Methyl-3-indolyacetate inhibits cancer cell invasion by targeting the MEK1/2-ERK1/2 signaling pathway

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Abstract

Epidemiologic studies have suggested an inverse correlation between dietary intake of cruciferous vegetables and cancer risk. It is thus of interest to investigate the anticancer potential of phytochemicals presented in cruciferous vegetables. In this study, methyl-3-indolyacetate (MIA), a cruciferous indole for which the bioactivity has not been previously reported, was found to significantly suppress the invasion of cancer cells stimulated by 12-O-tetradecanoyl-phorbol-13-acetate (TPA). Our data show that MIA pretreatments inhibited matrix metalloproteinase 9 (MMP-9) expression in a concentration-dependent manner, resulting in decreased MMP-9 activity. By using real-time reverse transcription-PCR, luciferase reporter gene assay, and electrophoretic mobility shift assay, we provided convincing evidence that MIA suppresses MMP-9 gene transcription via targeting the activator protein-1 signaling but not the nuclear factor-kB pathway. The TPA-induced mitogen-activated protein kinase (MAPK) activation cascade was also analyzed. Despite extensive activation of major MAPKs [c-Jun NH2-terminal kinase, p38, and extracellular signal-regulated kinase-1/2 (ERK1/2)] under TPA stimulation, only the ERK1/2 activation and its consequent nuclear translocation were found to be diminished by MIA. Interestingly, MIA did not affect the TPA-induced phosphorylation of either c-Raf or MAPK/ERK kinase-1/2 (MEK1/2), two upstream kinases of ERK. Moreover, using the in vitro kinase assay, MIA was shown to inhibit the kinase activity of MEK1/2, the upstream kinases of ERK, suggesting that MEK is the major molecular target of MIA. In conclusion, data from this study provided new insight into the anticancer potential of MIA, a cruciferous vegetable–derived indole compound. [Mol Cancer Ther 2006;5(12):3285–93]

Introduction

Cancer development is a multistage process, which includes the early stages of carcinogenesis as well as the advanced stages of metastasis, invasion, and angiogenesis. Among these stages, the prevention of cancer metastasis is particularly important as it is the leading cause of cancer mortality (1). Cancer cell invasion, which refers to the translocation of neoplastic cells across the extracellular matrix barriers, is an essential step in cancer metastasis (2). Due to the exceptional ability to degrade all known components of the extracellular matrix, the matrix metalloproteinases (MMP), a large family of zinc-dependent endopeptidases, are believed to be highly related to cancer invasion and metastasis (3, 4). Among all the MMPs, MMP-2 and MMP-9 are the two key enzymes that degrade type IV collagen (4). Although MMP-2 and MMP-9 share structural and functional similarities, their expressions are regulated at different transcriptional levels. MMP-9 is highly inducible by various growth factors, cytokines, oncogenes, hormones, and various chemical agents through the nuclear factor-kB (NF-kB) and activator protein-1 (AP-1) pathways (5). On the contrary, due to the lack of inducible promoter elements, MMP-2 is mostly constitutively expressed in tissues with only modest up-regulation or down-regulation under various conditions (6). Therefore, the regulation of MMP-9 expression can be a feasible approach for the development of antimitastatic agents.

On the other hand, numerous epidemiologic studies have provided strong evidence that a vegetable-rich diet reduces cancer risk in humans (7). Of special interest is the Cruciferae family, which contains various phytochemicals with potent anticancer activity (8, 9). In the last decade, extensive studies have been focused on the anticancer properties of cruciferous isothiocyanates such as sulforaphane and β-phenylethyl isothiocyanate (10, 11). In contrast, the anticancer potential of indole derivatives presented in these vegetables is still largely unknown. Recently, we observed that methyl-3-indolyacetate (MIA; Fig. 1A), an indole derivative, was present in a large variety of cruciferous vegetables, including cabbage, broccoli, Brussels sprout, mustard, Thai kale, etc., with concentrations ranging from 20 to 100 µg/g (dried weight of vegetables).3 Thus far,
the biological activity of this phytochemical in mammalian systems has not been reported. In this study, we showed that MIA could effectively prevent cancer cell invasion by suppressing MMP-9 expression via targeting MAPK/ERK kinase-1/2 (MEK1/2) activity and subsequent extracellular signal-regulated kinase-1/2 (ERK1/2) signaling pathway.

Materials and Methods

Chemicals and Reagents

MIA, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), parthenolide, PD98059, SB20358, U0126, and gelatin-agarose beads were purchased from Sigma-Aldrich (Singapore). SP600125 was from Merck (Singapore). The dual-luciferase reporter assay system was purchased from Promega (Madison, WI). Anti-MMP-9 antibody was purchased from BioMol International (Plymouth, PA). Antibody against c-Jun, c-Jun NH2-terminal kinase (JNK), p38 phospho-c-Jun, phospho-JNK, phospho-ERK1/2, phospho-p38, phospho-c-Raf (Ser338), and phospho-MEK1/2 (Ser217/221) were obtained from Cell Signaling Technology (Danvers, MA). Anti-phospho-ERK antibody (E-4) used in immunostaining was from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-MEK antibody used in kinase assays and unactivated GST-ERK2 protein were from Upstate, Inc. (Charlottesville, VA).

Cell Culture and Treatments

The human breast cancer cell line (MDA-MB-231), human liver cancer cells (HepG2), human cervical cancer cells (HeLa), and human cutaneous squamous carcinoma cell line (HSC-5) were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured at 37 °C, 5% CO2 in DMEM supplemented with 10% fetal bovine serum (FBS), 100 μg/mL of streptomycin, and 100 units/mL of penicillin. The stock solution of MIA (200 mmol/L) was prepared in DMSO. Cells were always pretreated for 1 h with the designated concentration of MIA before TPA stimulation, and the vehicle control group was always balanced with the same concentration of DMSO.

Cell Viability Assay

The general cytotoxicity of MIA on cancer cell proliferation was detected by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test as described previously (12), and the results are presented as the relative cell viability as compared with the control group.

Cell Invasion Assay

The cell invasion assay was conducted using BD BioCoat Tumor Invasion System as previously described (13). Briefly, 2 × 10⁴ cells (MDA-MB-231) were seeded into the top chamber. After pretreatment with different concentrations of MIA for 1 h, 80 nmol/L of TPA was added into the bottom wells as chemoattractant for 12 h. At the end of the treatment, the cells were post-stained with 4 μg/mL of Calcein-AM in Hank’s buffered salt solution at 37 °C, 5% CO2 for 1 h. The labeled cells that invaded the BD Matrigel Matrix and passed through the pores of the BD FluoroBlok membrane were detected and counted under an inverted fluorescent microscope (Nikon ECLIPSE TE2000-S, Nikon, Singapore).

Gelatin Zymography

Gelatin zymography was done as reported earlier with modifications (13). MDA-MB-231 cells were seeded onto six-well plates in DMEM with 10% FBS, and were cultured to 80% confluence. Cell were then washed and maintained in serum-free medium for 12 h prior to designated treatments with MIA and TPA. After the cells were treated with TPA for 12 h, the medium was then collected (conditioned medium), standardized by cell number in each well, and subjected to electrophoresis on a 10% SDS-PAGE gel containing 0.1% (w/v) gelatin. The resulting gels were washed in renaturing buffer [10 mmol/L Tris (pH 8.0) and 2.5% Triton X-100] and then incubated overnight in developing buffer [50 mmol/L Tris-HCl (pH 7.5), 200 mmol/L...
NaCl, 10 mmol/L CaCl₂, and 1 μmol/L ZnCl₂] at 37°C. Gels were subsequently stained using 0.5% Coomassie blue R-250 in 5% acetic acid and destained with destaining solution (10% methanol, 5% acetic acid). The image was captured by Kodak Image Station 440CF (Kodak, Rochester, NY).

**Western Blot**

For the detection of secreted MMP-9 protein levels in the cell culture medium, the conditioned medium was standardized by cell number and gelatinases were concentrated by incubation with gelatin-agarose beads for 2 h. The gelatin-agarose beads were washed and resuspended in gel loading buffer and subjected to SDS-PAGE and Western blot. For the detection of other proteins, the cells were lysed by M2 cell lysis buffer [20 mmol/L Tris (pH 7.0), 0.5% NP40, 250 mmol/L NaCl, 3 mmol/L EDTA, 3 mmol/L EGTA, 2 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L β-glycerol phosphate, 1 mmol/L sodium vanadate, and protease inhibitor cocktail] or SDS sample buffer [62.5 mmol/L Tris-HCl (pH 6.8), 2% w/v SDS, 10% glycerol, 50 mmol/L DTT, and protease inhibitor cocktail]. Equal amount of proteins were loaded with SDS sample buffer and fractionated on SDS-PAGE, and then transferred onto a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). After blocking with 5% nonfat milk in TBST [10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, and 0.1% Tween 20], the membrane was probed with the designated first and second antibodies and developed with enhanced chemiluminescence method (Pierce, Rockford, IL) and visualized by Kodak Image Station 440CF (Kodak).

**RNA Extraction and Quantification by Real-time PCR**

RNA extraction was carried out using RNAqueous total RNA isolation kit (Ambion, Austin, TX), following the instructions from the manufacturer. Two micrograms of total RNA from each sample was subjected to reverse transcription using moloney murine leukemia virus reverse transcriptase (Promega). The cDNA was subjected to PCR amplification using QuantiTect SYBR PCR mastermix (Qiagen, Valencia, CA) and real-time detection on Bio-Rad Opticon 2 real-time PCR machine. The PCR primers used were MMP-9 forward, 5′-TGTGTGCTGGGCTGCTGC-3′; MMP-9 reverse, 5′-CTGCCACCCCGATGTAAACCA-3′; MMP-2 forward, 5′-CTTTCAAGTCTTGAGCCGA TG-3′; MMP-2 reverse, 5′-TACGGTCAAGGGGATATCAT-3′; G3PDH forward, 5′-CATGAAAGTATGACACAGCTT3′; and G3PDH reverse, 5′-AGTCCCTCCACGA TACCAAATG-3′.

**Plasmids, Transient Transfection, and Reporter Gene Assay**

pAP-1-luc and pNF-κB-luc vectors were from Clontech (Mountain View, CA). Renilla luciferase reporter vector (phRL-TK) was from Promega. The transient transfection of luciferase vectors were done in MBA-MD-231 cells using LipofectAMINE 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The Renilla luciferase vector was also cotransfected with the above vectors as an internal control. The luciferase activity was measured in the cellular extracts using a dual-luciferase reporter assay system (Promega, Madison, WI). Briefly, cells were lysed and collected from each well after the addition of a cell lysis reagent (50 μL/well in 24-well plate). After adding the luciferase assay substrate, the firefly luciferase activity (relative light units) was determined in a luminometer (Lumi-One; Trans Orchid, Tampa, FL) for a total period of 10 s after a 5-s delay time. Then the Renilla luciferase activity was measured for another 10 s by adding 100 μL of Stop&Glo substrate.

**Electrophoretic Mobility Shift Assay**

The DNA-binding activity of the nuclear protein was tested as previously described (14). Briefly, at the end of the treatments, nuclear proteins were extracted with nuclear extraction buffer [20 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 450 mmol/L NaCl, 25% glycerol, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail], NF-κB, AP-1, and SP-1 consensus oligonucleotides (Promega) were labeled with T4 kinase and purified through a G50 column. Equal amounts of nuclear protein (5 μg) were incubated with 100,000 cpm of labeled NF-κB oligonucleotides in 5× reaction buffer [100 mmol/L HEPES/KOH (pH 7.9), 20% glycerol, 1 mmol/L DTT, and 300 mmol/L KCl] for 30 min at room temperature in the presence of 2 μg of poly(deoxyinosinic-deoxyctydilic acid) and 2 μg of bovine serum albumin in a total volume of 20 μL. The DNA-protein complexes were resolved on a 5% polyacrylamide gel using a vertical gel electrophoresis apparatus (model v16-2; GE Healthcare, Piscataway, NJ) at 150 V for 1.5 h. Gels were then dried and exposed to X-ray film at −80°C for 4 h (or overnight).

**Immunofluorescence Microscopy**

Immunostaining was carried out as described previously (15). Briefly, MDA-MB-231 cells were plated on eight-well chamber slides (Nunc, Rochester, NY) and cultured in FBS-free condition overnight. At the end of the designated treatments, cells on the slides were washed once with PBS and fixed in 3.75% paraformaldehyde for 1 h in room temperature, then permeabilized with 0.1% Triton-X in PBS and blocked in blocking solution (2% bovine serum albumin, 0.2% Tween 20 in PBS) for at least 1 h. Cells were further incubated with anti-phospho-ERK (E-4) antibody overnight in PBS and blocked in blocking solution (2% bovine serum albumin, 0.2% Tween 20 in PBS) for at least 1 h. Cells were then washed three times with PBS and fixed in 3.75% paraformaldehyde for 1 h in room temperature, then permeabilized with 0.1% Triton-X in PBS. Cells were further incubated with anti-phospho-ERK (E-4) antibody overnight in 4°C. After washing with PBS thrice and incubation with FITC-labeled secondary antibody for 1 h, slides were mounted with ProLong Gold Anti-Fade mounting solution (Invitrogen), and visualized by an Olympus FLOVIEW V500 confocal microscope.

**In vitro Kinase Assay**

Cells were lysed with M2 cell lysis buffer [20 mmol/L Tris (pH 7.0), 0.5% NP40, 250 mmol/L NaCl, 3 mmol/L EDTA, 3 mmol/L EGTA, 2 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L β-glycerol phosphate, 1 mmol/L sodium vanadate, and protease inhibitor cocktail]. Equal amounts of protein were immunoprecipitated with anti-MEK antibody (Upstate) and protein A-Sepharose beads (Roche, Palo Alto, CA) for 4 h at 4°C. After extensive washes, the immunoprecipitates were used.
for the kinase assay in a complete kinase buffer [20 mmol/L HEPES (pH 7.5), 20 mmol/L β-glycerol phosphate, 10 mmol/L MgCl2, 1 mmol/L DTT, 20 μmol/L ATP, and 5 μCi (γ-32P)-ATP] with the presence of unactivated substrates of MEK (GST-ERK2) and designated concentrations of MIA and U0126 (pretreated for 30 min before the addition of ATP) in a total reaction volume of 30 μL. After 30 min of incubation at 30°C, the reaction was stopped by the addition of SDS sample buffer. The samples were then separated on a 4% to 20% gradient SDS-PAGE and stained with Coomassie blue R-250 for the validation of equal loading. The phosphorylation of GST-ERK2 was visualized by autoradiography.

Statistical Analyses
All numerical data were presented as means ± SD from at least three independent experiments and analyzed by Student’s t test.

Results

Effect of MIA on Cancer Cell Viability
As shown in Fig. 1A, several indole derivatives could be detected in a typical cruciferous vegetable extract, including MIA, indole-3-carbinol, indole-3-acetonitrile, and 3,3'-dindolylmethane. Compared with indole-3-carbinol and 3,3',dindolylmethane, which have attracted much attention in recent years (16–18), the bioactivity of MIA is completely unknown. In this study, we first evaluated the general cytotoxicity of MIA on various cancer cell lines, including human breast cancer cell line (MDA-MB-231), human liver cancer cell line (HepG2), human cervical cancer cell line (HeLa), and human cutaneous squamous carcinoma cell line (HSC-5). As shown in Fig. 1B, the relative cell viability of human cancer cells was detected by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test. Compared with the strong cytotoxicity of a sesquiterpene lactone, parthenolide (25 μmol/L), MIA showed no significant effect on cancer cell viability up to 100 μmol/L, suggesting the weak cytotoxicity of MIA.

Inhibition of MIA on TPA-Induced Cancer Cell Invasion
Although MIA does not seem to strongly inhibit cancer cell proliferation, we wondered whether this compound could prevent metastasis during tumor formation. It was interesting to note that MIA, at noncytotoxic concentrations, could significantly inhibit cancer cell invasion stimulated by TPA, as detected using a BioCoat transwell invasion assay. As shown in Fig. 2A, the TPA-stimulated MDA-MB-231 cells invaded to the bottom of the wells as detected by fluorescence staining. This invasion could be effectively inhibited by MIA in a concentration-dependent manner (Fig. 2B). A similar trend could also be observed in HSC-5 cells, although this cell line is not as invasive as MDA-MB-231 cells (Supplementary Fig. S1).4 Owing to its highly invasive property, MDA-MB-231 breast cancer was used in the subsequent studies as the model system to examine the effect of MIA on cancer cell invasion.

Inhibition of TPA-Induced MMP-9 Activity and Protein Expression by MIA
Because MMP-9 and MMP-2 are the two major secreted enzymes responsible for the degradation of the extracellular matrix components such as gelatin, we decided to perform a gelatin zymography experiment to examine the effects of MIA on the activity of MMPs. MDA-MB-231 cells cultured in serum-free medium were first pretreated with designated concentrations of MIA and then followed by TPA. As shown in Fig. 2C, TPA significantly enhanced MMP-9 activity, whereas MMP-2 activity remained unchanged. For cells pretreated with MIA (up to 100 μmol/L), there was a significant concentration-dependent reduction of TPA-stimulated MMP-9 activity whereas no effect was observed on MMP-2 activity. Meanwhile, all the known inhibitors of MMP-9 gene transcription, including inhibitors for the AP-1 pathway (PD98059, SP600125, and SB203580) and an inhibitor for NF-κB pathway (parthenolide), also effectively suppressed the MMP-9 activity (Fig. 2C, bottom). In addition, when 100 μmol/L of MIA was added directly into the collected medium following TPA treatment, we failed to observe the suppression of the MMP-9 activity (data not shown). This finding suggests that MIA is unlikely to act directly on the MMP-9 to inhibit its activity. We also determined the MMP-9 protein level in the conditioned medium secreted by MDA-MB-231 cells. Parallel to the MMP zymography assay, TPA enhanced the MMP-9 protein level, and there was a significant reduction of MMP-9 protein levels when cells were pretreated with MIA (Fig. 2D). Therefore, results from this part of the study provide clear evidence that MIA is likely to target the regulatory pathways that control MMP-9 protein expression.

Inhibition of MMP-9 mRNA Transcription by Targeting the AP-1 Pathway
To further explore the possible effects of MIA on the transcriptional regulation of MMP-9, we determined the mRNA levels of MMP-9 using real-time reverse transcription-PCR. As shown in Fig. 3A, the mRNA transcription machinery responded quickly to the TPA stimulation, resulting in a 4-fold increase of MMP-9 mRNA levels over the control. More importantly, this increase could be effectively suppressed by MIA pretreatments. In contrast, no significant changes were detected in the MMP-2 mRNA levels in either the TPA or MIA-TPA combined treatment groups. It has been reported that the MMP-9 gene promoter region contains binding sites of multiple transcription factors. Among them, transcription factors AP-1 and NF-κB are believed to be particularly important (19). In order to further elucidate the major target(s) affected by MIA, we transfected the MDA-MB-231 cells with either AP-1 or NF-κB luciferase constructs, followed by treatments with TPA with or without MIA pretreatments. As shown in Fig. 3B, MIA could only inhibit AP-1 transcriptional activity (Fig. 3B), but not that of NF-κB (Fig. 3C), suggesting that the AP-1 pathway is the main molecular target of MIA.

To further understand the inhibitory mechanisms of MIA on MMP-9 transcriptional regulation, we examined

4 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
the effect of MIA on the DNA-binding capability of nuclear transcription factors using the electrophoretic mobility shift assay. As shown in Fig. 3D, the DNA-binding activities of both AP-1 and NF-κB were significantly stimulated by TPA treatment. However, consistent with the luciferase reporter gene assay shown earlier, MIA only interferes with the DNA binding of AP-1, but not that of NF-κB. Therefore, it is believed that MIA works mainly via the AP-1 signaling pathway to suppress the MMP-9 transcription.

**Effects of MIA on MAPK Signaling**

The MAPK family members are the main signaling molecules responsible for AP-1 activation in response to a variety of external physical and chemical stressors, by means of protein phosphorylation (20). To further determine which MAPK subfamily members were actively involved in MIA-mediated AP-1 inhibition, the phosphorylation status of major MAPK members were evaluated by Western blotting. As shown in Fig. 4A, TPA treatment led to a significant phosphorylation/activation of all the three subfamilies of MAPKs, including JNK, p38 kinase, and ERK. Interestingly, MIA pretreatment failed to affect the TPA-induced phosphorylation of JNK or p38. In contrast, MIA shows a concentration-dependent inhibition of ERK1/2 phosphorylation (Fig. 4A). In view of the potential nuclear shuttling of activated ERK1/2 under various stimulations (21, 22), we further determined the effect of MIA on ERK1/2 translocation in our system. By tracing the phosphorylated ERK1/2 using immunofluorescence microscopy, we observed an enhanced staining of phospho-ERK1/2, and the accumulation of activated ERK1/2 in the nucleus following TPA stimulation. This enhancement was significantly prevented by MIA pretreatment (Fig. 4B). Consistent with this observation, MIA pretreatment also concentration-dependently suppressed the phosphorylation of Elk-1, one of the important nuclear substrates of activated ERK1/2 (Fig. 4C; ref. 23). These results further supported the notion that the inhibition of the activation and translocation of ERK1/2 and the subsequent AP-1 transcription activity are the major targets of MIA.

We next examined the effect of MIA on MEK1/2 and MEK kinase (c-Raf), which function upstream of the ERK1/2. As shown in Fig. 5A, treatment with TPA induced hyperphosphorylation of c-Raf on Ser338, which is important for the interaction between c-Raf and MEK-1 (24). Consequently, increased phosphorylation of MEK1/2 on Ser217/221 was observed. However, to our surprise,
the pretreatment of MIA had no detectable effects on TPA-induced phosphorylation on either c-Raf or MEK1/2 (Fig. 5A). In addition, it was interesting to note that MIA was able to diminish the phosphorylation of ERK1/2 (Fig. 4A) without affecting the phosphorylation status of its upstream kinase, MEK1/2 (Fig. 5A). In order to further elucidate the mechanism of MIA’s inhibitory effects, an in vitro MEK1/2 kinase assay was done. Different concentrations of MIA were incubated directly with immunoprecipitated MEK1/2 protein in the presence of its substrate GST-ERK2 protein. A known MEK1/2 inhibitor, U0126, which is highly specific to MEK1/2 and noncompetitively inhibits its catalytic activity (25), was used as a positive control. The results in Fig. 5B showed that MIA suppressed the kinase activity of MEK1/2, which could be detected at concentrations as low as 25 μmol/L, as evidenced by a decrease of phosphorylated GST-ERK2 level. Therefore, these findings suggest that MIA inhibits the MEK1/2 catalytic activity independent of the phosphorylation of MEK1/2.

Based on these findings, the potential anti-invasion mechanisms of MIA are illustrated in Fig. 6. This compound was found to be effective in blocking the TPA-induced MMP-9 gene transcription (Figs. 2 and 3). It was able to suppress the AP-1 luciferase (Fig. 3B) and DNA-binding activity (Fig. 3D). Furthermore, it could directly inhibit the MEK1/2 enzyme activity and ERK activation (Figs. 4 and 5). The overall findings suggest that the MEK1/2...
2-ERK1/2 signaling pathway has a critical role in TPA-stimulated cell invasion. Our data also offered convincing evidence that the MEK1/2-ERK1/2 signaling pathway is the main molecular target of MIA.

Discussion

Suppression of cancer cell invasion has been shown to contribute to the anticancer properties of several bioactive compounds derived from the diet, such as (−)-epigallocatechin 3-gallate (from green tea; ref. 26) and resveratrol (from grapes; ref. 27). During the past two decades, various phytochemicals found in cruciferous vegetables have been shown to possess anticancer activities via either inducing phase 2 detoxification enzymes or inducing apoptosis (9, 11, 28). However, thus far there have been few studies reporting the antitumor invasion and metastasis potential of phytochemicals derived from cruciferous vegetables. In this study, we showed that MIA, at a similar dose-range as that of epigallocatechin 3-gallate and resveratrol, has convincing activity against cancer cell invasion. This phytochemical was found to target the MEK1/2-ERK1/2 signaling pathway, leading to the suppression of AP-1 activation and the decrease of MMP-9 protein expression and secretion, and eventually inhibits cancer cell invasion.

Cancer cell invasion, a process of translocation of the tumor cells across extracellular matrix barriers, is an essential step for tumor metastasis, which requires the degradation of matrix proteins by MMPs (29). An unusually high expression of MMPs, especially MMP-9, have been found in invasive breast cancers and were considered to be one of the major mechanisms leading to the poor survival rate of patients (30). In this study, we first excluded the possible direct inhibition of MMP-9 enzyme activity in the cell culture medium (data not shown) and then observed a concentration-dependent decrease of secreted MMP-9 protein level presented in the conditioned medium, suggesting that some of the major transcriptional regulatory pathways responsible for MMP-9 gene regulation may be involved in the anti-invasion mechanism of MIA. The MMP-9 promoter region contains the fast response cis-regulatory elements, including one NF-κB and two AP-1 binding sites, which are absent in the promoter region of MMP-2. Due to the different structures of 5′-flanking regulatory sequences of MMP-2 and MMP-9 genes, the MMP-9 protein is highly inducible (19, 29, 31). In our study, MMP-9 mRNA transcription was markedly induced by TPA stimulation (Fig. 3A), leading to an increased protein secretion (Fig. 2D) and enzyme activity, as shown in gelatin zymography (Fig. 2C). In contrast, TPA treatment did not stimulate MMP-2 transcription. Based on the electrophoretic mobility shift assay experiments, it seems that only AP-1 and NF-κB are involved in TPA-stimulated MMP-9 gene expression (Fig. 3D). We thus

Figure 4. Effects of MIA on MAPK protein phosphorylation. **A**, effects of MIA on TPA-induced MAPK phosphorylation. MDA-MB-231 cells cultured in FBS-free medium overnight were pretreated with designated concentrations of MIA for 1 h prior to TPA exposure (80 nmol/L × 1 h). Various known MAPK inhibitors (SP200125, 20 μmol/L for JNK; SB20358, 10 μmol/L for p38; PD98059, 40 μmol/L for ERK) were also included as positive controls. Phosphorylation of JNK, p38, and ERK1/2 after TPA stimulation was detected by Western blot. **B**, inhibitory effects of MIA on phospho-ERK1/2 nuclear translocation determined by immunofluorescence staining. MDA-MB 231 cells cultured overnight in FBS-free medium were treated with TPA (80 nmol/L) for 1 h with/without MIA (100 μmol/L) or PD98059 (40 μmol/L) pretreatment. (i) 4',6-Diamidino-2-phenylindole staining, (ii) anti-phospho-ERK1/2, (iii) combined image. **C**, inhibition of TPA-induced phosphorylation of ERK nuclear substrate Elk-1 by MIA. MDA-MB 231 cells were treated as in **A** and phosphorylation of Elk-1 was detected by Western blot.
further examined the molecular mechanisms by which MIA inhibits the TPA-stimulated MMP-9 transcription. Although the two transcription pathways, AP-1 and NF-κB, have been implicated in MMP-9 regulation, our data indicate that MIA was only able to interfere with the AP-1 DNA binding and transcription, but not NF-κB (Fig. 3D). It was reported earlier that AP-1 might play a regulatory role in TPA-induced MMP-9 expression in MDA-MB-231 cells (32). Our data show that AP-1 is the main molecular target for MIA in the suppression of TPA-stimulated MMP-9 gene expression.

The regulation of AP-1 activation is a complex process. Upon external stimuli, such as TPA, a group of MAPKs could be quickly activated, which then trigger the signal transduction pathway converging on the AP-1 transcription factors (20, 33). By examining the phosphorylation of major groups of MAPKs (JNK, p38, and ERK1/2), we observed that only the phosphorylation of ERK1/2 and subsequent nuclear translocation induced by TPA (Fig. 4) were noticeably inhibited by MIA, whereas the other two MAPK pathways (JNK and p38) were not affected. These results suggest that MIA may target the kinases upstream of ERK1/2 with high specificity. It has been established that TPA-induced ERK1/2 activation is mediated by the PKCα/c-Raf/MEK cascade (34). However, from our results, it is unlikely that the suppressive activity of MIA on ERK1/2 activation originated from its effects on PKCα because MIA did not affect the phosphorylation status of c-Raf (Ser328) induced by TPA (Fig. 5A). In addition, it is interesting to note that MIA pretreatment failed to alter the phosphorylation status of MEK1/2 stimulated by TPA (Fig. 5A, middle). Because MEK1/2 is the direct upstream kinase responsible for ERK1/2 phosphorylation and activation (35), the discrepancy of the phosphorylation status of MEK1/2 and ERK1/2 under MIA pretreatment implies the following two possibilities: (a) MIA acts as a specific inhibitor of MEK kinase catalytic activity or (b) MIA inhibits ERK1/2 phosphorylation via a MEK-independent pathway (36). Nevertheless, the results from the MEK in vitro kinase assay (Fig. 5B) seem to support the first hypothesis, although at this stage, we could not completely rule out the possible effect of MIA on the MEK-independent pathway. Based on all the data elucidated in this study, it is thus believed that MEK1/2 is the key target of MIA on the TPA/PKCα/c-Raf/MEK/ERK signaling cascade in suppressing cancer cell invasion.

Taken together, in this study, we systematically investigated the anti-invasion mechanisms of MIA (Fig. 6), an indole compound found in a wide variety of cruciferous vegetables. By targeting MEK1/2 kinase activity, MIA inhibits ERK signaling, down-regulates MMP-9 transcription, and consequently, prevents cancer cell invasion.

Acknowledgments
We thank Dr. Jiaping Lai, Min Zhao, and Yeong-Bing Ong for their technical support.

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Mol Cancer Ther 2006;5:3285-3293.

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