cells were divided into five groups as follows: i) Serum-free group (control group); ii) cells treated with 2 U/ml thrombin; iii and iv) cells pretreated with madanin-1 WT or madanin-1 2S (10 μg/ml) for 30 min followed by thrombin treatment (2 U/ml); and v) cells treated with 2% FBS. All groups were incubated for 24 h at 37˚C. Phase-contrast images were acquired after scratching and after 24 h of incubation at 37˚C using a DP70 fluorescence microscope (Olympus Corporation). The wound area was measured using ImageJ software 6.0 (National Institutes of Health), and each experiment was repeated three times. Relative wound area was calculated using the following formula: Relative wound area (%)=100x (24 h area of wound/initial area of wound).

Transwell cell invasion assay. The invasion assay was carried out using an 8-μM pore, 24-well Transwell (Costar; Corning, Inc.) coated with PBS buffer containing 25 μg Matrigel (Sigma-Aldrich; Merck KGaA) and 0.1% gelatin (Sigma-Aldrich; Merck KGaA) for 5 h at 25˚C. Cells were grown to 80% confluence in growth medium, followed by starvation in serum-free RPMI-1640 for 24 h at 37˚C, and were then seeded ($1x10^5$ cells/ml) in the upper chamber (26).

Table I. Sequences of madanin-1 nucleotides and amino acids.

Name	Type	Sequence[a]
Madanin-1 WT	Nucleotide	(AAAGAATTC) TACCCAGAGCGTGATTCCGCAAAAGAAGGTAACC AGGAACAAGAGCGGGCGTTGCATGTAAAGGTGCAAAAGCGCAC TGACGGCGATGCTGACTACGATGAATACGAAGAGGATGGCACAA CTCCAACGCCCGATCCGACTGCACCTACGGCCAAGCCCAGACTG CGTGGTAACAAGCCTTAA (AAGCTTAAA)
Madanin-1 2S	Nucleotide	(AAAGAATTC) TACCCAGAGCGTGATTCCGCAAAAGAAGGTAACC AGGAACAAGAGCGGGCGTTGCATGTAAAGGTGCAAAAGCGCAC TGACGGCGATGCTGACTAGGATGAATAGGAAGAGGATGGCACAA CTCCAACGCCCGATCCGACTGCACCTACGGCCAAGCCCAGACTG CGTGGTAACAAGCCTTAA (AAGCTTAAA)
Madanin-1 WT	Amino acid	YPERDSAKEGNQEQERALHVKVQKRTDGDADYDEYEEDGTTPTP DPTAPTAKPRLRGNKP
Madanin-1 2S	Amino acid	YPERDSAKEGNQEQERALHVKVQKRTDGDAD_[b]DE_[b]EEDGTTPTP DPTAPTAKPRLRGNKP

[a]Nucleotides are ordered from 5' to 3', and amino acids are ordered from N to C terminus. [b]Sulfotyrosine site. The brackets indicate nucleotides that were added to make the *Eco*RI and *Hin*dIII restriction sites. WT, wild-type; 2S, 2 sulfation.

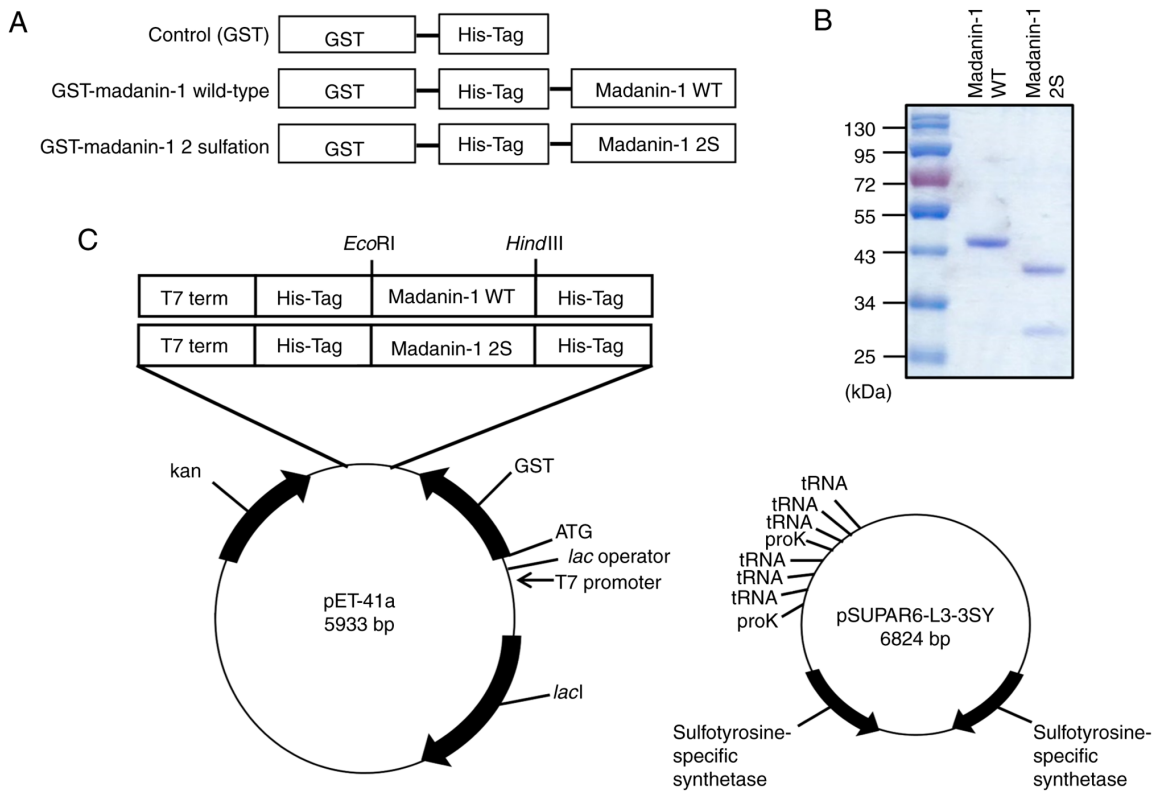

Figure 1. Cloning and purification of madanin-1 WT and madanin-1 2S proteins. (A) Diagram of the expressed control, madanin-1 WT and madanin-1 2S proteins. Madanin-1 was cloned into the *Eco*RI and *Hin*dIII restriction sites of the pET-41a vector. (B) Purified fusion proteins were detected by Coomassie brilliant blue staining after SDS-PAGE on a 10% gel. (C) Along with pET-41a plasmid encoding madanin-1 WT and madanin-1 2S, pSUPAR6-L3-3SY plasmid encoding the components necessary for translational incorporation of sulfotyrosine in response to the TAG codon was used. WT, wild-type; 2S, 2 sulfation; GST, glutathione S-transferase.

The lower chamber contained serum-free medium with or without madanin-1 WT or madanin-1 2S (10 μg/ml). The cells were divided into four groups as follows: i) Untreated cells (control group); ii) cells treated with 2 U/ml thrombin (2 U/ml thrombin was also added to the lower chamber); iii) cells treated with 2 U/ml thrombin and 10 μg/ml madanin-1 WT

(2 U/ml thrombin and 10 μg/ml madanin-1 WT were also added to the lower chamber); and vi) cells treated with 2 U/ml thrombin and 10 μg/ml madanin-1 2S (2 U/ml thrombin and 10 μg/ml madanin-1 2S were also added to the lower chamber). All groups were incubated for 24 h at 37˚C. After which, the cells were fixed in 100% methanol for 10 min at 25˚C and stained with 0.1% crystal violet (Thermo Fisher Scientific, Inc.) for 10 min at 25˚C. After which, a cotton swab was used to remove cells on the upper side of the filter. Images of cells that migrated to the underside of the filter were captured with a DP70 fluorescence microscope (Olympus Corporation); three fields per sample were captured (magnification, 10x).

Western blotting. To evaluate the changes in protein expression induced by thrombin after treatment with madanin-1, cells were divided into four groups as follows: i) Untreated cells as the control group; ii) cells treated with 2 U/ml thrombin; and iii and iv) cells pretreated with madanin-1 WT or madanin-1 2S (10 μg/ml) for 30 min followed by thrombin treatment (2 U/ml). All groups were incubated at 37˚C for 15 min or 24 h. Proteins were extracted from cells in cold 1X Cell Lysis Buffer (Cell Signaling Technology, Inc.) containing 1 mM PMSF and 50 mM NaF. The protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Equal amounts of protein (30 μg/lane) were resolved by SDS-PAGE on 6-10% gels. The proteins were transferred to nitrocellulose membranes (MilliporeSigma). Following blocking with 5% skim milk (BD Biosciences) for 1 h at 25˚C, membranes were probed with primary antibodies (1:1,000) against phosphorylated (p)-Akt (cat. no. 4060), Akt (cat. no. 9272), β-actin (cat. no. 4967), E-cadherin (cat. no. 5296) (all Cell Signaling Technology, Inc.), p-extracellular signal-regulated kinase (ERK; cat. no. SC-7383), ERK (cat. no. SC-1647), N-cadherin (cat. no. SC-393933) and vimentin (cat. no. SC-6260) (all Santa Cruz Biotechnology) overnight at 4˚C. Then, membranes were incubated with horseradish peroxidase-linked anti-mouse IgG (cat. no. 7076; 1:1,000) and anti-rabbit IgG (cat. no. 7074; 1:1,000) secondary antibodies (both Cell Signaling Technology, Inc.) for 1 h at 25˚C. The specific immunoreaction was detected using Western Blotting Luminol Reagent (cat. no. SC-2048; Santa Cruz Biotechnology). The relative protein amount was quantified through densitometry using the Multi Gauge software version 2.2 (FUJIFILM Corporation).

Cell viability assay. SKOV3 and MDA-MB-231 cells were seeded into 24-well culture plates at a density of $5x10^4$ cells/well and were incubated for 48 h at 37˚C with or without thrombin (2 U/ml) following pretreatment with madanin-1 WT or madanin-1 2S at different concentrations (0, 1, 10 and 20 μg/ml) for 1 h at 37˚C. Following exposure, cells were cleaned and incubated with 5 mg/ml MTT solution (Sigma-Aldrich; Merck KGaA) for 2 h at 37˚C. A microplate reader (EL 340 Bio Kinetics Reader; BioTek Instruments; Agilent Technologies, Inc.) was used to measure the absorbance of the dye at 560 nm after it had been solubilized with DMSO (Sigma-Aldrich; Merck KGaA) and background subtraction at 630 nm. Cell viability was expressed as a percentage compared with control cells.

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining. SKOV3 and MDA-MB-231 cells were plated in 6-well plates at $2x10^5$ cells/well. After serum starvation, the cells were treated for 48 h at 37˚C with or without thrombin (2 U/ml) following pretreatment with madanin-1 WT or madanin-1 2S at different concentrations (0, 1, 10 and 20 μg/ml) for 1 h at 37˚C. Subsequently, the supernatant and cells were harvested and washed with PBS. The samples were stained with Annexin V and PI using the FITC Annexin V Apoptosis kit (BD Biosciences) according to the manufacturer's protocol. BD FACSymphony A5 (BD Biosciences) and BD FACSDiva software version 9.3.1 (BD Biosciences) were used for data analysis.

Statistical analysis. Data are presented as the mean ± standard deviation of at least three independent experiments. To identify statistically significant differences in the experimental data, a one-way analysis of variance was used. Tukey's post hoc analysis was performed for pairwise comparisons between conditions. IBM SPSS Statistics 20.0 (IBM Corp.) was used to analyze the data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inhibition of cancer cell migration by madanin-1 2S. The effects of madanin-1 2S compared with madanin-1 WT on the migration of SKOV3 and MDA-MB-231 cells were examined through the wound healing assay (Fig. 2). Cells were pretreated with 10 μg/ml madanin-1 WT or madanin-1 2S for 30 min before treatment with 2 U/ml thrombin for 24 h. Compared with the serum-free group, SKOV3 and MDA-MB-231 cells treated with thrombin (2 U/ml) exhibited significant increased migration. Thrombin-induced migration was significantly inhibited by madanin-1, and the inhibitory effect was more effective when cells were treated with madanin-1 2S (the relative wound area was 93.3% in SKOV3 and 75% in MDA-MB-231 cells) compared with madanin-1 WT (the relative wound area was 38.2% in SKOV3 and 47.1% in MDA-MB-231 cells).

Inhibition of cancer cell invasion by madanin-1 2S. The invasive capacity of cancer cells was analyzed using a Transwell cell invasion assay (Fig. 3). Cells were treated with thrombin (2 U/ml) with or without 10 μg/ml madanin-1 WT or madanin-1 2S for 24 h. The results showed that thrombin treatment significantly enhanced cell invasion in both SKOV3 and MDA-MB-231 cells compared with untreated control cells. Madanin-1 2S significantly suppressed thrombin-induced cell invasion in both cell lines; the inhibitory effect was 427% higher in SKOV3 cells and 190% higher in MDA-MB-231 cells compared with madanin-1 WT.

Effect of madanin-1 WT and madanin-1 2S on thrombin-associated ERK expression and the Akt signaling pathway. Western blot analysis was performed to investigate the potential effects of madanin-1 WT and madanin-1 2S on thrombin-associated signaling pathways (Fig. 4). Cells were left untreated or were pretreated with 10 μg/ml madanin-1 WT or madanin-1 2S for 30 min, followed by treatment with thrombin (2 U/ml for 15 min). The expression levels of p-ERK and p-Akt were significantly induced by thrombin; madanin-1 2S significantly inhibited the thrombin-induced expression of

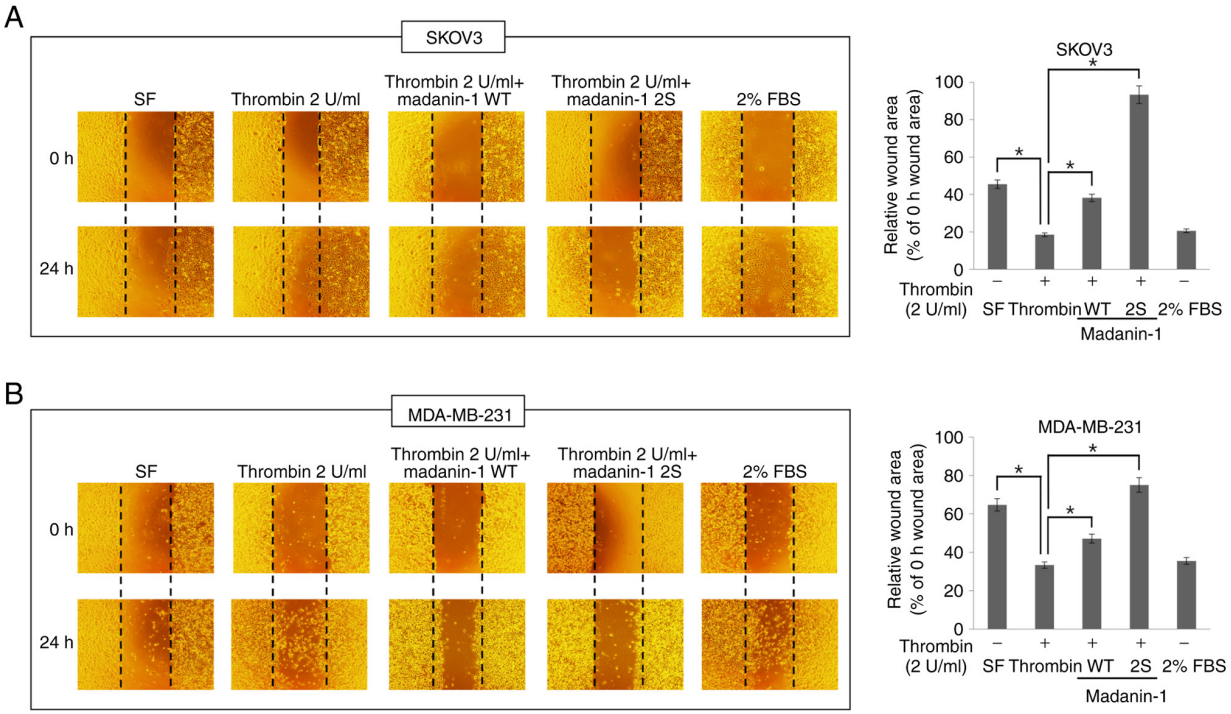

Figure 2. Effect of madanin-1 WT and madanin-1 2S on cancer cell migration. A monolayer of confluent cells was scraped with a sterile pipette tip after being preincubated with 10 μg/ml madanin-1 WT or madanin-1 2S. (A) SKOV3 and (B) MDA-MB-231 cells were left untreated or were pretreated with madanin-1 WT or madanin-1 2S for 30 min, followed by treatment with or without thrombin (2 U/ml) for 24 h, and images of the wound were captured (magnification, 4x). The wound area was measured in three independent experiments. Madanin-1 2S significantly inhibited thrombin-induced migration more effectively than madanin-1 WT. Data are presented as the mean ± standard deviation of three independent experiments (*P<0.05). WT, wild-type; 2S, 2 sulfation; SF, serum free.

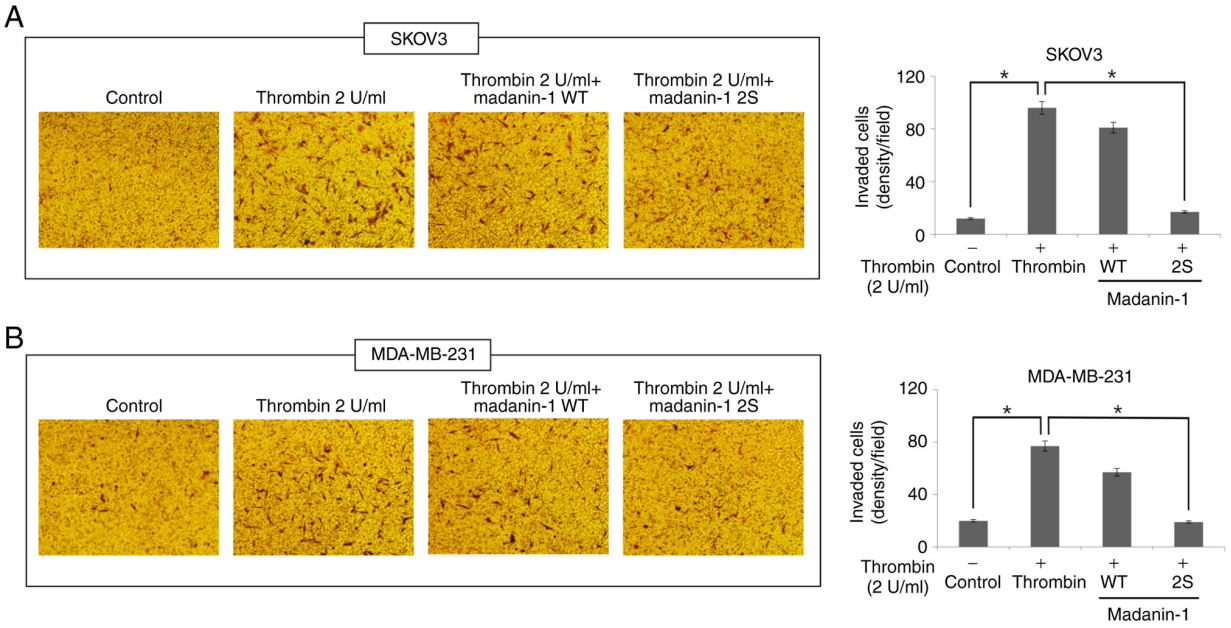

Figure 3. Effect of madanin-1 WT and madanin-1 2S on the invasion of cancer cells. Invasion of (A) SKOV3 and (B) MDA-MB-231 cells were investigated using a Transwell invasion assay with Matrigel for 24 h. The lower chamber contained serum-free medium with or without madanin-1 WT or madanin-1 2S (10 μg/ml). Representative images and graphs indicating the density of invaded cells per field 24 h after seeding are shown (magnification, 10x). Only madanin-1 2S treatment significantly inhibited thrombin-induced cell invasion. Data are presented as the mean ± standard deviation of three independent experiments completed in triplicate (*P<0.05). WT, wild-type; 2S, 2 sulfation.

p-ERK and p-Akt in both SKOV3 and MDA-MB-231 cells, whereas madanin-1 WT did not significantly inhibit p-ERK and p-Akt expression in MDA-MB-231 cells.

Effects of madanin-1 WT and madanin-1 2S on thrombin-related E-cadherin, N-cadherin and vimentin protein expression. Protein expression, including cadherin and

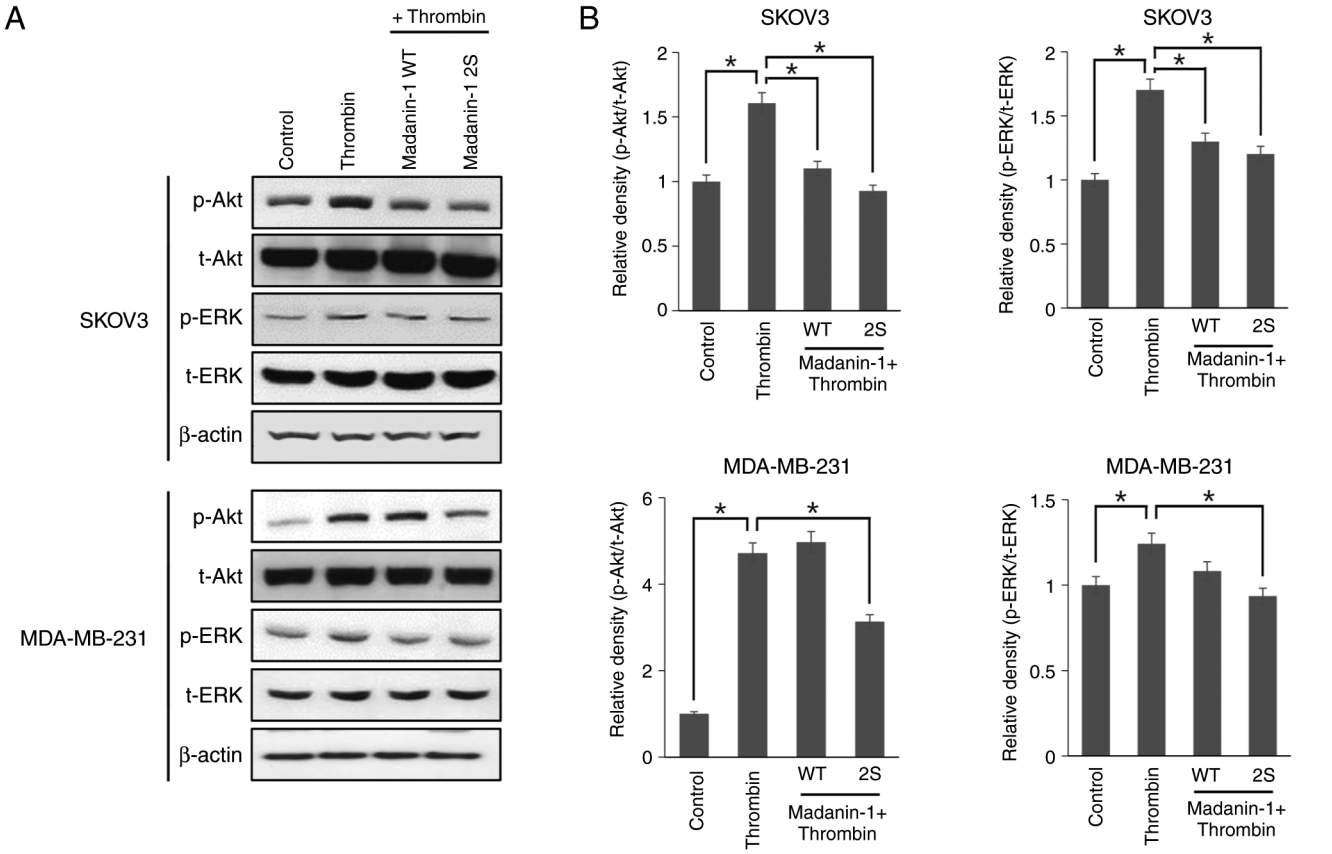

Figure 4. Effects of madanin-1 WT and madanin-1 2S on signaling pathways. SKOV3 and MDA-MB-231 cells were pretreated with madanin-1 WT or madanin-1 2S (10 μg/ml for 30 min) prior to treatment with thrombin (2 U/ml for 15 min). The protein expression levels of p-Akt, t-Akt, p-ERK and t-ERK in SKOV3 and MDA-MB-231 cells were analyzed by western blotting. (A) Representative gel images. (B) In SKOV3 cells, thrombin-induced p-Akt and p-ERK expression was significantly attenuated by both madanin-1 WT and madanin-1 2S treatment. In MDA-MB-231 cells, thrombin-induced p-Akt and p-ERK expression was significantly inhibited only by madanin-1 2S treatment. The protein expression levels were normalized to the level of β-actin in the same sample. Data are presented as the mean relative density ratio of each protein (phosphorylated form/total form) ± standard deviation of three independent experiments completed in triplicate (*P<0.05). WT, wild-type; 2S, 2 sulfation; p, phosphorylated; t, total; ERK, extracellular signal-regulated kinase.

vimentin expression, was analyzed using western blotting (Fig. 5). To investigate the effect of madanin-1 on the epithelial-mesenchymal transition (EMT) process, experiments were performed using highly aggressive MDA-MB-231 cells, well characterized for their high metastatic phenotype. Cells were left untreated or were pretreated with 10 μg/ml madanin-1 WT or madanin-1 2S for 30 min, followed by treatment with thrombin (2 U/ml for 24 h). The results showed that thrombin significantly suppressed E-cadherin expression, and the expression was significantly increased by madanin-1 2S but not by madanin-1 WT. Furthermore, thrombin-induced N-cadherin and vimentin expression was significantly suppressed by madanin-1 2S but not by madanin-1 WT.

Effects of madanin-1 WT and madanin-1 2S on cancer cell viability and apoptosis. Cell viability (Fig. S1), and apoptosis in SKOV3 (Fig. S2) and MDA-MB-231 (Fig. S3) cells upon treatment with madanin-1 WT or madanin-1 2S were detected using the MTT assay and apoptosis assay, respectively. Cells were pretreated with madanin-1 WT or madanin-1 2S for 1 h, followed by treatment with or without thrombin (2 U/ml). The results showed that no significant levels of apoptosis and no significant changes in cell viability were induced in SKOV3 and MDA-MB-231 cells by treatment with madanin-1 WT or madanin-1 2S, either in the presence or absence of thrombin.

Discussion

The present study investigated whether tyrosine-sulfated madanin-1 2S, a thrombin inhibitor, affected malignant tumor cell behavior compared with madanin-1 WT, using SKOV3 and MDA-MB-231 cells. Using a wound healing assay and Transwell cell invasion assay, it was demonstrated that madanin-1 2S significantly attenuated thrombin-induced cancer cell migration and invasion. To the best of our knowledge, this is the first study to investigate the effects of madanin-1 on thrombin-related signaling pathways as well as cancer cell behavior.

Knowledge of the association between hypercoagulability and the poor prognosis of cancer has grown considerably (7,28). There are a number of mechanisms that occur in cancer that can induce thrombin production (29). This is demonstrated in invasive breast cancer where fibroblast and epithelial expression of thrombin are significantly increased (30). Thrombin activates signaling pathways, including the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) cascade via PAR-1 (31); and stimulation of PAR-1

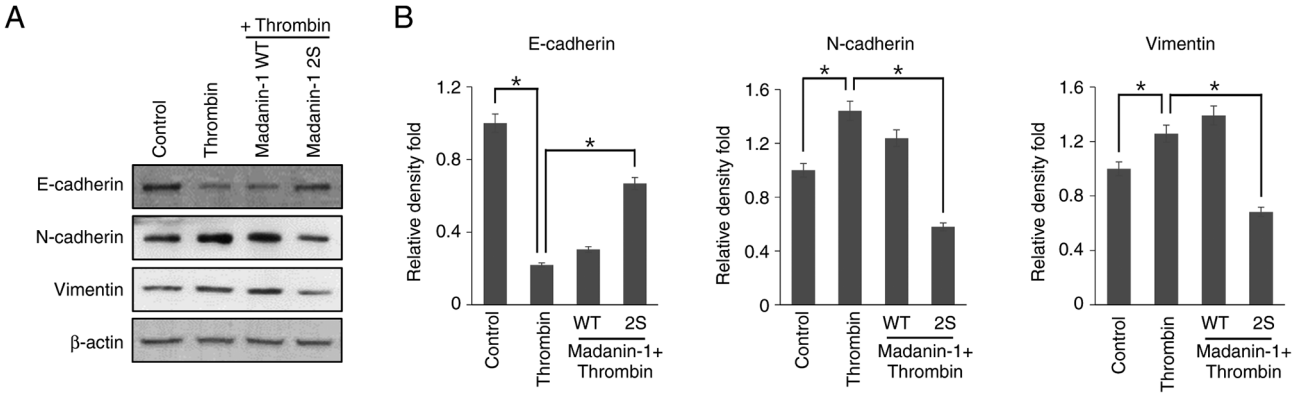

Figure 5. Effects of madanin-1 WT and madanin-1 2S on the expression of E-cadherin, N-cadherin and vimentin in MDA-MB-231 cells. MDA-MB-231 cells were pretreated with madanin-1 WT or madanin-1 2S (10 μg/ml for 30 min) prior to treatment with thrombin (2 U/ml for 24 h). The protein expression levels of E-cadherin, N-cadherin and vimentin were analyzed in MDA-MB-231 cells by western blotting. (A) Representative gel images. (B) Madanin-1 2S significantly increased E-cadherin expression, which was suppressed by thrombin treatment. The expression of N-cadherin and vimentin induced by thrombin was significantly decreased in response to madanin-1 2S. The protein expression levels were normalized to the level of β-actin in the same sample. Data are presented as the mean relative density ratio ± standard deviation of three independent experiments completed in triplicate (*P<0.05). WT, wild-type; 2S, 2 sulfation.

on tumor cells and stromal cells mediates diverse effects, including cancer progression, inflammation and immunosuppression (32,33). Moreover, Zhong *et al* (25) reported that thrombin treatment of SKOV3 cells not only increases invasion but also upregulates PAR-1. The therapeutic effects of inhibiting thrombin action via PAR-1 inhibitors have been reported in cell experiments; in inflammatory breast cancer (34), PAR-1 antagonist SCH 79797 reduced thrombin-induced invasion of cell lines, and in epithelial ovarian cancer (21), PAR-1 antagonist vorapaxar reversed thrombin-induced proliferation of cell lines. Furthermore, in previous animal experiments, the group injected with PAR-1-deficient lung cancer cells showed a higher survival rate and lower tumor volume (35). Likewise, the data from the present study showed that thrombin treatment increased p-Akt and p-ERK expression, as well as cancer migration and invasion in SKOV3 and MDA-MB-231 cells. It is hypothesized that thrombin inhibition by madanin-1 2S could effectively block PAR-1 signaling in the tumor microenvironment.

The process of tyrosine sulfation, which is a post-translational modification, is involved in various biological processes, including hemostasis (36). For example, sulfation of a tyrosine residue within the acidic tail of the anticoagulant hirudin increases the affinity of hirudin for thrombin by >10-fold (37). A total of two tyrosine residues (Y32 and Y35) of madanin-1 are found within acidic stretches and provide suitable sites for sulfation by tyrosylprotein sulfotransferase enzymes that catalyze the transfer of the sulfuryl group (17,38). Thompson *et al* (17) demonstrated that disulfated variants of madanin-1 at Y32 and Y35 can enhance the magnitude of thrombin inhibition relative to unsulfated madanin-1. The disulfated madanin-1 has been shown to prolong TT to a similar degree at 500-fold lower concentrations than unsulfated madanin-1 (28.7±0.9 sec at 0.01 and 5 μM, respectively) (17). In the present study, recombinant tyrosine-sulfated madanin-1 protein was manufactured using pET-41a and pSUPAR6-L3-3SY plasmid in an *E. coli* cell system, and this purified madanin-1 2S protein was used for the experiments. The present study showed that the migration and invasion of SKOV3 and MDA-MB-231 cells induced by thrombin were more effectively suppressed by madanin-1 2S treatment than by madanin-1 WT.

EMT is a reversible cellular process that allows epithelial cells to acquire mesenchymal features, which increases the metastatic potential of cancer cells and confers resistance to chemotherapy and immunotherapy (39). EMT activation triggers the downregulation of E-cadherin, and upregulation of vimentin and N-cadherin, which are considered to play vital roles in inducing invasion and metastasis (39,40). E-cadherin is known to be a potent tumor suppressor, and the upregulated expression of N-cadherin negatively affects the overall survival and progression of patients with cancer (41,42). There is mounting evidence that targeting E-cadherin and N-cadherin could be a promising approach for cancer treatment (41-44). In the context of thrombin, several studies have reported that thrombin induces EMT in various cells, including gastric (45) and ovarian cancer cells (25). The data from the present study were consistent with these studies whereby thrombin significantly reduced E-cadherin expression in MDA-MB-231 cells, whereas its expression was significantly increased in response to madanin-1 2S treatment, but not madanin-1 WT treatment. Moreover, madanin-1 2S significantly reduced thrombin-induced expression of vimentin and N-cadherin, while madanin-1 WT showed no effect. The switch in expression from E-cadherin to N-cadherin by thrombin indicates activation of the EMT process, and these findings suggested that madanin-1 2S may have a therapeutic effect by inhibiting metastasis.

The PI3K/Akt/mammalian target of rapamycin (mTOR) and MAPK/ERK signaling pathways, which regulate fundamental cellular functions, are activated downstream from cell surface receptors, such as receptor tyrosine kinases and GPCRs (46). Mutations in the molecules involved in these pathways have been found in various types of cancer, and dysregulated signaling transduction plays an important role in mediating oncogenic signals, enhancing cancer cell growth, survival and metabolism (47). Moreover, activated Akt and ERK are also implicated in orchestrating EMT by regulating EMT-inducing transcription factors (39,48). Accordingly, research and clinical

trials for anticancer agents targeting molecules in these pathways are being actively conducted (46,47,49). Several agents are used in clinical practice, such as alpelisib (PI3K p110α inhibitor) and everolimus (mTOR complex 1 inhibitor), which have been approved by the US Food and Drug Administration for the treatment of advanced breast cancer (50). In the present study, thrombin-induced expression of p-Akt and p-ERK was significantly inhibited by madanin-1 2S in both SKOV3 and MDA-MB-231 cells. These results suggested a potential molecular mechanism wherein madanin-1 2S inhibits thrombin-induced Akt and ERK signaling pathways, subsequently impeding cancer development, including EMT.

The exact mechanism of madanin-1 in the pathogenesis of cancer has yet to be elucidated, and further studies on its mode of action are needed. While other cysteine-less thrombin inhibitors, such as variegin and anophelin, occupy the active site of thrombin with strong affinity, madanin-1 competitively binds to thrombin with low affinity (15). Madanin-1 is cleaved by clotting factor Xa as well as thrombin, and thrombin-cleaved madanin-1 consequently loses its inhibitory function (15), limiting its effectiveness as an inhibitor. Thrombin rapidly cleaves both unsulfated and sulfated madanin-1 at specific sites (17). However, unlike cleaved fragments from unmodified madanin-1, synthesized disulfated madanin-1 fragments have been shown to exert a similar inhibitory activity to the full-length molecule (17). These findings indicated that the inhibitory activity of disulfated madanin-1 is not substantially affected by thrombin processing (17), and madanin-1 2S might be a more effective therapeutic agent than madanin-1 WT. To the best of our knowledge, the present study demonstrated the therapeutic potential of madanin-1 2S for cancer for the first time *in vitro*.

Notably, there are some limitations to the present study. Firstly, the lack of reported preclinical studies or therapeutic applications related to madanin-1 results in an insufficient safety profile and pharmacological information. Secondly, madanin-1 2S did not induce significant levels of apoptosis and significant changes in cell viability in cancer cells. However, as the mechanism of madanin-1 2S involves inhibiting the interaction between thrombin and cancer cell receptors, rather than directly inducing cell apoptosis, it is hypothesized to have potential as a novel therapeutic agent for inhibiting the spread of cancer. Finally, as a tumor microenvironment closely related to cancer development (51) was not sufficiently reflected in the present study, further *in vivo* studies and clinical trials are necessary to verify the actual effects and biological mechanisms of sulfated madanin-1.

In conclusion, post-translational sulfation of madanin-1, a small cysteine-free anticoagulant, showed enhanced inhibitory activity on thrombin-induced cancer cell behavior *in vitro*. The strengthened inhibitory effect of madanin-1 2S on thrombin may have resulted in the inhibition of the EMT process, and Akt and ERK phosphorylation, leading to the suppression of breast and ovarian cancer cell migration and invasion. Taken together, the present study suggested the possibility of tyrosine-sulfated madanin-1 as a therapeutic candidate in the field of cancer therapy.

Acknowledgements

The authors would like to thank Ms Soo Hyun Choi (Yonsei University College of Medicine; Seoul, Republic of Korea) for technical assistance.

Funding

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (grant no. 2022R1F1A1060081).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

JHL and JSY conceived and designed the study. GHJ and SAJ performed the experiments. JSY and THR contributed to the data analysis, interpretation, and manuscript writing. JHL and JSY confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Larsen JB and Hvas AM: Thrombin: A pivotal player in hemostasis and beyond. Semin Thromb Hemost 47: 759-774, 2021.
2. Chen LB and Buchanan JM: Mitogenic activity of blood components. I. Thrombin and prothrombin. Proc Natl Acad Sci USA 72: 131-135, 1975.
3. Maruyama I, Shigeta K, Miyahara H, Nakajima T, Shin H, Ide S and Kitajima I: Thrombin activates NF-kappa B through thrombin receptor and results in proliferation of vascular smooth muscle cells: Role of thrombin in atherosclerosis and restenosis. Ann N Y Acad Sci 811: 429-436, 1997.
4. Zhou S, Xiao W, Pan X, Zhu M, Yang Z, Zhang F and Zheng C: Thrombin promotes proliferation of human lung fibroblasts via protease activated receptor-1-dependent and NF-κB-independent pathways. Cell Biol Int 38: 747-756, 2014.
5. Szaba FM and Smiley ST: Roles for thrombin and fibrin(ogen) in cytokine/chemokine production and macrophage adhesion in vivo. Blood 99: 1053-1059, 2002.
6. Chiang HS, Yang RS and Huang TF: Thrombin enhances the adhesion and migration of human colon adenocarcinoma cells via increased beta 3-integrin expression on the tumour cell surface and their inhibition by the snake venom peptide, rhodostomin. Br J Cancer 73: 902-908, 1996.
7. Nierodzik ML and Karpatkin S: Thrombin induces tumor growth, metastasis, and angiogenesis: Evidence for a thrombin-regulated dormant tumor phenotype. Cancer Cell 10: 355-362, 2006.
8. Franchini M and Mannucci PM: Thrombin and cancer: From molecular basis to therapeutic implications. Semin Thromb Hemost 38: 95-101, 2012.
9. Cantrell R and Palumbo JS: The thrombin-inflammation axis in cancer progression. Thromb Res 191 (Suppl 1): S117-S122, 2020.
10. Covic L and Kuliopulos A: Protease-activated receptor 1 as therapeutic target in breast, lung, and ovarian cancer: Pepducin approach. Int J Mol Sci 19: 2237, 2018.

11. Liu X, Yu J, Song S, Yue X and Li Q: Protease-activated receptor-1 (PAR-1): A promising molecular target for cancer. Oncotarget 8: 107334-107345, 2017.
12. Jacobs JW, Cupp EW, Sardana M and Friedman PA: Isolation and characterization of a coagulation factor Xa inhibitor from black fly salivary glands. Thromb Haemost 64: 235-238, 1990.
13. Waxman L, Smith DE, Arcuri KE and Vlasuk GP: Tick anticoagulant peptide (TAP) is a novel inhibitor of blood coagulation factor Xa. Science 248: 593-596, 1990.
14. Salzet M: Leech thrombin inhibitors. Curr Pharm Des 8: 493-503, 2002.
15. Figueiredo AC, de Sanctis D and Pereira PJ: The tick-derived anticoagulant madanin is processed by thrombin and factor Xa. PLoS One 8: e71866, 2013.
16. Iwanaga S, Okada M, Isawa H, Morita A, Yuda M and Chinzei Y: Identification and characterization of novel salivary thrombin inhibitors from the ixodidae tick, *Haemaphysalis longicornis*. Eur J Biochem 270: 1926-1934, 2003.
17. Thompson RE, Liu X, Ripoll-Rozada J, Alonso-Garcia N, Parker BL, Pereira PJB and Payne RJ: Tyrosine sulfation modulates activity of tick-derived thrombin inhibitors. Nat Chem 9: 909-917, 2017.
18. Khorana AA, Mackman N, Falanga A, Pabinger I, Noble S, Ageno W, Moik F and Lee AYY: Cancer-associated venous thromboembolism. Nat Rev Dis Primers 8: 11, 2022.
19. Asanuma K, Wakabayashi H, Okamoto T, Asanuma Y, Akita N, Yoshikawa T, Hayashi T, Matsumine A, Uchida A and Sudo A: The thrombin inhibitor, argatroban, inhibits breast cancer metastasis to bone. Breast Cancer 20: 241-246, 2013.
20. Alexander ET, Minton AR, Peters MC, van Ryn J and Gilmour SK: Thrombin inhibition and cisplatin block tumor progression in ovarian cancer by alleviating the immunosuppressive microenvironment. Oncotarget 7: 85291-85305, 2016.
21. Chanakira A, Westmark PR, Ong IM and Sheehan JP: Tissue factor-factor VIIa complex triggers protease activated receptor 2-dependent growth factor release and migration in ovarian cancer. Gynecol Oncol 145: 167-175, 2017.
22. Chaffer CL and Weinberg RA: A perspective on cancer cell metastasis. Science 331: 1559-1564, 2011.
23. Radjabi AR, Sawada K, Jagadeeswaran S, Eichbichler A, Kenny HA, Montag A, Bruno K and Lengyel E: Thrombin induces tumor invasion through the induction and association of matrix metalloproteinase-9 and beta1-integrin on the cell surface. J Biol Chem 283: 2822-2834, 2008.
24. Kim J, Lee JW, Kim SI, Choi YJ, Lee WK, Jeong MJ, Cha SH, Lee HJ, Chun W and Kim SS: Thrombin-induced migration and matrix metalloproteinase-9 expression are regulated by MAPK and PI3K pathways in C6 glioma cells. Korean J Physiol Pharmacol 15: 211-216, 2011.
25. Zhong YC, Zhang T, Di W and Li WP: Thrombin promotes epithelial ovarian cancer cell invasion by inducing epithelial-mesenchymal transition. J Gynecol Oncol 24: 265-272, 2013.
26. Justus CR, Leffler N, Ruiz-Echevarria M and Yang LV: In vitro cell migration and invasion assays. J Vis Exp: 51046, 2014.
27. Liu CC, Cellitti SE, Geierstanger BH and Schultz PG: Efficient expression of tyrosine-sulfated proteins in *E. coli* using an expanded genetic code. Nat Protoc 4: 1784-1789, 2009.
28. Kirwan CC, Descamps T and Castle J: Circulating tumour cells and hypercoagulability: A lethal relationship in metastatic breast cancer. Clin Transl Oncol 22: 870-877, 2020.
29. Reddel CJ, Tan CW and Chen VM: Thrombin generation and cancer: Contributors and consequences. Cancers (Basel) 11: 100, 2019.
30. Shaker H, Bundred NJ, Landberg G, Pritchard SA, Albadry H, Nicholson SL, Harries LJ, Heah JYE, Castle J and Kirwan CC: Breast cancer stromal clotting activation (tissue factor and thrombin): A pre-invasive phenomena that is prognostic in invasion. Cancer Med 9: 1768-1778, 2020.
31. Ossovskaya VS and Bunnett NW: Protease-activated receptors: Contribution to physiology and disease. Physiol Rev 84: 579-621, 2004.
32. Wojtukiewicz MZ, Hempel D, Sierko E, Tucker SC and Honn KV: Protease-activated receptors (PARs)-biology and role in cancer invasion and metastasis. Cancer Metastasis Rev 34: 775-796, 2015.
33. Alexander ET and Gilmour SK: Immunomodulatory role of thrombin in cancer progression. Mol Carcinog 61: 527-536, 2022.
34. Ohshiro K, Bui-Nguyen TM, Divijendra Natha RS, Schwartz AM, Levine P and Kumar R: Thrombin stimulation of inflammatory breast cancer cells leads to aggressiveness via the EGFR-PAR1-Pak1 pathway. Int J Biol Markers 27: e305-e313, 2012.
35. Zhao B, Wu M, Hu Z, Wang T, Yu J, Ma Y, Wang Q, Zhang Y, Chen D, Li T, *et al*: A novel oncotherapy strategy: Direct thrombin inhibitors suppress progression, dissemination and spontaneous metastasis in non-small cell lung cancer. Br J Pharmacol 179: 5056-5073, 2022.
36. Yang YS, Wang CC, Chen BH, Hou YH, Hung KS and Mao YC: Tyrosine sulfation as a protein post-translational modification. Molecules 20: 2138-2164, 2015.
37. Corral-Rodriguez MA, Macedo-Ribeiro S, Pereira PJ and Fuentes-Prior P: Leech-derived thrombin inhibitors: From structures to mechanisms to clinical applications. J Med Chem 53: 3847-3861, 2010.
38. Ouyang YB and Moore KL: Molecular cloning and expression of human and mouse tyrosylprotein sulfotransferase-2 and a tyrosylprotein sulfotransferase homologue in Caenorhabditis elegans. J Biol Chem 273: 24770-24774, 1998.
39. Dongre A and Weinberg RA: New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer. Nat Rev Mol Cell Biol 20: 69-84, 2019.
40. Huber MA, Kraut N and Beug H: Molecular requirements for epithelial-mesenchymal transition during tumor progression. Curr Opin Cell Biol 17: 548-558, 2005.
41. Zhu X, Wang X, Gong Y and Deng J: E-cadherin on epithelial-mesenchymal transition in thyroid cancer. Cancer Cell Int 21: 695, 2021.
42. Mrozik KM, Blaschuk OW, Cheong CM, Zannettino ACW and Vandyke K: N-cadherin in cancer metastasis, its emerging role in haematological malignancies and potential as a therapeutic target in cancer. BMC Cancer 18: 939, 2018.
43. Song Y, Ye M, Zhou J, Wang ZW and Zhu X: Restoring E-cadherin expression by natural compounds for anticancer therapies in genital and urinary cancers. Mol Ther Oncolytics 14: 130-138, 2019.
44. Song Y, Ye M, Zhou J, Wang Z and Zhu X: Targeting E-cadherin expression with small molecules for digestive cancer treatment. Am J Transl Res 11: 3932-3944, 2019.
45. Otsuki T, Fujimoto D, Hirono Y, Goi T and Yamaguchi A: Thrombin conducts epithelial-mesenchymal transition via protease-activated receptor-1 in human gastric cancer. Int J Oncol 45: 2287-2294, 2014.
46. Cao Z, Liao Q, Su M, Huang K, Jin J and Cao D: AKT and ERK dual inhibitors: The way forward? Cancer Lett 459: 30-40, 2019.
47. Asati V, Mahapatra DK and Bharti SK: PI3K/Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways inhibitors as anticancer agents: Structural and pharmacological perspectives. Eur J Med Chem 109: 314-341, 2016.
48. Karimi Roshan M, Soltani A, Soleimani A, Rezaie Kahkhaie K, Afshari AR and Soukhtanloo M: Role of AKT and mTOR signaling pathways in the induction of epithelial-mesenchymal transition (EMT) process. Biochimie 165: 229-234, 2019.
49. Li Q, Li Z, Luo T and Shi H: Targeting the PI3K/AKT/mTOR and RAF/MEK/ERK pathways for cancer therapy. Mol Biomed 3: 47, 2022.
50. Narayan P, Prowell TM, Gao JJ, Fernandes LL, Li E, Jiang X, Qiu J, Fan J, Song P, Yu J, *et al*: FDA approval summary: Alpelisib plus fulvestrant for patients with HR-positive, HER2-negative, PIK3CA-mutated, advanced or metastatic breast cancer. Clin Cancer Res 27: 1842-1849, 2021.
51. Yuan Y, Jiang YC, Sun CK and Chen QM: Role of the tumor microenvironment in tumor progression and the clinical applications (review). Oncol Rep 35: 2499-2515, 2016.