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Inhibitory effects of a benz[*f*]indole-4,9-dione analog on cancer cell metastasis mediated by the down-regulation of matrix metalloproteinase expression in human HT1080 fibrosarcoma cells

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Abstract

In our previous study, a synthetic benz[*f*]indole-4,9-dione analog, 2-amino-3-ethoxycarbonyl-*N*-methylbenz[*f*]indole-4,9-dione (SME-6), exhibited a potential anti-tumor activity. We, in this study, further explored the anti-metastatic and anti-invasive effect of SME-6 by determining the regulation of matrix metalloproteinases (MMPs). MMPs, zinc-dependent proteolytic enzymes, play a pivotal role in tumor metastasis by cleavage of extracellular matrix as well as non-matrix substrates. On this line, we examined the influence of SME-6 on the expressions of MMP-2, -9, membrane type 1-MMP (MT1-MMP), tissue inhibitor of metalloproteinases (TIMP-1, -2), and in vitro invasiveness of human fibrosarcoma cells. Dose-dependent suppressions of MMPs and TIMP-2 mRNA levels were observed in SME-6-treated HT1080 human fibrosarcoma cells detected by reverse transcriptase–polymerase chain reaction. TIMP-1 mRNA level, however, was induced in a dose-dependent manner. Gelatin zymographic analysis also exhibited a significant down-regulation of MMP-2 and -9 expression in HT1080 cells treated with SME-6 compared to controls. Furthermore, SME-6 inhibited the invasion, motility, and migration of tumor cells. Taken together, these data provide a possible role of SME-6 as a potential antitumor agent with the markedly inhibition of the metastatic and invasive capacity of malignant cells.

Keywords: Benz[*f*]indole-4,9-dione; 2-Amino-3-ethoxycarbonyl-*N*-methylbenz[indole-4,9-dione; Extracellular matrix; Matrix metalloproteinase; Tissue inhibitor of metalloproteinase; Membrane type 1-matrix metalloproteinase

1. Introduction

Matrix metalloproteinases (MMPs) are a multigene family of zinc-dependent endopeptidases capable of degrading essentially all extracellular matrix components, and MMPs have been considered to play an important role in matrix degradation for tumor growth, invasion and tumor-induced angiogenesis (Westermarck and Kähäri, 1999). Among MMPs, MMP-2 and -9 are thought to play critical roles during tumor invasion and metastasis (Birkedal-Hansen et al., 1993; Itoh et al., 1998; Coussen et al., 2000).

Heterocyclic quinones with nitrogen atoms have been reported to exhibit antitumor and antibacterial activities (Silver and Holmes, 1968; Zee-Cheng and Cheng, 1982; Zee-Cheng et al., 1987). In our continuous efforts to develop novel antitumor agents we extended to synthesize benz[*f*]indole-4,9-dione analogs, heterocyclic pyrrole ring derivatives attached to 1,4-naphthoquinone based on the cytotoxic potential of 1,4-naphthoquinones (Fig. 1; Suh and Shin, 1992; SME-6). Benz[*f*]indole-4,9-dione analogs showed potential cell growth inhibitory activity against human cancer cells in our previous study. One probable mechanism of these compounds was suggested the catalytic inhibition of topoisomerase activity (Park et al., 2003). In addition, SME-6 induced G_2/M cell cycle arrest and apoptosis in cultured human lung cancer cells (Lee et al., 2004). However, the exact mechanism of action of this compound remains still unclear.

In this study we further explore to investigate the influence of SME-6, a synthetic benz[*f*]indole-4,9-dione analogue, on the

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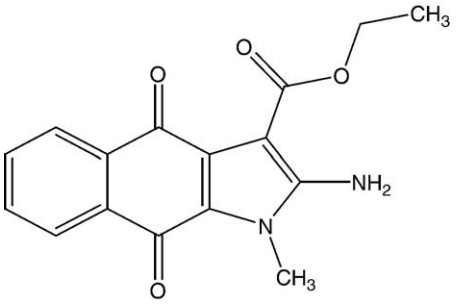

Fig. 1. Chemical structure of 2-amino-3-ethoxycarbonyl-*N*-methylbenz[*f*]indole-4,9-dione (SME-6).

gene expression of MMP-2,-9, membrane type 1-MMP (MT1-MMP), tissue inhibitor of metalloproteinase-1,-2 (TIMP-1,-2), and in vitro invasiveness of human fibrosarcoma cells.

2. Materials and methods

2.1. Materials

All media for cell culture were purchased from Gibco BRL (Grand Island, NY, USA). Human type I collagen, bovine serum albumin, and gelatin were purchased from Sigma (St. Louis, MO, USA). Matrigel was purchased from Becton Dickinson (Bedford, MA, USA). All other chemicals used were of reagent grade. SME-6 was synthesized as described previously (Suh and Shin, 1992) and dissolved in dimethyl sulfoxide (DMSO) for use.

2.2. Cell culture

HT1080 human fibrosarcoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin, and 10% heat-inactivated fetal bovine serum. Cultures were maintained at 37 °C in a 5% CO_2/95% air atmosphere.

2.3. Reverse transcriptase–polymerase chain reaction (RT-PCR)

Total RNA was extracted by the use of TRI reagent (Sigma, St. Louis, MO, USA) and reverse-transcribed at 42 °C for 60 min in 20 μl of 5 mM $MgCl_2$, 10 mM Tris–HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1 mM dNTP, 1 unit/μl of recombinant RNasin ribonuclease inhibitor, 15 units/μg of AMV reverse transcriptase, and 0.5 μg of oligo$(dT)_{15}$ primer. For the determination of target genes, sequences of the primer pairs were used as shown in Table 1. The PCR mixture consisted of a Taq PCR Master Mix Kit (Qiagen, Valencia, CA, USA), 2 μl of the reverse transcriptase reaction, and 20 pmol of primer pairs in a total volume of 50 μl. Thermocycling was performed according to the following profile: 94 °C for 30 s; followed by 94 °C for 30 s, 55 °C for 30 s, and 72 °C 30 s, repeated 25 times. Amplification was linear within the range of 20–30 cycles. PCR products were separated by 2% agarose gel electrophoresis, stained with SYBR® Green I (Molecular Probes, Eugene, OR, USA) and visualized using phosphoimaging technology (FLA-2000, Fuji, Stamford, CN, USA).

2.4. Zymography

MMP-2 and MMP-9 enzymatic activities were assayed by gelatin zymography (Herron et al., 1986). All experiments, including zymography, were performed in the absence of serum. Samples were electrophoresed on a gelatin containing 10% SDS-polyacrylamide gel. After electrophoresis, the gel was washed twice with washing buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 2.5% Triton X-100), followed by a brief rinsing in washing buffer without Triton X-100. The gel was incubated with incubation buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, 1 μM $ZnCl_2$) at 37 °C. After incubation, the gel was stained and destained. A clear zone of gelatin digestion was represented with the MMP activity.

2.5. Cell invasion and motility assay

Cells (5×10^4/chamber) were used for each invasion assay. The lower and upper parts of Transwell (Corning Coaster, Cambridge, MA, USA) were coated with 10 μl of type I collagen (0.5 mg/ml) and 20 μl of 1:2 mixture of Matrigel/DMEM, respectively. Cells were plated on the Matrigel-coated Transwell. The medium of the lower chambers was also contained 0.1 mg/ml bovine serum albumin. The inserts were incubated for 18 h at 37 °C. The cells that had invaded the lower surface of the membrane were fixed with methanol and stained with hematoxylin and eosin, and photographed.

To determine the effect of the agents on cell motility, cells were seeded into Transwell on membrane filters coated with 10

Table 1
Sequences of the primer pairs employed in the RT-PCR reactions

Studied gene	Sequences of the primer pairs (5′→3′)	Product size (bp)
MMP-2	Forward: AGATCTGCAAACAGGACATTGTATT Reverse: TTCTTCTTCACCTCATTGTATCTCC	400
MMP-9	Forward: CTGGGCTTAGATCATTCCTCAGT Reverse: AGTACTTCCCATCCTTGAACAAATA	400
TIMP-1	Forward: TGGGGACACCAGAAGTCAAC Reverse: TTTTCAGAGCCTTGGAGGAG	400
TIMP-2	Forward: GTCAGTGAGAAGGAAGTGGACTCT Reverse: ATGTTCTTCTCTGTGACCCAGTC	401
MT1-MMP	Forward: GGGCCTGCCTGCGTCCATCAACA Reverse: GCCGCCCTCCTCGTCCACCTCAAT	400
β-Actin[a]	Forward: AGCACAATGAAGATCAAGAT Reverse: TGTAACGCAACTAAGTCATA	188

[a] Ballester et al. (1998).

μl of type I collagen (0.5 mg/ml) at the bottom of the membrane. Motility in the absence or presence of agents was measured as described in the invasion assay. In addition, cell motilities were measured using a wound-healing method. Briefly, for a wound-healing assay, cells were grown almost confluently, and a wound was created with the blunt end of a yellow tip. This was documented through time-lapse photography.

2.6. Statistical analysis

Statistical analyses were performed using the Student's *t*-test and one-way analysis of variance (ANOVA). $*P<0.05$ was considered statistically significant. All the statistical analyses were performed using SPSS 10.0 software.

3. Results

To investigate whether SME-6 can inhibit the expression of MMP-2 and -9, HT1080 human fibrosarcoma cells were treated

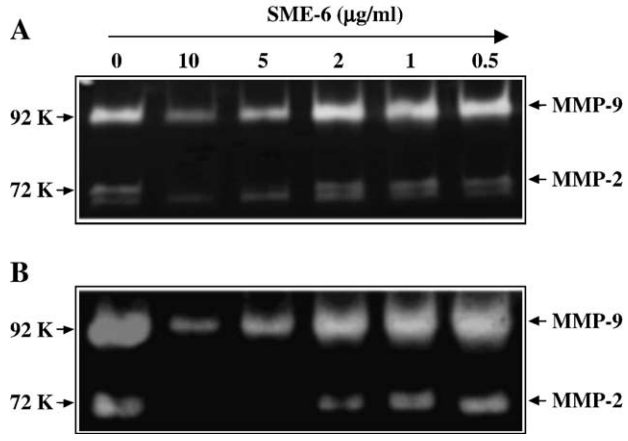

Fig. 3. Effects of SME-6 on the activities of MMP-2 and -9. HT1080 cells were treated with various concentrations of SME-6. After (A) 48 h or (B) 72 h, conditioned media were collected and analysed by gelatin zymography. Data shown are representatives of three independent experiments.

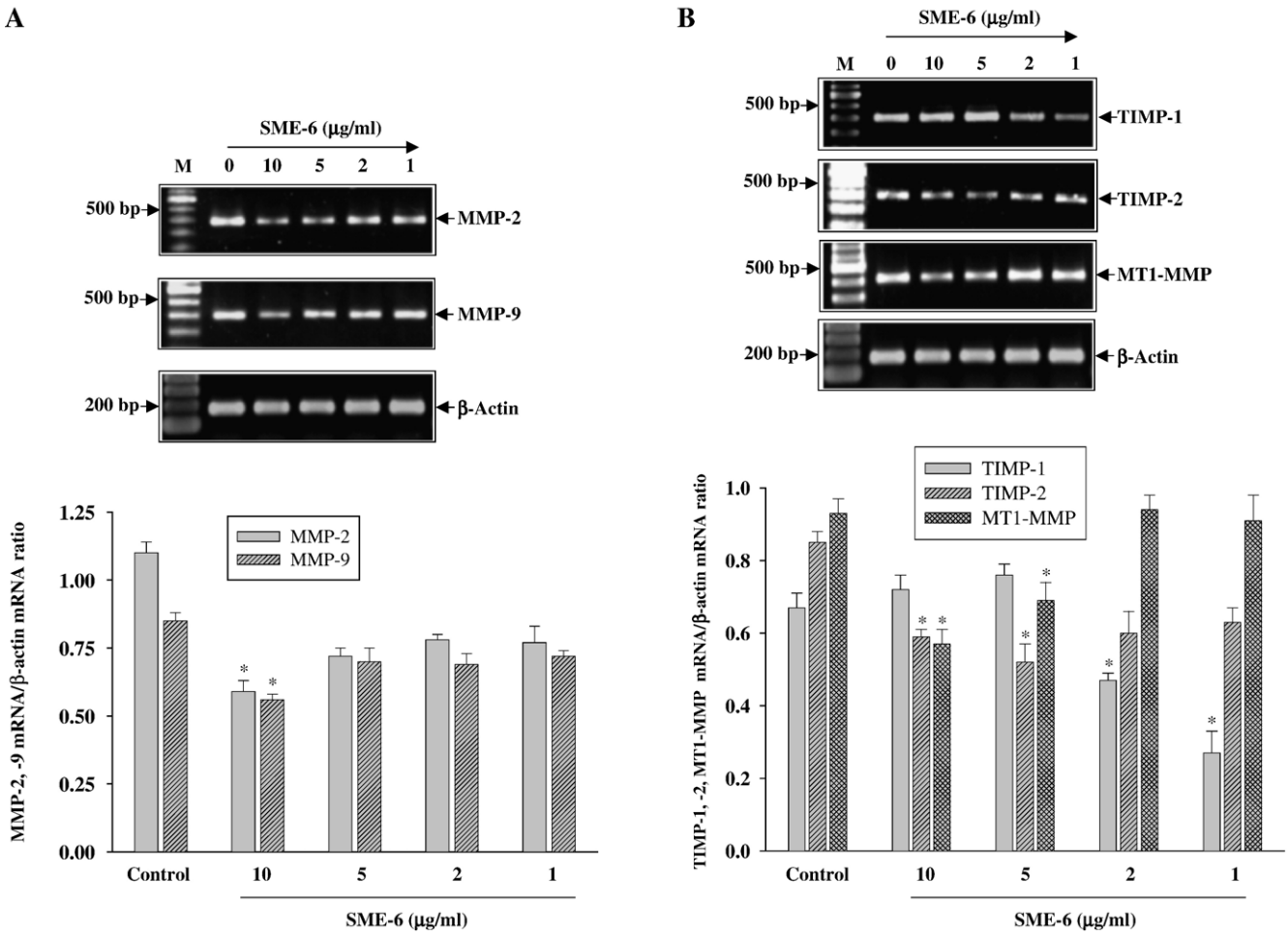

Fig. 2. Analysis of the (A) MMP-2 and -9 and (B) TIMP-1, -2, and MT1-MMP mRNA in HT1080 cells by SME-6 using RT-PCR analysis. HT1080 cells were treated with various concentrations of SME-6 for 24 h. PCR products were analyzed by 2% agarose gel electrophoresis and visualized using phosphoimaging (upper panel), followed by densitometric measurements (lower panel). The predicted sizes of RT-PCR products for (A) MMP-2 and -9 and (B) TIMP-1, -2, MT1-MMP and β-actin are (A) 400 bp, 401 bp and (B) 400 bp, 401 bp, 400 bp, and 188 bp, respectively. M, molecular weight markers (100-bp DNA ladder). Statistical analyses were performed using the Student's *t*-test and one-way ANOVA. $*P<0.05$ was considered statistically significant.

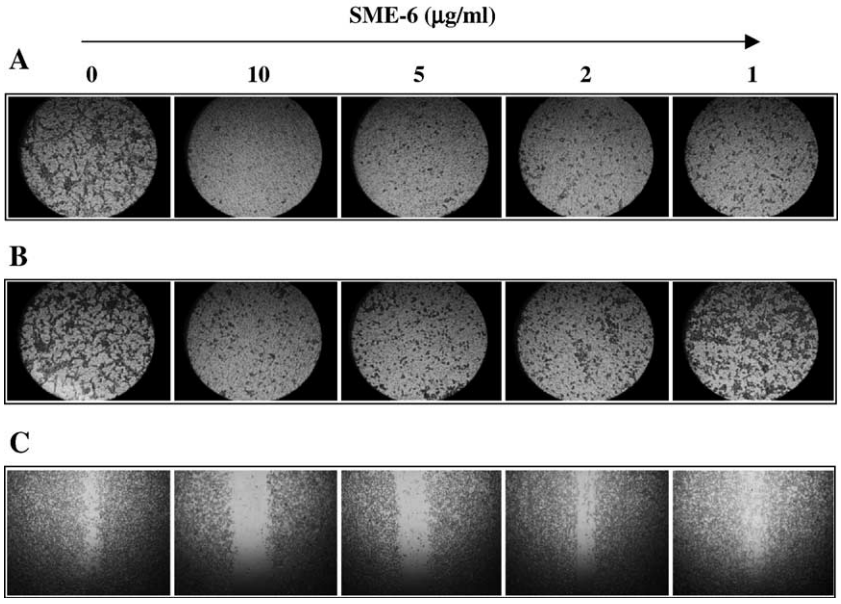

Fig. 4. Effect of SME-6 on invasion, motility, and wound healing. (A) HT1080 cells were treated with 0, 1, 2, 5, or 10 μg/ml SME-6 for 18 h using Matrigel-coated Transwell. (B) The same method used in (A) except Matrigel-coated on upper parts of Transwell. (C) Confluent cultures of HT1080 cells were wounded with a tip. The cells were incubated with increasing concentrations of SME-6 for 16 h, fixed and stained. Data shown are representatives of three independent experiments.

with the compound for various concentrations, and MMP-2 and MMP-9 mRNA levels were determined by semi-quantitative RT-PCR technique. As shown in Fig. 2A, mRNA transcripts for MMP-2 and -9 were clearly inhibited in SME-6 treated cells. Down-regulation of MMP-2 and -9 expression by treatment of SME-6 was observed in a dose-dependent manner. To further explore the modulation of activation of pro-MMPs mediated by SME-6, TIMPs and MT1-MMP gene expressions were investigated. As shown in Fig. 2B, interestingly, SME-6 increased TIMP-1 mRNA in a dose-dependent manner, but weakly decreased TIMP-2 mRNA level. MT1-MMP mRNA levels were also suppressed by the treatment of SME-6, and dose-dependent down-regulation was observed at 24 h (Fig. 2B), demonstrating that SME-6 suppresses the expression of TIMP-2 and MT1-MMP mRNA levels. In addition, gelatin zymographic analysis revealed that MMP-2 and -9 proteins were constitutively expressed in untreated HT1080 cells, but the cells treated with SME-6 for 48 h (Fig. 3A) and 72 h (Fig. 3B) were markedly down-regulated in a dose-dependent manner.

HT1080 cells have an ability to invade through Matrigel. Treatment of SME-6 for 16 h exhibited the significant inhibition of cell invasion in a dose-dependent manner (Fig. 4A). Cell motility through Transwell (Fig. 4B) and spreading onto plasticware were also inhibited by the treatment of SME-6 (Fig. 4C).

4. Discussion

The aminoquinones are an interesting class of compounds with their remarkable anticancer activities (Skibo et al., 1994). Moreover, the planar tri- and tetracyclic quinones such as adriamycin have been found to act as intercalating agents (Marshall and Ralph, 1985). It is also known that benzo[*f*] indole-4,9-dione derivatives possess a variety of pharmacological activities including anticancer, virus-static, antibacterial, tuberculostatic, antianaphylastatic, fungicide, and anticoagulant effects (Nagai et al., 1990; Lee et al., 2003). In our previous study, we also suggested that the anticancer activity of benzo[*f*] indole-4,9-dione derivatives might be related to the inhibitory effect of topoisomerase activity.

In the present study, we explored whether SME-6 modulates cancer invasion and metastasis in cultured HT-1080 cells using the representative biomarkers MMP expressions. As a result, SME-6 significantly suppressed the gene expression of MMPs (MMP-2, MMP-9, MT1-MMP) and TIMP-2 but increased TIMP-1 mRNA levels in HT1080 human fibrosarcoma cells (Fig. 2A,B), and also nontoxic ranges of SME-6 markedly decreased MMPs activity, cell migration, motility, and invasiveness (Figs. 3 and 4).

MMPs mediate invasion and metastasis through degradation of the extracellular matrix and basement membrane, allowing tumor cells to invade surrounding tissues and enter the blood stream to travel to distant sites (Liotta et al., 1991). The MMP activity is also finely regulated on many levels including transcriptional control, proenzyme activation, and inhibition of activated MMPs by nonspecific inhibitors such as α2-macroglobulin (Watanabe et al., 1993), or more specific endogenous inhibitors, tissue inhibitors of metalloproteinase (TIMPs) (Gomez et al., 1997). TIMPs bind to the active site of MMPs and block access of MMPs to their substrates. As suggested, MMPs are also regulated by the endogenous modulators with various mechanisms (Denhardt et al., 1993). In particular, MMP-2 is secreted from cells as a zymogen (pro-MMP-2) and is activated post-translationally by a transmembrane MMP designated as membrane type 1-MMP (MT1-MMP) (Butler et al., 1998). The activation of pro-MMP-2 is regulated by a complex mechanism involving formation of a trimolecular complex with

MT1-MMP and TIMP-2 (Strongin et al., 1995; Butler et al., 1998; Sato et al., 1994; Forget et al., 1999; Sternlicht and Werb, 2001). TIMP-2 bridges the interaction between the MMP-2 zymogen and MT1-MMP via N-terminal binding to the active site of MT1-MMP with the concomitant C-terminal binding to the pro-MMP-2 hemopexin domain (Strongin et al., 1995; Zucker et al., 1998). In this situation, the balance between MT1-MMP and TIMP-2 is important. At low concentration, TIMP-2 binds to the catalytic site of some activated MT1-MMP molecules, generating receptors for pro-MMP-2, thereby promoting MMP-2 activation. Furthermore, knockout mice for either gelatinase have reduced tumor burden, decreased metastasis, as well as reduced tumor angiogenesis without developmental abnormalities (Itoh et al., 1998; Itoh et al., 1999). On this line, SME-6-mediated suppression of MMP-2, -9 and MT1-MMP gene expression (Fig. 2A,B) suggests that SME-6 might inhibit the proteinase expression (Fig. 3) giving reasonable explanation for the inhibition of cancer cell invasion (Fig. 4).

TIMPs play an important role in regulating the activity of the secreted metalloproteases. In addition to their ability to bind at the active site, TIMP-1 and TIMP-2 can form complexes with specific metalloproteinases. TIMP-1, a 28-kDa *N*-glycosylated protein, forms a 1:1 complex with the 92-kDa procollagenase (proMMP-9) (Drummond et al., 1999). TIMP-2, a 21-kDa non-glycosylated protein, selectively forms a complex with the 72-kDa procollagenase (proMMP-2). In the present study, SME-6 exerts its action through regulation of inhibitor of MMP-9, and TIMP-1. Interestingly, SME-6 increased TIMP-1 mRNA but decreased TIMP-2 level (Fig. 2B). Although TIMP-1 and TIMP-2 are considered to be inhibitors of MMP-9 and MMP-2, respectively, expression of these inhibitors is differentially regulated in vivo as well as cell culture system (Stetler-Stevenson et al., 1990). As TIMP-1 is a natural inhibitor of MMP-9, the increase in its mRNA can inhibit tumor cell invasion (Nakano et al., 1995; George et al., 1998). These kinds of effects on MMPs and TIMPs are not just confined to SME-6. Genistein and selenite decrease MMP-2 and -9 mRNA level, whereas these compounds increase TIMP-1 mRNA levels in MDA-MB-231, MCF-7 and HT1080 fibrosarcoma cells (Shao et al., 1998; Yoon et al., 2001). Ursolic acid decreases MMP-9 but MMP-2 and membrane-type MMP were constantly expressed, and TIMP-1 and -2 also were not changed after 3 and 6 days of treatment with ursolic acid in HT1080 cells (Cha et al., 1996). 1α,25-Dihydroxyvitamin D_3 and its analogues also down-regulate MMP-9 and uPA, whereas it up-regulates TIMP-1 and uPAI-1 levels in MDA-MB-231 cells (Koli and Keski-Oja, 2000). Silibinin, a flavonoid antioxidant, markedly decreased MMP-2 and uPA level and increased TIMP-2 protein level without affecting the mRNA level, and also not affecting TIMP-1 protein or mRNA levels (Chu et al., 2004).

Collectively, SME-6 down-regulated gene expression of MMP-2 and -9, but up-regulated that of TIMP-1 is, at least in part, mediated at the level of transcription. In addition, gelatin zymographic analysis showed that down-regulation of the MMP-2 and -9 gene by SME-6 is well correlated with a significant and dose-dependent suppression of MMP-2 and -9 protein in HT1080 cells (Fig. 3). Moreover, SME-6-treated HT1080 cells, at least in part, decreased pro-MMP-2 activation through the suppression of MT1-MMP and not adequate expression of TIMP-2. Therefore, as shown in Fig. 4, SME-6 inhibited an in vitro invasion of HT1080 cells in the Matrigel model.

In conclusion, we here report that SME-6 inhibited invasion- or metastasis-associated proteases activities. The inhibition was also related to the suppression of transcriptional level, however, its mechanism of action still remains elusive. In addition, SME-6 inhibits degradation and cellular invasion of extracellular matrix and basement membrane. This study provides an additional activity of anti-metastatic potential beyond anti-tumor activity mediated by SME-6.

Acknowledgments

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