J Nat Med (2015) 69:513–521
DOI 10.1007/s11418-015-0916-6

ORIGINAL PAPER

2,3,5-Trimethoxy-4-cresol, an anti-metastatic constituent from the solid-state cultured mycelium of *Antrodia cinnamomea* and its mechanism

Chin-Chung Lin[1,2] · Ching-Chun Chen[1] · Yueh-Hsiung Kuo[3,4] · Jong-Tar Kuo[2] · K. J. Senthil Kumar[5] · Sheng-Yang Wang[5,6,7]

Received: 28 January 2015 / Accepted: 20 April 2015 / Published online: 8 May 2015

Abstract *Antrodia cinnamomea* is a valuable and unique edible fungus originating from the forests of Taiwan. In this study, an anti-metastatic compound, 2,3,5-trimethoxy-4-cresol (TMC), was isolated from the solid-state cultured mycelium of *A. cinnamomea*. According to the results obtained from cell wound healing, cell migration and invasion assays, TMC effectively suppressed movement, migration and invasion of lung cancer cells at the dosage of 5–40 μM, which was non-toxic to A549 cells. In addition, TMC reduced protein expression of Akt, MMP-2 and MMP-9 and enhanced E-cadherin and TIMP-1 protein expression, which are known to regulate cell adhesion, migration and invasion. Taken together, TMC effectively suppresses movement, migration and invasion of lung cancer cells, and achieves an anti-cancer metastasis effect.

✉ Jong-Tar Kuo
jtk0901@cc.cust.edu.tw

✉ Sheng-Yang Wang
taiwanfir@dragon.nchu.edu.tw

1 Taiwan Leader Biotech Company, Taipei, Taiwan

2 Department of Biological Science and Technology, China University of Science and Technology, Taipei, Taiwan

3 Graduate Institute of Chinese Pharmaceutical Science, China Medical University, Taichung, Taiwan

4 Department of Biotechnology, Asia University, Taichung, Taiwan

5 Department of Forestry, National Chung Hsing University, 250-Kuo-Kung Road, Taichung 402, Taiwan

6 Agricultural Biotechnology Center, National Chung-Hsing University, Taichung, Taiwan

7 Agricultural Biotechnology Research Institute, Academia Sinica, Taipei, Taiwan

Keywords *Antrodia cinnamomea* · 2,3,5-Trimethoxy-4-cresol · Anti-metastasis · EMT

Abbreviations

DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
ECM	Extracellular matrix
EMT	Epithelial–mesenchymal transition
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
GA	Gallic acid
MMP	Matrix metalloproteinase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5,-diphenyltetrazolium bromide
NSCLC	Non-small-cell lung cancer
SDS	Sodium dodecyl sulfate
TEMED	*N,N,N',N'*-Tetra-methylethylenediamine
TIMP-2	Tissue inhibitor of metalloproteinase-2
TNF-α	Tumor necrosis factor alpha

Introduction

Non-small-cell lung cancer (NSCLC) is the leading cause of cancer death worldwide, and one of the most metastatic tumors. Although the 5-year survival rate for patients with localized NSCLC is more than 50 %, 57 % of patients have advanced/metastatic disease at the time of diagnosis, which carries a survival rate of less than 5 %. The primary cause of death in patients with lung cancer is tumor metastasis [1, 2]. Currently, surgery is the treatment of choice for early stage NSCLC to improve patient survival [3], but surgery also increases the risk to the patient, and therefore

chemotherapy, i.e., anti-metastatic and/or anti-invasion drugs, may be recommended to prevent local recurrence of the primary tumor and spread of the tumor cells.

Antrodia cinnamomea (syn. *Antrodia camphorata* and *Taiwanofungus camphorata*) is a valuable and unique edible fungus that originates from the forests of Taiwan. It has long been used as a folk remedy for treating various diseases including liver diseases, hypertension, abdominal pain and cancer [4]. Due to its high commercial value, *A. cinnamomea* has attracted much research and development interest in Taiwan over the past decade, and has been awarded a "National Health Food" certificate by Taiwan's Department of Health. Numerous studies have been conducted on the physiology and biochemical and pharmacological properties of *A. cinnamomea* [4–11], and many compounds identified from *A. cinnamomea* have been reported to possess health-promoting activities [12, 13]. Among the various activities reported, the anti-cancer activities of *A. cinnamomea* are of particular interest. Recently, increasing importance has been attached to the anti-metastatic activity of *A. cinnamomea* and its bioactive compounds [14–16]. Our previous study demonstrate that antrodin C, a melamide derivative isolated from the mycelia of *A. cinnamomea*, inhibits the metastatic potential of human breast carcinoma (MCF-7 cells) through the inhibition of epithelial–mesenchymal transition via suppression of the Smad2/3 signaling pathway [17]. Antrodon, a glycoprotein isolated from the mycelia of *A. cinnamomea*, inhibits the metastatic potential of Lewis lung carcinoma (LLC) in vitro via down-regulation of matrix metalloproteinase (MMP)-2, MMP-9, tumor necrosis factor alpha (TNF-α), interleukin (IL)-6 and IL-8, and up-regulation of tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 [18]. However, studies on anti-metastatic compounds of the fruiting body or mycelium of *A. cinnamomea* are limited.

In the present study, a potent anti-metastatic constituent, 2,3,5-trimethoxy-4-cresol (TMC) (Fig. 1), was isolated from the solid-state cultural mycelium product of *Antrodia cinnamomea*. The biochemical steps including the role of TMC in the metastatic process in lung cancer cells (A549) have been elucidated. Curcumin has been reported to be a potent anti-metastatic phyto-compound [19], so in the present study a non-cytotoxic concentration of curcumin was used as a positive drug control.

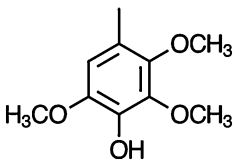

Fig. 1 2,3,5-Trimethoxy-4-cresol (TMC)

Results and Discussion

Most cancer cells can acquire metastatic capability leading to secondary tumors. Here we first used a low dose of purified extract, pure compound TMC, to explore the cytotoxicity of the treatment. A549 cells treated with different concentrations of TMC underwent MTT assay and were analyzed after 24 h. Cell viability was not significantly different in cells treated with TMC at concentrations of 5–40 μM compared to the control group (DMSO-treated A549 cells) (Fig. 2). This data is consistent with a previous report stating that below the concentration of 20 μM, TMC (previously known as 2,3,6-trimethoxy-4-methylphenol) was not cytotoxic to murine colorectal CT26 and human leukemia cancer cell lines, with an IC_{50} value of >20 μM [20]. TMC was not cytotoxic to A549 cells at these dosages. Therefore, in subsequent experiments, TMC was applied in this concentration range. In addition, the positive drug control curcumin (10 μM) does not show cytotoxicity to A549 cells (data not shown).

Wound healing is generally characterized by cell proliferation, remodeling of the extracellular matrix, cell invasion and migration [21]. In this study, a wound-healing assay was conducted to investigate cell migration ability. A wound line was created by scraping seeded monolayer A549 cells, which were then cultured in low serum-free medium plus TMC. The wound-healing activity of cells was observed between 0 and 12 h. The results indicated that TMC significantly decreased the wound-healing ability of A549 cells in a dose-dependent manner (Fig. 3). Next, in order to investigate the invasive and migratory capability of tumor cells, a trans-well migration assay was performed. The results showed that inhibition occurred in a concentration-dependent manner with higher doses of TMC

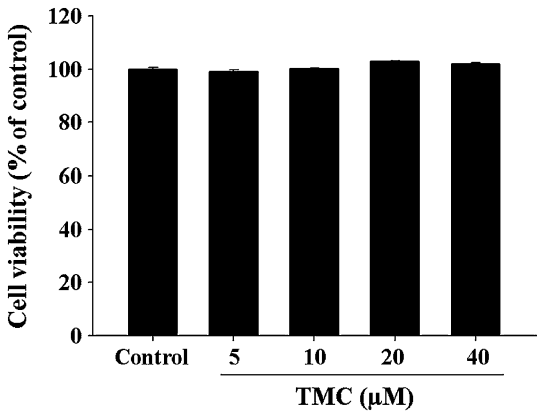

Fig. 2 Effect of TMC on the viability of A549 cells. Cells were treated with or without TMC at various concentrations (0, 5, 10, 20, 40 μM) for 24 h. Cell viability was analyzed by MTT assay. The results represent the average of three independent experiments ± SD. $*P < 0.05$, $**P < 0.005$ represent significant difference relative to the control group

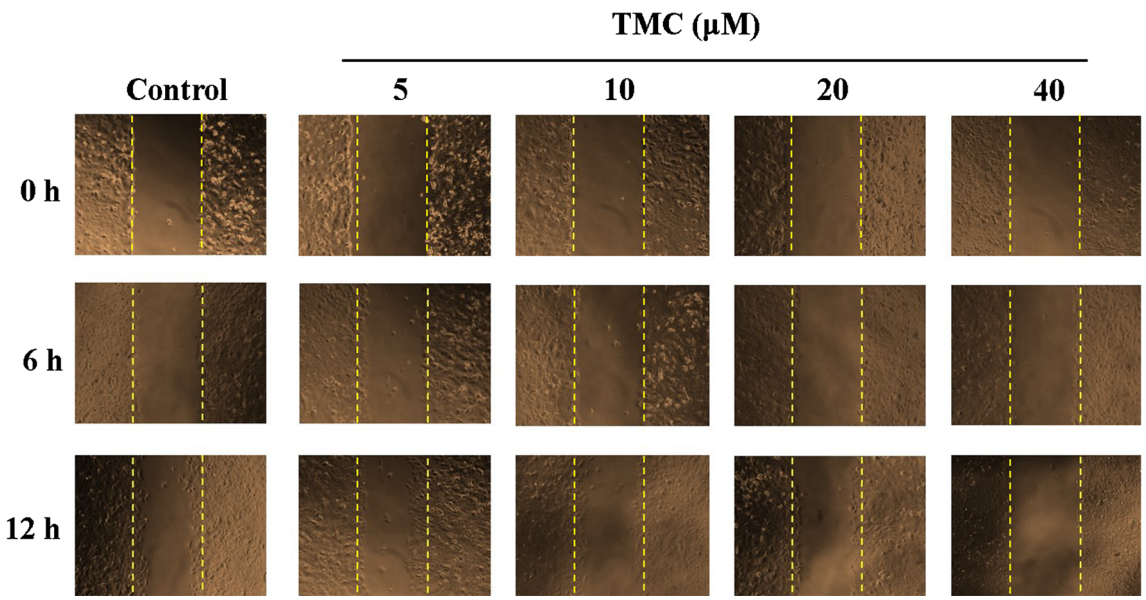

Fig. 3 Effects of TMC on the morphology and migration of A549 cells in wound healing assay. A549 cell monolayers treated with or without different dosages of TMC (0, 5, 10, 20, 40 μM) were scraped and the number of cells in the denuded zone (i.e., the wound) was quantitated after 0, 6 and 12 h through light microscopy observation. The results represent the average of three independent experiments ± SD. $*P < 0.05$, $**P < 0.005$ represent significant difference relative to the control group

resulting in fewer cells migrating to the lower chamber (Fig. 4a). When TNF-α was added to induce A549 cell growth, the same dose of TMC was used to explore the inhibition of A549 cell migration capability (Fig. 4b). We also used a trans-well chamber coated with Matrigel to analyze the invasion capability of cells. As shown in Fig. 5, significantly fewer cells passed into the lower chamber with higher doses of TMC, in a dose-dependent manner. Previous studies have suggested that enhanced tumor cell adhesiveness correlates with metastatic ability [22]. When A549 cells were treated with different dosages of TMC (0–40 μM), after 8 h the adhesion ability of cells was inhibited in a dose-dependent manner (Fig. 6). The inhibition ratio of adhesion activity of cells on the Matrigel membrane was 70 % of that of the control group. This result indicates that TMC significantly reduced the cell adhesion ability of A549 cells. With reference to previous reports [23, 24], the positive drug control of this study, a non-cytotoxic concentration of curcumin (10 μM), showed a strong inhibition of migration, invasion and adhesion of A549 cells; the inhibitory effect of TMC was highly comparable with curcumin.

Matrix metalloproteinases (MMPs) belong to a zinc-dependent family (ECM) of endopeptidases and are the principal mediators of the alterations observed in the microenvironment during cancer progression [25]. MMPs are implicated in a variety of physiological processes, including wound healing, uterine involution and organogenesis, as well as in pathological conditions such as inflammation, vascular and auto-immune disorders, and carcinogenesis [26, 27]. MMPs have been considered to be important therapeutic targets for cancer because the metastatic potential of various cancers is correlated with the ability of cancer cells to degrade the basement membrane [27]. The other key protease involved in degrading the components of the basement membrane is urokinase plasminogen activator (u-PA). u-PA is a serine protease that cleaves the ECM and converts the inactive plasminogen to active plasmin, which can directly mediate the invasion by degrading matrix components [27]. In order to investigate whether MMPs and u-PA are involved in the TMC inhibition of cancer cell invasion and motility, a gelatin and casein zymography assay was performed to check MMP and u-PA activity. As shown in Fig. 7, TMC significantly reduced the MMP-2 (Fig. 7a) and MMP-9 activity (Fig. 7b). The inhibition of MMP-2 and MMP-9 expression in A549 cells was 52 and 35 %, respectively, at the dosage of 40 μM. The u-PA activity in A549 cells was also inhibited by TMC (Fig. 8).

Epithelial–mesenchymal transition (EMT) has an important role in the progression of tumor metastasis [28]. It has been reported that protein kinase Akt induces EMT through down-regulation of the epithelial marker E-cadherin and upregulation of the mesenchymal marker vimentin in cancer progression [29]. E-cadherin is responsible for epithelial cell–cell adhesion and maintenance of cytoskeleton organization, and it is the major transmembrane component of adherens junctions. Cancer cell proliferation, invasion and metastasis increases with loss of function of E-cadherin [30]. During the EMT process, most of the tumor suppressor proteins (E-cadherin) are down-regulated, and the

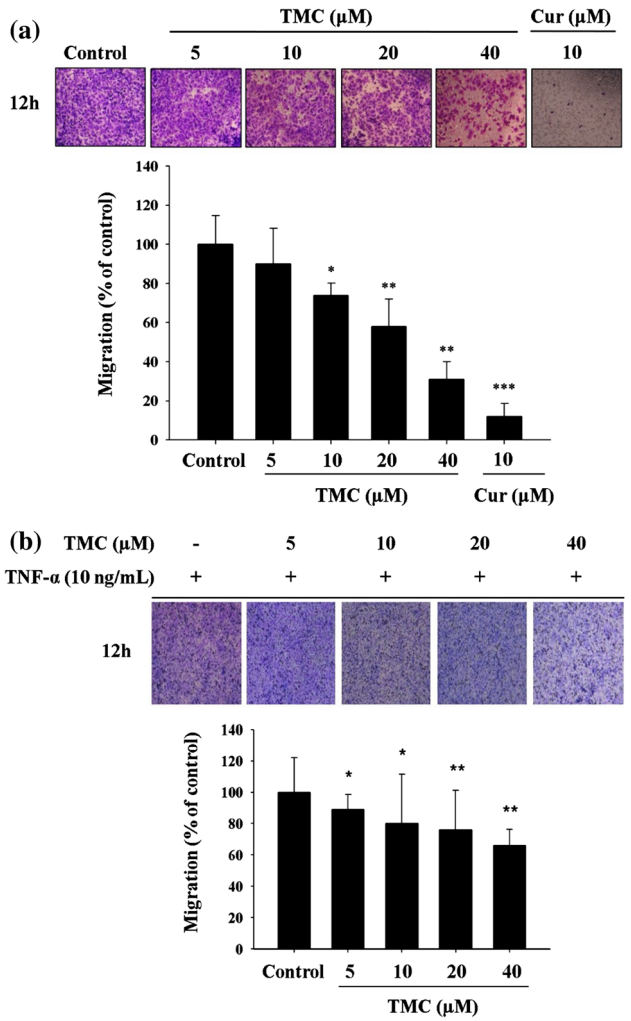

Fig. 4 TMC inhibited the migration of A549 cells in trans-well assay. **a** A549 cells were seeded in the upper chamber of the trans-well filters (pore size, 8 μm) after treatment with various concentrations of TMC (0, 5, 10, 20, 40 μM) and curcumin (10 μM). Chemoattractant was placed in the bottom chamber. A549 cells migrate through the small pores in the transmembrane in response to the chemoattractant. FBS (20 %) was used as the inducer. **b** A549 cells treated with TMC (0, 5, 10, 20, 40 μM) for 24 h, then seeded in the upper chamber of the trans-well and incubated for 12 h; 20 % FBS and TNF-α were used as the inducer. $*P < 0.05$, $**P < 0.005$ represent significant difference relative to the control group. *Cur* curcumin

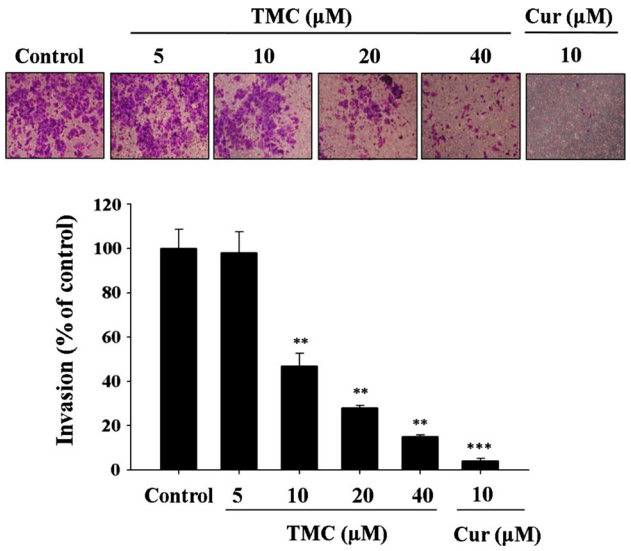

Fig. 5 TMC inhibited the invasive ability of A549 cells. A549 cells were treated with TMC (0, 5, 10, 20, 40 μM) or curcumin (10 μM) and seeded in the upper chamber of the trans-well which was coated Matrigel, and incubated for 12 h; 20 % FBS was used as the inducer. $*P < 0.05$, $**P < 0.005$ show significant difference relative to the control group. *Cur* curcumin

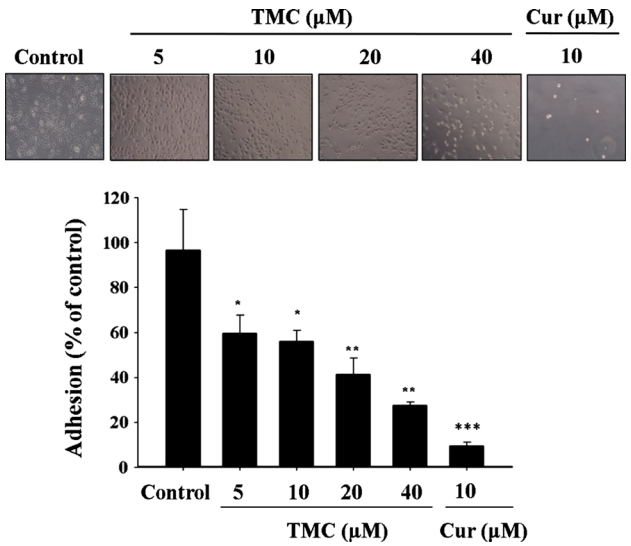

Fig. 6 TMC inhibited the adhesion of A549 cells through Matrigel. Matrigel was coated in the 24-well incubator for 24 h, and A549 cells were treated with TMC (0, 5, 10, 20, 40 μM) or curcumin (10 μM) for 24 h, then cells were subtracted and seeded in the 24-well incubator for 8 h. Results are presented as mean ± SD of three assays. $*P < 0.05$, $**P < 0.005$ show significant difference relative to the control group. *Cur* curcumin

oncoproteins (vimentin, N-cadherin) are up-regulated [31]. E-cadherin promotes homotypic tumor cell adhesion function to maintain intercellular contacts that confine cells to the primary tumor site and prevent metastatic potential [32]. To further confirm the inhibition mechanism of TMC in metastasis, the effects of TMC on E-cadherin, p-AKT and TIMP-1 were examined. As shown in Fig. 9a, TMC significantly enhanced levels of the epithelial marker E-cadherin, and decreased expression of p-Akt. Moreover, TIMP-1 expression was also increased (Fig. 9b). In conclusion, our data suggest that treatment of lung cancer cells with TMC induces molecular changes, which are consistent with a less motile, migratory and invasive phenotype of lung cancer cell. In conclusion, 2,3,5-trimethoxy-4-cresol (TMC) effectively suppresses movement, migration and invasion of lung cancer cells, and achieves an anti-cancer metastasis effect.

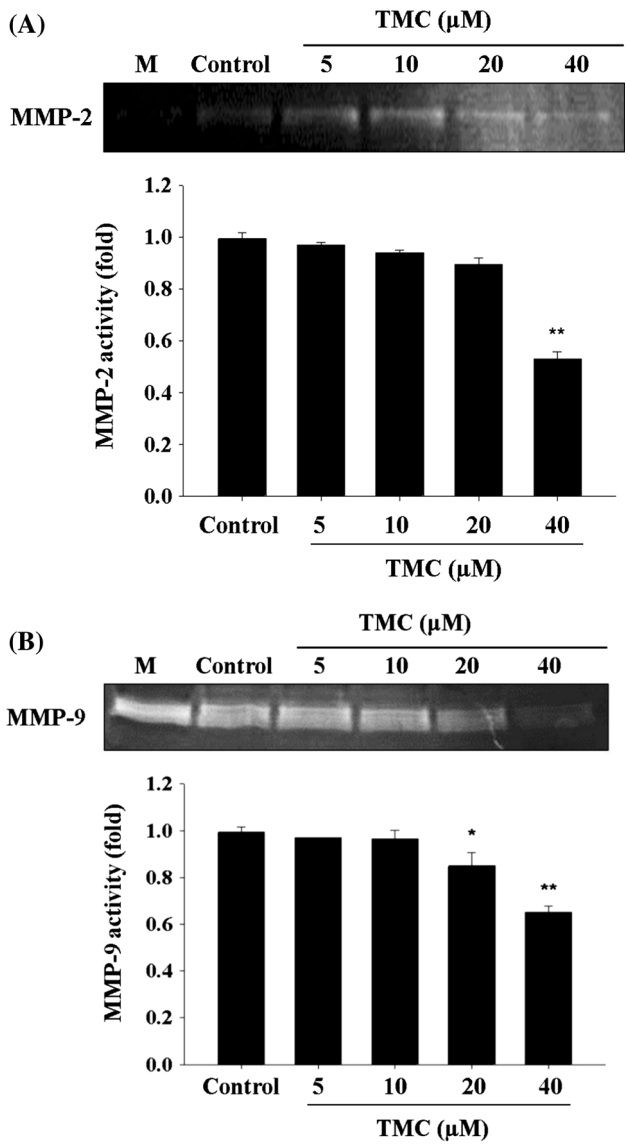

Fig. 7 Effect of TMC-dependent MMP activity inhibition on A549 cells. A549 cells were treated with TMC (0, 5, 10, 20, 40 μM) in culture medium. After 24 h, the cells were cultured in serum-free medium and then subjected to gelatin zymography to analyze the activities of **a** MMP-2 and **b** MMP-9. The activities of these proteins were subsequently quantified by densitometric analysis. Data from 3 independent experiments are shown. $*P < 0.05$, $**P < 0.005$ represent significant difference relative to the control

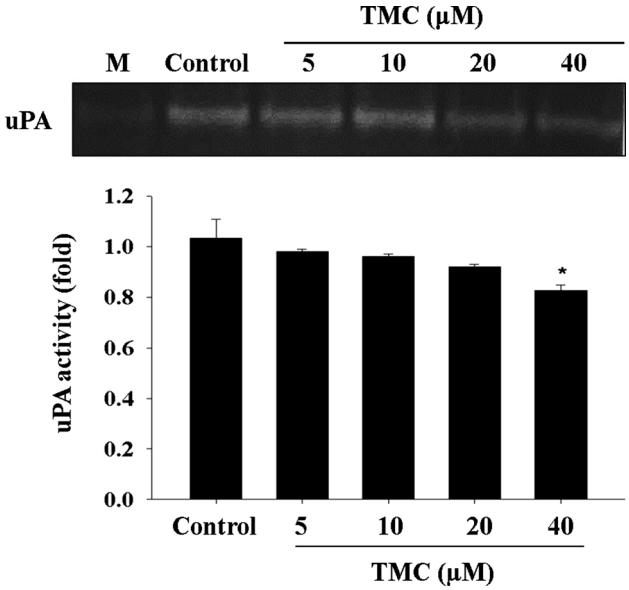

Fig. 8 TMC inhibited uPA activity on A549 cells. Cells were treated with up to 40 μM TMC for 24 h and were then subjected to casein zymography to analyze uPA activity, which was subsequently quantified by densitometry analysis. Data from 3 independent experiments are shown. $*P < 0.05$, $**P < 0.005$ represent significant difference relative to the control group

Experimental section

Chemical and reagents

2,3,5-Trimethoxy-4-cresol was previously isolated and identified from the solid-state cultured mycelium of *A. cinnamomea* and was known as 2,3,6-trimethoxy-4-methylphenol [20]. In this study, we also isolated it from the solid-state cultured mycelium of *A. cinnamomea* (strain no. LEACS-002) obtained from the R&D Center of Taiwan Leader Biotechnology Corp. (Taichung, Taiwan). The purity of TMC was above 99 %, as confirmed by HPLC and ^{1}H-NMR analysis. Ham's F12 medium was purchased from Amimed/BioConcept (Allschwil, Switzerland); fetal bovine serum (FBS) was obtained from Gibco-BRL (China). Antibodies against MMP-2, MMP-9, and β-actin were purchased from Santa Cruz Biotechnology (Heidelberg, Germany); Akt, cadherin, NF-κB, and TIMP1 were purchased from Cell Signaling (Danvers, MA, USA). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and curcumin were obtained from Sigma–Aldrich (St. Louis, MO, USA). Millicell hanging cell culture insert was obtained from Millipore (Billerica, MA, USA); Matrigel was purchased from BD Biosciences (San Jose, CA, USA).

Cell culture

The human non-small-cell lung cancer cell line A549 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cell line was cultured in Ham's F12 medium supplemented with 10 % heat-inactivated FB and, 2 mM glutamine, and cell cultures were maintained at 37 °C in an incubator with 5 % CO_2.

MTT assay

Cell viability was determined by MTT colorimetric assay as described previously [33]. A549 cells (2×10^5 cells/24-

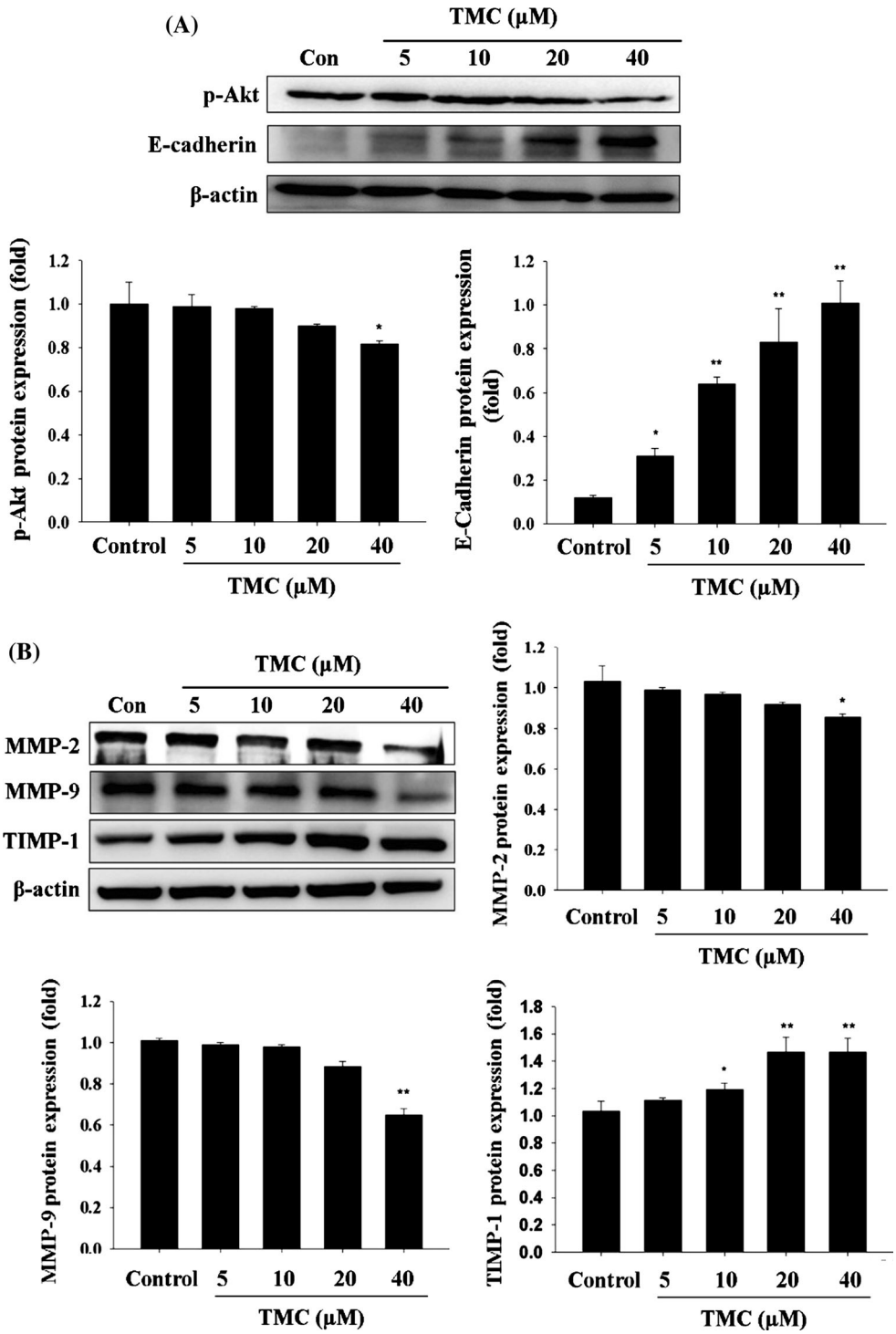

Fig. 9 Inhibition effects of TMC on expression of proteins involved in regulation of cell motility and invasion of A549 cells. A549 cells were treated with TMC (0, 5, 10, 20, 40 μM) for 24 h. Immunoblots are shown for **a** p-Akt, E-cadherin, **b** MMP-2, MMP-9, TIMP-1 and β-actin (control) in A549 cell lines. The cell lysates were prepared for Western blotting as described in Experimental Section. Data from 3 independent experiments are shown. $*P < 0.05$, $**P < 0.005$ represent significant difference relative to the control

well plates) were seeded and incubated overnight. Cells were then treated with various concentrations of TMC (5, 10, 20 and 40 μM) for 24 h. After incubation, the medium-deprived cells were incubated with MTT (10 μg/mL) in 100 μL of fresh DMEM for 1 h at 37 °C. The MTT-generated violet formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and the absorbance was measured at 570 nm (A_{570}) using an ELISA microplate reader (μQuant, Bio-Tek Instruments, Winooski, VT, USA). Cell viability (%) was calculated as: (A_{570} of treated cells/A_{570} of untreated cells) ×100.

Cell wound-healing repair assay

Cell proliferation and migration was observed in A549 cells using the wound-healing assay [34]. A549 cells

(2×10^7 cells/cm^2) were seeded in a 12-well culture plate and grown to confluence or near (>90 %) confluence in Ham's F12 medium containing 10 % FBS. Using a sterile 200 μL pipet tip, separate wounds were scratched, and cellular debris and FBS were removed by washing with PBS; cells were then starved in low-serum medium (0.5–0.1 % serum in Ham's F12 medium). The culture medium was then removed and cells were treated with TMC (5, 10, 20 and 40 μM) and cell movement was observed at 0, 12, 24 and 48 h to monitor the migration of cells into the wounded area. Images were captured and the results analyzed.

Cell migration and invasion assay

A Millipore cell culture insert was used to examine the cell migration and invasion [35]. For the cell invasion assay prior to preparation, A549 cells were pretreated with TMC (5, 10, 20 and 40 μM) or curcumin (10 μM) with/without TNF-α for 24 h, then A549 cells (2×10^5 cells/cm^2) were seeded in the upper chamber, and Ham's F12 medium was added to the lower chamber with 10 % FBS as inducer. For the cell invasion assay prior to preparation, BD Matrigel was thawed overnight at 4 °C and kept on ice; the Millicell insert and plate was chilled to 4 °C and kept in ice; Matrigel was diluted using ice-cold serum-free Ham's F12 medium to a final concentration of 2 mg/ml, After Matrigel solidification, A549 cells (2×10^5 cells/cm^2) were seeded in the upper chamber, and Ham's F12 medium was added to the lower chamber with 10 % FBS as inducer. Both migration and invasion assays were conducted after incubation at 37 °C with 5 % CO_2 for 12 h. Non-invaded cells in the upper chamber were removed with a cotton swab, and invaded cells were washed twice with PBS and fixed in methanol for 15 min. Fixed cells were stained with Giemsa. Three random fields were analyzed for each chamber.

Cell adhesion assay

BD Matrigel was used for the cell adhesion assay. Prior to preparation, BD Matrigel was thawed overnight at 4 °C and kept on ice. Matrigel was diluted using ice-cold serum-free Ham's F12 medium to a final concentration of 2 mg/ml. A549 cells were pretreated with TMC (5, 10, 20 and 40 μM) or curcumin (10 μM) for 24 h. On the first day, Matrigel was added to the plate and incubated overnight at 37 °C with 5 % CO_2. On the second day, after Matrigel solidification, A549 cells (2×10^5 cells/cm^2) were seeded onto the plate. After incubation at 37 °C with 5 % CO_2 for 12 h, non-invaded cells and the culture medium were removed and cells were washed twice with PBS and fixed in paraformaldehyde for 15 min. Fixed cells were stained with Giemsa. Three random fields were analyzed for each chamber.

Zymography assay

Gelatin zymography and casein zymography protease assays were used to measure the activities of MMP-2, MMP-9 and uPA in the medium of A549 cells. A549 cells (2×10^5 cells/cm^2) were seeded in a 6-well culture dish and incubated at 37 °C with 5 % CO_2 overnight. Cells were then treated with TMC (5, 10, 20 and 40 μM) for 24 h before being starved in low-serum medium (0.5–0.1 % serum in Ham's F12 medium) for 24 h. The culture medium was then collected. Gelatin SDS-PAGE (8 % gel with 1 mg/ml gelatin) and casein SDS-PAGE electrophoresis (8 % gel with 1 mg/ml casein and plasminogen) was conducted. After electrophoresis, gels were washed with 2.5 % Triton X-100 and incubated in developing buffer at 37 °C for 24 h. The developing buffer was then removed and gels were stained with Coomassie blue R-250.

Western blot analysis

Metastasis-associated proteins were measured by Western blotting. A549 cells (2×10^5 cells/cm^2) were seeded in a 10-cm dish and grown in Ham's F12 medium containing 10 % FBS. When cells attached, they were treated with TMC (5, 10, 20 and 40 μM) for 24 h. Total cell lysates were prepared using Mammalian Protein Extraction Reagent (Cayman Chemicals, Ann Arbor, MI, USA). The cytosolic and nuclear protein fractions were obtained using NE-PER Nuclear and Cytoplasmic Protein Extraction Reagent Kit (Pierce Biotechnology, Rockford, IL, USA). The protein content in each fraction was determined by Bio-Rad protein assay reagent, with BSA as the standard. Equal amounts (50 μg) of denatured protein samples were electrophoresed by 8–15 % SDS-PAGE, followed by transfer onto PVDF membranes overnight. The membranes were blocked with 5 % non-fat dry milk for 30 min at room temperature and then reacted with primary antibodies for 2 h. They were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody for 2 h and developed using the enhanced chemiluminescence substrate (Millipore). Images were captured with a VL Chemi-Smart 3000 gel documentation system (Vilber Lourmat, France).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's test for multiple comparisons and Student's *t* test for single

comparison. The data are reported as mean ± SD. The numbers of independent experiments assessed are given in the figure legends.

Acknowledgments Parts of this study were supported by the Ministry of Science and Technology, Taiwan, Republic of China (NSC-103-2911-I-005-301, NSC-102-2911-I-005-301) and the Ministry of Education, Taiwan, R.O.C. under the ATU plan.

Conflict of interest No competing financial interests exist.

References

1. Simon GR (2014) nab-Paclitaxel for the treatment of advanced squamous none small-cell lung cancer: a comprehensive update. Clin Lung Cancer 15:391–397
2. Riihimäki M, Thomsen H, Hemminki A, Sundquist K, Hemminki K (2013) Comparison of survival of patients with metastases from known versus unknown primaries: survival in metastatic cancer. BMC Cancer 13:36
3. Nichols L, Saunders R, Knollmann FD (2012) Causes of death of patients with lung cancer. Arch Pathol Lab Med 136:1552–1557
4. Wang HC, Chu FH, Chian SC, Liao JW, Hsieh HW, Li WH, Lin CC, Shaw JF, Kuo YH, Wang SY (2012) Establishment of the metabolite profile for an *Antrodia cinnamomea* health food product and investigation of its chemoprevention activity. J Agric Food Chem 61:8556–8564
5. Hsieh YH, Chu FH, Wang YS, Chien SC, Chang ST, Shaw JF, Chen CY, Hsiao WW, Kuo YH, Wang SY (2010) Antrocamphin A, an anti-inflammatory principal from the fruiting body of *Taiwanofungus camphoratus*, and its mechanisms. J Agric Food Chem 58:3153–3158
6. Gokila Vani M, Senthil Kumar KJ, Liao JW, Chien SC, Mau JL, Chiang SS, Lin CC, Kuo YH, Wang SY (2013) Antcin C from *Antrodia cinnamomea* protects liver cells against free radical-induced oxidative stress and apoptosis in vitro and in vivo through Nrf2-dependent mechanism. Evid Based Complement Alternat Med 2013:296082
7. Lin TY, Chen CY, Chien SC, Hsiao WW, Chu FH, Li WH, Lin CC, Shaw JF, Wang SY (2011) Metabolite profiles for *Antrodia cinnamomea* fruiting bodies harvested at different culture ages and substrates from different wood. J Agric Food Chem 59:7626–7635
8. Senthil Kumar KJ, Chu FH, Hsieh HW, Liao JW, Li WH, Lin CC, Shaw JF, Wang SY (2011) Antroquinonol from ethanolic extract of mycelium of *Antrodia cinnamomea* protects hepatic cells from ethanol-induced oxidative stress through Nrf-2 activation. J Ethnopharmacol 136:168–177
9. Lin TY, Chien SC, Kuo YH, Wang SY (2012) Distinguishing between R- and S-antcin C and their cytotoxicity. Nat Prod Commun 7:835–836
10. Lu MC, El-Shazly M, Wu TY, Du YC, Chang TT, Chen CF, Hsu YM, Lai KH, Chiu CP, Chang FR, Wu YC (2013) Recent research and development of *Antrodia cinnamomea*. Pharmacol Ther 139:124–156
11. Chen YC, Liu YL, Li FY, Chang CI, Wang SY, Lee KY, Li SL, Chen YP, Jinn TR, Tzen JT (2011) Antcin A, a steroid-like compound from *Antrodia camphorata*, exerts anti-inflammatory effect via mimicking glucocorticoids. Acta Pharmacol Sin 32:904–911
12. Geethangili M, Tzeng YM (2011) Review of pharmacology effects of *Antrodia camphorata* and its bioactivities compounds. Evid Based Complement Alternat Med 2011:212641
13. Ao ZH, Xu ZH, Lu ZM, Xu HY, Zhang XM, Dou WF (2009) Niuchangchih (*Antrodia camphorata*) and its potential in treating liver diseases. J Ethnophrarmcol 121:194–212
14. Liu FC, Lai MT, Chen YY, Lin WH, Chang SJ, Sheu MJ, Wu CH (2013) Elucidating the inhibitory mechanisms of the ethanolic extract of the fruiting body of the mushroom *Antrodia cinnamomea* on the proliferation and migration of murine leukemia WEHI-3 cells and their tumorigenicity in a BALB/c allograft tumor model. Phytomedicine 20:874–882
15. Chen YY, Chou PY, Chien YC, Wu CH, Wu TS, Sheu MJ (2012) Ethanol extracts of fruiting bodies of *Antrodia cinnamomea* exhibit anti-migration action in human adenocarcinoma CL1-0 cells through the MAPK and PI3 K/AKT signaling pathways. Phytomedicine 19:768–778
16. Yang HL, Kuo YH, Tsai CT, Huang YT, Chen SC, Chang HW, Lin E, Lin WH, Hseu YC (2011) Anti-metastatic activities of *Antrodia camphorata* against human breast cancer cells mediated through suppression of the MAPK signaling pathway. Food Chem Toxicol 49:290–298
17. Senthil Kumar KJ, Vani MG, Chueh PJ, Mau JL, Wang SY (2015) Antrodin C inhibits epithelial-to-mesenchymal transition and metastasis of breast cancer cells via suppression of Smad2/3 and β-Catenin signaling pathways. PLoS One 10:e0117111
18. Fa KN, Yang CM, Chen PC, Lee YY, Chyau CC, Hu ML (2015) Anti-metastatic effect of antrodan, the *Antrodia cinnamomea* mycelia glycoprotein in lung carcinoma cells. Int J Biol Macromol 74:476–482
19. Shishodia S, Chaturvedi MM, Aggarwal BB (2007) Role of curcumin in cancer therapy. Curr Probl Cancer 31:243–305
20. Lee TH, Chen CC, Chen JJ, Liao HF, Chang HS, Sung PJ, Tseng MH, Wang SY, Ko HH, Kuo YH (2014) New cytotoxic components from *Antrodia camphorate*. Molecules 19:21378–21385
21. Dauer DJ, Ferraro B, Song L, Yu B, Mora L, Buettner R, Enkemann S, Jove R, Haura EB (2005) Stat3 regulates genes common to both wound healing and cancer. Oncogene 24:3397–3408
22. Albelda S (1993) Role of integrins and other cell adhesion molecules in tumor progression and metastasis. Lab Invest J Tec Meth Pathology 68:4–17
23. Chen HW, Lee JY, Huang JY, Wang CC, Chen WJ, Su SF, Huang CW, Ho CC, Chen JJW, Tsai MF, Yu SL, Yang PC (2008) Curcumin inhibits lung cancer cell invasion and metastasis through the tumor suppression HLJ1. Cancer Res 68:7428–7438
24. Chen QY, Zheng Y, Jiao DM, Chen FY, Hu HZ, Wu YQ, Song J, Yan J, Wu LJ, Lv GY (2014) Curcumin inhibits lung cancer cell migration and invasion through Rac1-dependent signaling pathway. J Nutr Biochem 25:177–185
25. Kessenbrock K, Plaks V, Werb Z (2010) Matrix metalloproteinases: regulators of the tumor microenviroment. Cell 141:52–67
26. Gialeli C, Theocharis AD, Karamanos NK (2010) Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. FEBS J 278:16–27
27. Roy DM, Walsh LA (2014) Candidate prognostic markers in breast cancer: focus on extracellular proteases and their inhibitors. Breast Cancer 6:81–91
28. Thiery JP (2002) Epithelial–mesenchymal transitions in tumour progression. Nat Rev Cancer 2:442–454
29. Grille SJ, Bellacosa A, Upson J, Klein-Szanto AJ, van Roy F, Lee-Kwon W, Donowitz M, Tsichlis PN, Larue L (2003) The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness of squamous cell carcinoma lines. Cancer Res 63:2172–2178
30. Xiao D, He J (2010) Epithelial mesenchymal transition and lung cancer. J Thoracic Disease 2:154–159
31. Tania M, Khan MA, Fu J (2014) Epithelial to mesenchymal transition inducing transcription factors and metastatic cancer. Tumour Biol 35:7335–7342

32. Zetter BR (1993) Adhesion molecules in tumor metastasis. Semin Cancer Biol 4:219–229
33. Lin CT, Senthil Kumar KJ, Tseng YH, Wang ZJ, Pan MY, Xiao JH, Chien SC, Wang SY (2009) Anti-inflammatory activity of Flavokawain B from *Alpinia pricei* Hayata. J Agric Food Chem 57:6060–6065
34. Wu YY, Peck K, Chang YL, Pan SH, Cheng YF, Lin JC, Yang RB, Hong TM, Yang PC (2011) SCUBE3 is an endogenous TGF-β receptor ligand and regulates the epithelial-mesenchymal transition in lung cancer. Oncogene 30:3682–3693
35. Meng XN, Jin Y, Yu Y, Bai J, Liu GY, Zhu J, Zhao YZ, Wang Z, Chen F, Lee KY, Fu SB (2009) Characterisation of fibronectin-mediated FAK signalling pathways in lung cancer cell migration and invasion. Br J Cancer 101:327–334