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ORIGINAL ARTICLE

A curcumin analog CA-5f inhibits urokinase-type plasminogen activator and invasive phenotype of triple-negative breast cancer cells

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Abstract

Triple-negative breast cancer (TNBC) is one of the most aggressive types of breast cancer with poor outcomes. Patients with TNBC cannot benefit from targeted therapies such as Tamoxifen and Herceptin. The aim of the present study was to seek a preventive or therapeutic agent with a potential inhibitory effect on aggressive progression of TNBC. Anticancer effect of a natural compound curcumin have been demonstrated, however, development of more effective curcumin analogs with better bioavailability is needed. We investigated if a curcumin analog CA-5f could inhibit the invasive phenotype of TNBC cell lines in the present study. Treatment with CA-5f inhibited the viability of MDA-MB-231 and Hs578T TNBC cells, possible by inducing apoptosis. The invasive phenotypes of these cells were inhibited by CA-5f in a concentration-dependent manner. Protein expression of urokinase-type plasminogen activator (uPA), a serine protease known to degrade the extracellular matrix and lead to invasion, was markedly decreased by CA-5f in Hs578T cells. However, mRNA level of uPA was not altered by CA-5f, implicating that the effect of CA-5f was not through transcriptional regulation. Of note, CA-5f upregulated plasminogen activator inhibitor type (PAI)-1, which is known to inhibit uPA by interacting with urokinase-type plasminogen receptor, in TNBC cells. Taken together, these results demonstrated that CA-5f significantly inhibited the invasive phenotype of TNBC cells, possibly by decreasing the protein level of uPA through upregulating PAI-1. Our results may provide useful information on developing CA-5f as a potential therapeutic agent against malignant progression of TNBC.

Keywords Triple-negative breast cancer · CA-5f · uPA · PAI-1 · Invasion

Abbreviations

ECM	Extracellular matrix
ER	Estrogen receptor
HER2	Human epidermal growth factor receptor 2
MMP	Matrix metalloproteinase
NSCLC	Non-small-cell lung cancer
PAI-1	Plasminogen activator inhibitor type 1
PARP	Poly (ADP-ribose) polymerase
PR	Progesterone receptor
ROS	Reactive oxygen species
TNBC	Triple-negative breast cancer
uPA	Urokinase-type plasminogen activator

Introduction

Breast cancer is the most common cause of cancer death for women worldwide [1]. It has a high likelihood to invade other organs and cause metastasis [2]. Triple-negative breast cancer (TNBC) is an aggressive invasive type of breast cancer that is negative for estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) expression [3]. Hormone therapy is not available for TNBC. Currently there is no clear targeted chemotherapy regimen for TNBC either.

Metastasis is a highly complex process involving cancer cell proliferation and invasion, both of which are remarkably correlated with tumor aggressiveness and poor prognosis [4]. A poor prognosis of TNBC is associated with metastasis. Many TNBC patients develop metastasis disease much earlier than estrogen receptor-positive patients [5]. Therefore, preventing metastasis of TNBC is important for the treatment of TNBC.

The key step in metastasis is the process of invasion, which is associated with matrix-degrading activity [6].

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Critical proteases involved in the degradation of extracellular matrix (ECM) include serine proteases (plasmin), matrix metalloproteinase (MMP), and urokinase plasminogen activator (uPA) [7, 8]. Activated uPA protease can cleave inactive plasminogen to form enzymatically active plasmin [9], which in turn cleaves pro-MMP into active-MMP [10, 11]. The plasminogen activator inhibitor type (PAI)-1 inhibits uPA by interacting with urokinase-type plasminogen receptor [12]. It can effectively inhibit metastasis of cancer cells by inhibiting activation of uPA [13]. Although PAI-1 expression in the tumor stroma of patient specimens seems to promote angiogenesis, tumor metastasis, and tumor invasion, overexpression of PAI-1 directly in tumor cells can decrease cellular proteolysis, primary tumor growth, and metastasis [14].

Natural compounds and their analogs have been extensively used for medicinal chemistry and drug discovery. More than half of anti-tumor drugs are obtained from natural scaffolds [15]. It has been shown that curcumin can inhibit the proliferation of diverse tumor cells using in vitro cell culture models and inhibit the growth of human tumors in xenograft models [16]. In our previous study, we have shown that curcumin can inhibit the invasive phenotype of H-Ras-transformed MCF10A human breast epithelial cells [17]. A recent study reported that invasion and proliferation of TNBC cells can be inhibited by treatment with curcumin [18]. Although anticancer properties of curcumin have been highlighted in the treatment of several types of cancer, difficulties associated with its low bioavailability, inadequate distribution, and kinetics along with a poor solubility limit its routine use in patients [19]. Therefore, the design of more effective curcumin analogs has been required [20].

Among a series of curcumin analogs, (3E,5E)-3-(3,4-dimethoxybenzyliene)-5-[(1H-indol-3-yl)methylene]-1methylpiperidin-4-one (CA-5f) has shown promising anticancer effects [21]. CA-5f effectively suppressed tumor growth and induced apoptosis of A549 non-small cell lung cancer cells [21]. This compound exerted cytotoxic effects by increasing level of mitochondrial-derived reactive oxygen species (ROS). CA-5f-mediated blockade of autophagosome-lysosome fusion might be related with its inhibition of cytoskeletal and membrane vesicle trafficking protein expression [21]. CA-5f was reported to inhibit autophagy and suppress tumor growth in A549 NSCLC [21], while curcumin induced autophagy in breast cancer cells [22, 23]. Since autophagy contributes to progression of TNBC, inhibition of autophagy would be beneficial for anticancer strategies against TNBC [24–26].

In the present study, we aimed to investigate the anticancer effect of CA-5f against aggressive progression of TNBC cells. Here, we demonstrated the inhibitory effect of CA-5f on the invasive phenotype of TNBC cells. We further showed that CA-5f effectively inhibited uPA expression and upregulated its inhibitor PAI-1 in TNBC cells, providing information on therapeutic and/or preventive strategies against malignant progression of TNBC.

Materials and methods

Cell lines and culture conditions

Human breast carcinoma Hs578T cells were purchased form Korean Cell Line Bank (KCLB, Korea) and cultured in DMEM media (cat. no. SH30243.01; HyClone; Thermo Fisher Sceintific, Inc.) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. Human breast carcinoma MDA-MB-231 cells were purchased from the KCLB and cultured in RPMI-1640 media (cat. No. 10-041-CVR; Corning Life Sciences) supplemented with 10% FBS and 1% penicillin–streptomycin. These cells were maintained in a humidified atmosphere with 5% CO_2 at 37℃.

Chemicals

CA-5f was purchased from Med Chem Express (MCE, USA). Structure of CA-5f is depicted in Fig. 1. Powdered CA-5f was dissolved in dimethyl sulfoxide (DMSO, Sigma Aldrich Co., USA) to 10 mM stock solution and stored at − 80 °C. The stock solution was added to the cell culture medium immediately before the experiment. The concentration of DMSO in the media was maintained below 0.1% because DMSO at concentrations of 0.1% and 0.25% caused little or no damage in cell viability.

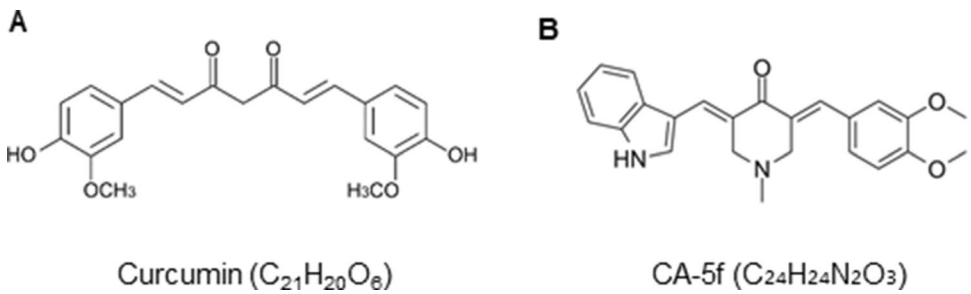

Fig. 1 **a** Chemical structure of curcumin with a molecular weight of 368.39 g/mol [17]. **b** Chemical structure of CA-5f with a molecular weight of 388.46 g/mol [21]

3-((4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay

To investigate the effect of CA-5f on cell viability, MTT assay was performed. Cells cultured in a 96-well plate were treated with various concentrations of CA-5f in serum-free media for 24 h and 48 h. MTT solution at 0.5 mg/mL was prepared by dissolving 3-((4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Sigma Aldrich Co., USA) in phosphate buffer saline. After 24 h and 48 h of treatment with CA-5f, 25 µL of MTT solution was added into each well and incubated for 4 h. Since metabolically active cells could convert MTT into purple formazan, the formation of purple formazan could suggest the extent of cell viability. After the medium in each well was removed, 100 µL of DMSO was added to dissolve formazan crystals produced. The optical density was measured at 540 nm using a micro-enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, USA) to quantify cell viability. The percentage of cell viability was defined as the relative absorbance of treated cells *versus* control cells. Assays were performed in triplicate.

In vitro invasion assay

In vitro invasion assay was performed using a 24-well transwell unit with polyethylene terephthalate filters (Corning Falcon®, USA). The lower side of the filter was coated with type I collagen (Sigma Aldrich Co., USA) and upper side was coated with Matrigel (BD Bioscience, USA). Hs578T cells (2×10^4 cells/well) or MDA-MB-231 cells (3×10^4 cells/well) were seeded onto the upper compartment of a 24-well transwell plate. These cells were cultured with CA-5f treated serum-free media (SFM), incubated for 24 h, fixed with methanol, and stained with 0.5% crystal violet (Sigma Aldrich Co., USA) for 20 min. Invasive phenotypes were determined for cells that migrated to the lower side of the filter under microscopy at × 100. Filters were cut, dissolved with 30% acetic acid, and measured at 595 nm using a micro-enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, USA) to quantify invasive cells. The percentage of cell invasion was defined as the relative absorbance of treated cells *versus* control cells. Assays were performed in triplicate.

Immunoblot analysis

Immunoblot analysis was performed as previously described [27]. Whole-cell lysates were prepared using sodium dodecyl sulfate (SDS) lysis buffer. Anti-uPA (cat. No. MAB1310; dilution 1:1000) was obtained from R&D System. Anti-PAI-1 (cat. No. #11907; dilution 1:1000) was purchased from Cell Signaling. Secondary antibodies that matched primary antibody's origin were used. A Western Bright ECL kit (Advansta Inc.) was used for band detection. Assays were performed in triplicate.

Reverse transcriptase (RT)-PCR

RT-PCR was performed as previously described [28]. For human *uPA* and *SERPINE1/PAI-1* detection, the following primers were used: *uPA* forward primer, 5'-AAAATGCTG TGTGCTGCTGACC-3'; *uPA* reverse primer, 5'-CCCTGC CCTGAAGTCGTTAGTG-3'; *SERPINE1/PAI-1* forward primer, 5'-CTCATCAGCCACTGGAAAGGCA-3'; *SERPINE1/PAI-1* reverse primer, 5'-GACTCGTGAAGTCAG CCTGAAAC-3'. PCR reactions were performed using an EmeraldAmp GT PCR master mix (Takara, Japan). Assays were performed in triplicate.

Statistical analysis

The data were analyzed with AlphaView SA software (version 3.4.0.0). Unpaired two-tailed Student's *t* test or one-way analysis of variance (ANOVA) was used to determine significant differences in in vitro experiments. Three independent experiments were performed. Data are expressed as mean ± standard deviation (SD).

Results

CA-5f exerts cytotoxicity to TNBC cells

We examined the cytotoxic effect of CA-5f (depicted in Fig. 1 [21]) on Hs578T and MDA-MB-231 TNBC cells by conducting MTT assays. As shown in Fig. 2A, the viability of Hs578T cells was significantly decreased by 20 µM CA-5f after treatment for 48 h (35% of control). Treatment with 20 µM CA-5f for 48 h inhibited the viability of MDA-MB-231 cells by 42% (Fig. 2B).

To investigate if CA-5f affected apoptosis, we detected the cleavage of poly (ADP-ribose) polymerase (PARP), an apoptotic marker [29] upon CA-5f treatment. There was no prominent change in PARP cleavage in Hs578T cells after treatment with CA-5f up to 10 µM concentration for 24 h or 48 h (Fig. 2C). A PARP cleavage was observed by treatment with 20 µM CA-5f, demonstrating that CA-5f at concentration of 20 µM exerted cytotoxic effect of TNBC cells, possibly by inducing apoptosis.

Treatment with CA-5f effectively inhibits invasion of TNBC cells

We next investigated the effect of CA-5f on the invasiveness of TNBC cells by performing the in vitro transwell

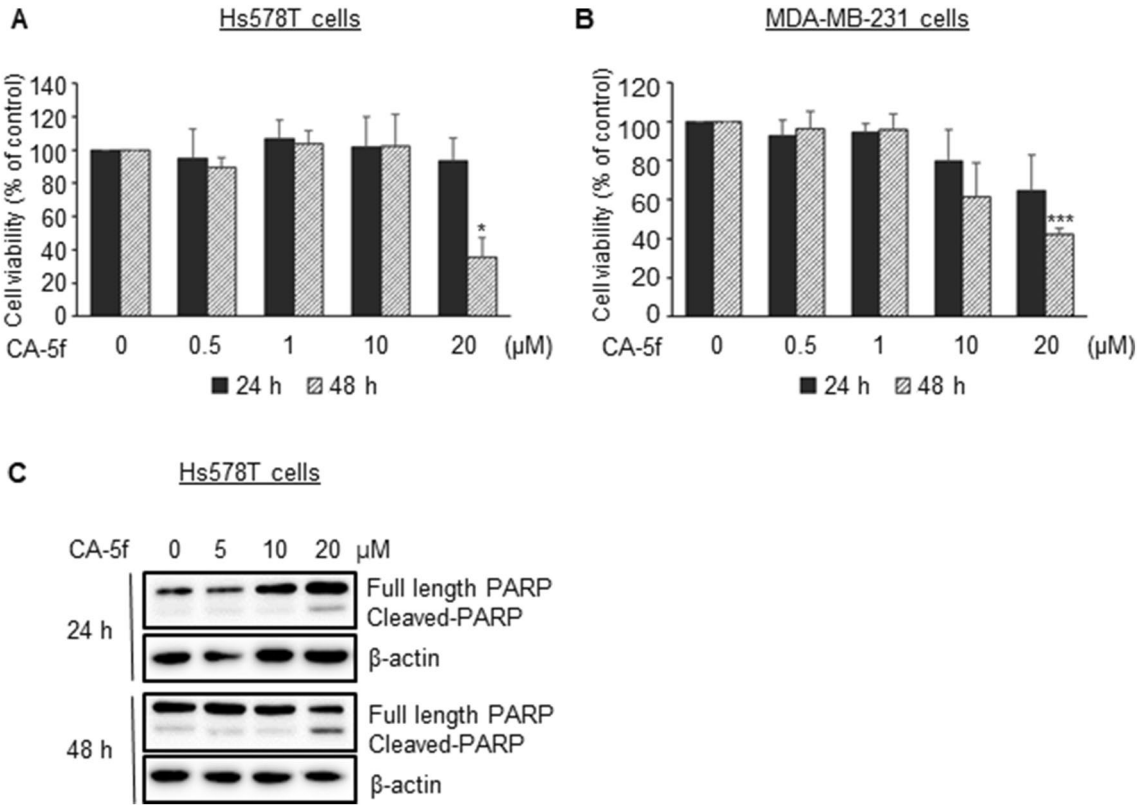

Fig. 2 Cell viabilities of Hs578T and MDA-MB-231 TNBC cells after treatment with CA-5f were determined. **a**, **b** Cells were treated with CA-5f and then analyzed by MTT assay. Hs578T cells (2×10^4) in a 96-well plate were treated with various concentrations of CA-5f for 24 h and 48 h (**a**). MDA-MB-231 cells (2×10^4) in a 96-well plate were treated with various concentrations of CA-5f for 24 h and 48 h (**b**). **c** Hs578T cells were treated with CA-5f at various concentrations for 24 h and 48 h. Control cells were treated with DMSO. Immunoblot analysis was conducted. Data are presented as mean ± SD ($n=3$; unpaired two-tailed Student's t test); *Statistically significant at $p<0.05$; **Statistically significant at $p<0.01$

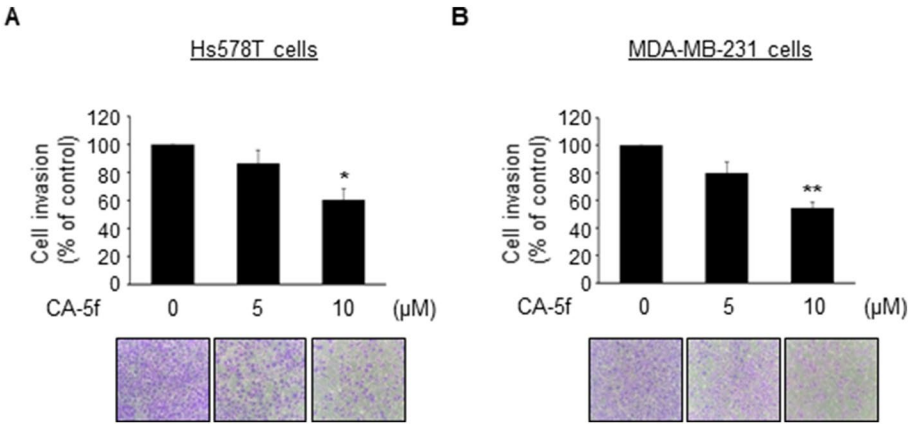

Fig. 3 CA-5f suppresses invasive phenotype of TNBC cells. **a**, **b** Transwell invasion assay was conducted on Hs578T cells (**a**) and MDA-MB-231 cells (**b**) treated with 5 μM or 10 μM CA-5f for 24 h. Data are presented as mean ± SD ($n=3$; unpaired two-tailed Student's t test); *Statistically significant at $p<0.05$; **Statistically significant at $p<0.01$. Representative photos of transwell filters were shown (bottom)

invasion assay. To exclude the cytotoxic effect of CA-5f, we treated the cells with 5 µM and 10 µM CA-5f, the concentrations that would not cause cell death (Fig. 2). As shown in Fig. 3A, CA-5f inhibited the invasive phenotype of Hs578T cells in a concentration-dependent manner. Similar results were obtained using MDA-MB-231 cells (Fig. 3B). These data clearly indicated that CA-5f effectively inhibited the invasive phenotype of TNBC cells.

CA-5f reduces protein expression of pro- and active-uPA, possibly by upregulating PAI-1

We aimed to elucidate the mechanism involved in the inhibitory effect of CA-5f on invasion of TNBC cells. To this end, we first examined if CA-5f inhibited MMP-2 and/or MMP-9 by conducting gelatin zymogram assay. Neither MMP-2 nor MMP-9 was detected in Hs578T and MDA-MB-231 cells (data not shown). We then investigated the effect of CA-5f on uPA expression. Expression levels of pro-uPA (55 kDa) and active uPA (32 kDa) were significantly decreased in Hs578T cells after treatment with CA-5f as evidenced by immunoblot analysis (Fig. 4A). To examine if CA-5f inhibited uPA expression by transcriptional suppression, we conducted RT-PCR analysis. As shown in Fig. 4B, the mRNA level of uPA was not significantly changed by CA-5f treatment, implying that the reduced uPA protein level by CA-5f was not through transcriptional regulation.

We examined the possible involvement of PAI-1, an inhibitor of uPA [30], in CA-5f-induced uPA downregulation. The protein level of PAI-1 in Hs578T cells was

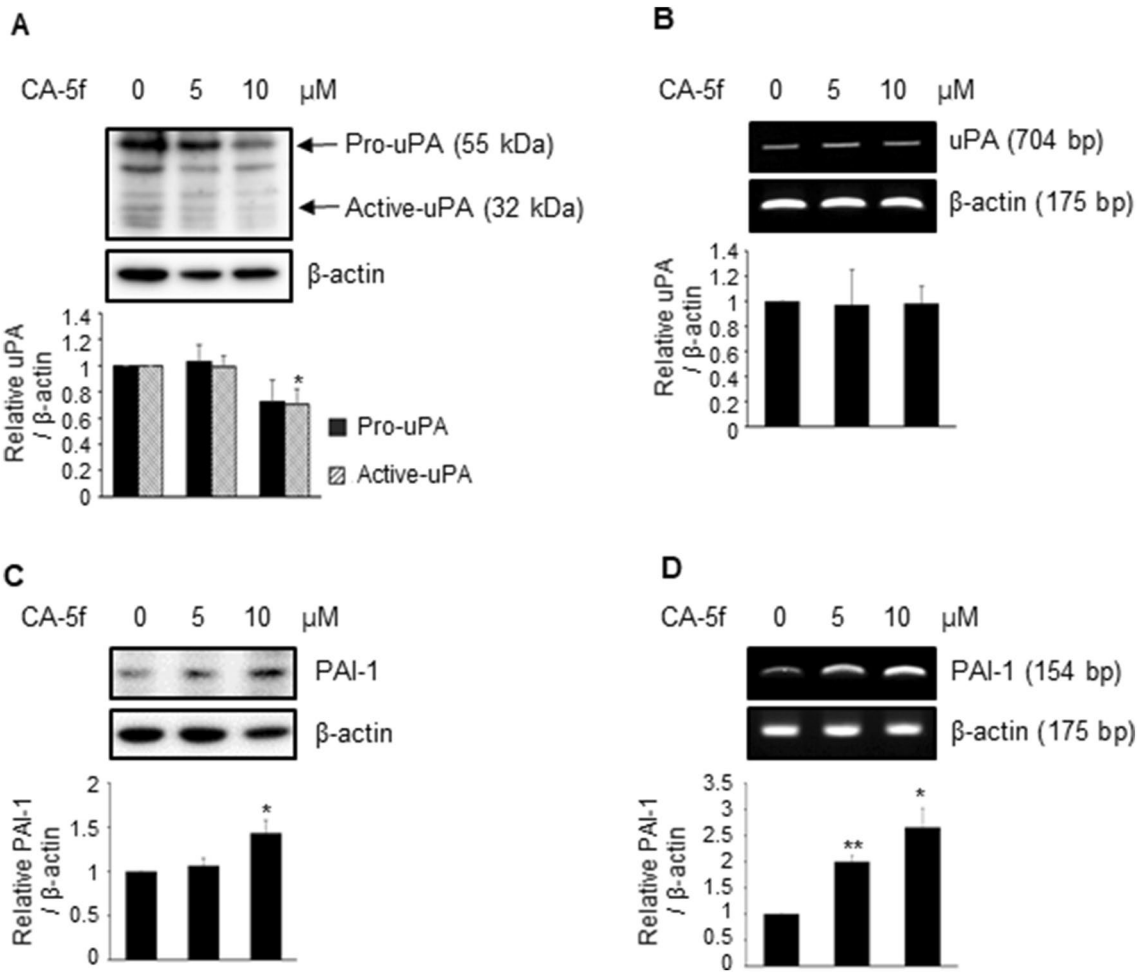

Fig. 4 CA-5f inhibits uPA and increases PAI-1. **a** Hs578T cells were treated with 5 µM or 10 µM CA-5f for 24 h, and subjected to immunoblot analysis to detect pro- and active forms of uPA. Control cells were treated DMSO. ß-actin was used as a loading control. Band intensities were quantitated and plotted. *Statistically significant at $p < 0.05$. **b** RT-PCR was performed to detect mRNA level of uPA. ß-actin was used as a loading control. **c** Hs578T cells were treated with 5 µM or 10 µM CA-5f for 24 h, and subjected to immunoblot analysis to detect PAI-1. Control cells were treated DMSO. ß-actin was used as a loading control. Band intensities were quantitated and plotted. *Statistically significant at $p < 0.05$. **d** RT-PCR was performed to detect mRNA level of PAI-1. ß-actin was used as a loading control. *Statistically significant at $p < 0.05$; **Statistically significant at $p < 0.01$

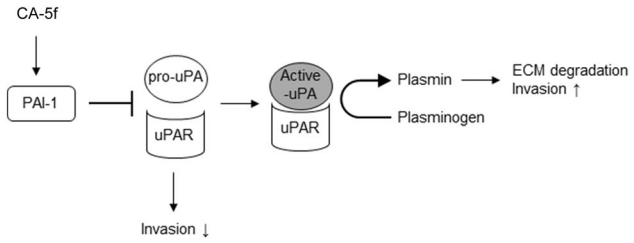

Fig. 5 A proposed model for anti-invasive effect of CA-5f. CA-5f decreased the protein level of uPA, possibly by upregulating PAI-1 in Hs578T TNBC cells

markedly increased by treatment with CA-5f (Fig. 4C). The mRNA level of PAI-1 was also increased by CA-5f in a concentration-dependent manner as evidenced by RT-PCR analysis (Fig. 4D). These data demonstrated that CA-5f decreased the protein level of uPA, possibly by upregulating PAI-1 in Hs578T TNBC cells (depicted in Fig. 5).

Discussion

Curcumin remains an outstanding lead compound for designing more effective analogs [20], despite problems associated with clinical use of curcumin such as its low efficacy and poor absorption characteristics [31]. The present study investigated cytotoxic effect of CA-5f on TNBC cell lines, Hs578T and MDA-MB-231 cells. Viabilities of these two cell lines were significantly decreased by treatment with 20 μM CA-5f for 48 h. We also showed that CA-5f induced apoptosis at a concentration of 20 μM. Consistent with our results, a recent study has revealed that 20 μM CA-5f suppressed cell growth and induced apoptosis in A549 non-small cell lung cancer cell [21].

TNBC has been associated with poor prognosis and metastatic growth [32]. Therefore, suppression of cancer cell invasion and metastasis is extremely important for effective therapies against TNBC [33, 34]. We showed that CA-5f efficiently inhibited the invasive phenotype of TNBC cells, suggesting a potential application of this compound against TNBC progression. Among matrix-degrading enzymes, MMP-2 and MMP-9 are play a crucial role in cancer cell invasion and metastasis [35]. In Hs578T and MDA-MB-231 cells, however, these MMPs were not detected.

uPA has been involved in several physiological and pathological procedures, including cell invasion, tumor growth, and metastasis [30, 36–39]. Calcitonin-mediated inhibition of MDA-MB-231 cell invasion depended on the downregulation of uPA [40]. A positive correlation between uPA expression and tumor stage in MDA-MB-231 TNBC cells was reported [41]. In the present study, our data clearly showed that the protein levels of pro- and active uPA were markedly decreased by CA-5f in Hs578T TNBC cells. The mRNA level of uPA was not altered, suggesting that transcriptional regulation may not be responsible for the inhibitory effect of CA-5f on uPA.

Subsequently, we investigated the possible involvement of PAI-1 which was known to play a crucial role in downregulating uPA in cancer cells [42]. CA-5f significantly increased both protein and mRNA levels of PAI-1 in Hs578T cells. Curcumin induces ROS in breast cancer cells when treated at high concentrations [43]. PAI-1 expression was increased by oxidative stress [44]. Since CA-5f was shown to induce oxidative stress by increasing ROS [21], it is possible that CA-5f may upregulate PAI-1 expression through ROS. Our results imply that CA-5f-induced PAI-1 may decrease uPA, causing inhibition of invasive phenotype of Hs578T cells. It was suggested that the change in the ratio of uPA to PAI-1 might result in a more aggressiveness of cancer cells and lead to poor outcomes of patients in vivo [45, 46]. Further studies would be needed to elucidate detailed molecular mechanisms for the effect of CA-5f on PAI-1, uPA and invasion of TNBC cells. Taken together, our findings present CA-5f as a promising chemopreventive and/or therapeutic agent that may inhibit or control more aggressive progression of TNBC.

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Declarations

Conflict of interest The authors have no conflict of interest to disclose.

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