Tautomycetin Induces Apoptosis by Inactivating Akt Through a PP1-Independent Signaling Pathway in Human Breast Cancer Cells

Mingshan Niu¹,†, Yan Sun¹,†, Xuejiao Liu¹, Li Tang¹, and Rongguo Qiu¹,*

¹Research Center for Molecular Medicine, School of Life Science and Biotechnology, Dalian University of Technology, Dalian 116023, China

Abstract. Tautomycetin (TMC), originally isolated from Streptomyces griseochromogenes, has been suggested as a potential drug retaining specificity toward colorectal cancer. However, we found that TMC exhibited inhibitory effects on cell proliferation of many cancer cell lines including adriamycin-resistant human breast adenocarcinoma. We investigated its anti-tumor activity and mechanisms in human breast cancer cells for the first time. In this study, we showed that TMC effectively inhibited breast cancer cell proliferation, migration, and invasion. TMC also induced apoptosis in MCF-7 cells. This apoptotic response was in part mediated by Bcl-2 cleavage, leading to the release of cytochrome c, which facilitates binding of Apaf-1 to caspase-9 in its presence and subsequent activation of caspase-7 in apoptosis induction signaling pathways. Furthermore, we identified that TMC induced apoptosis by suppressing Akt signaling pathway activation, which is independent of protein phosphatase PP1 inhibition. The levels of downstream targets of Akt, including phospho-forkhead transcription factor and Bad, were also reduced after TMC treatment. Overall, our results indicate that TMC could be used as a potential drug candidate for breast cancer therapy. More importantly, our study provides new mechanisms for the anticancer effects of TMC.

Keywords: tautomycetin, apoptosis, breast cancer, Akt pathway

Introduction

Breast cancer is a major cause of cancer-related death in women, second only to lung cancer (1, 2). In the United States, one of the most frequently used agents for treating breast cancer is the chemotherapeutic drug Adriamycin. When Adriamycin is given as a single-agent treatment, response rates are typically 40% – 60% and can be as high as 80% (3). Unfortunately, resistance to Adriamycin is a major clinical problem and an important cause for treatment failure. Therefore, novel agents for treatment of human breast cancers, especially hormone-independent breast cancers, are highly desirable. Natural products have received increasing attention in recent years for the discovery of novel cancer preventive and therapeutic agents (4).

The epidermal growth factor receptor (EGFR) plays a critical role in the control of cellular proliferation, differentiation, development, and oncogenesis. Activation of the EGFR recruits and activates components of the downstream intracellular-signaling cascade, including phosphatidylinositol-3-kinase (PI3K) and Akt proteins (5). Akt, also called as protein kinase B, is a serine-threonine kinase activated by phosphorylation of two critical residues: threonine 308 (T308) located in the activation loop and serine 473 (S473) at the COOH terminal portion of the protein. AKT activation is mediated by PI3K and phosphoinositide-dependent kinase-1 (PDK1) (6, 7). The mechanisms by which AKT functions to promote survival may include, among others, the phosphorylation of Bad, Glycogen Synthase Kinase-3 (GSK-3), Forkhead transcription factor (FKHR), caspase-9, and RelA/p65 subunit of NF-κB (8 – 10). The PI3K/Akt pathway is hyperactive and genetically selected during tumorigenesis in a variety of cancers, including ovarian (11), breast (12), prostate (13), lung, and colon (14, 15). Furthermore, constitutive activation or overex-
pression of Akt has been found to correlate with poor prognosis and drug resistance (16).

Tautomycetin (TMC) is classified as a type I PKS-derived compound and it was first isolated from Streptomyces griseochromogenes in 1989 (17). TMC was found to specifically inhibit the protein phosphatases PP1 and PP2A (18). In contrast to other naturally occurring PP1 and PP2A inhibitors, such as okadaic acid, calyculin-A, fostriecin, and cantharidin, TMC exhibits a high degree of PP1 selectivity (19 – 21). TMC preferentially inhibits PP1 by nearly 40-fold relative to PP2A and is the most selective PP1 inhibitor reported to date (18). Many human diseases are characterized by an altered interplay between phosphatases and kinases, and thus the selective inhibition of PP1 and PP2A has been proposed to be an attractive goal for rational anticancer drug design (22).

TMC has been identified as a potent immunosuppressor of activated T cells both in vivo and in vitro (23). TMC has also been suggested as a potential drug retaining specificity toward colorectal cancer because of its regulation of Raf-1 activity in a cell-type–specific manner. The effect of TMC in antiproliferation is acquired by regulation of Raf-1 activity in a cell-type–specific manner. The effect of TMC in antiproliferation is acquired by regulation of Raf-1 activity in a cell-type–specific manner.

Herein our data show that TMC inhibits cell proliferation of breast cancer, small cell lung cancer, and prostate cancer in addition to colorectal cancer. Further, we demonstrated that TMC inhibits proliferation of breast cancer via an ERK-independent pathway. These data indicate that there might be a distinct mechanism for its activity of inhibiting growth of breast cancer cells. In this study, the results clearly demonstrate that TMC could induce apoptosis and suggest that TMC-induced apoptotic death is mediated through blockade of the Akt pathways and subsequent activation of the mitochondrial-mediated pathway.

Materials and Methods

Cell culture and reagents

The MCF-7 human breast cancer cells were maintained in RPMI 1640 containing 10% fetal bovine serum at 37°C in a 5% CO2 humidified incubator. Antibodies for caspase-7, caspase-8, caspase-9, and phosphorylated Akt (Ser473) were obtained from Cell Signaling Technology (Danvers, MA, USA). Monoclonal anti-PP1 antibodies, which recognize all isoforms of PP1, were purchased from GE Healthcare. The antibodies against Bel-2, p53, β-catenin, GAPDH, p-Bad, p-ERK, p-EGFR, p-HER2, p-PDK1, and p-FKHR were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell proliferation assay

MCF-7 tumor cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) rapid colorimetric assay. Briefly, cells were seeded in quadruplicate on 96-well plates and incubated overnight. The cells were then treated with TMC in concentrations of 0 – 100 μM and incubated for up to 72 h. The MTT assay was performed by replacing the standard medium with 100 μL PBS containing 0.5 mg/mL MTT and incubating at 37°C for 4 h. After incubation, the crystals were dissolved with 200 μL dimethyl sulfoxide. The multiwell plates were then measured at 540 nm using a spectrophotometer.

Wound healing and invasion assay

The migration of cells was investigated using the ‘wound-healing’ assay. Briefly, cells were seeded in a 24-well microtiter plate. A ‘wound’ was created using a pipette tip and rinsed with PBS to remove detached cells. Medium with the indicated concentrations of TMC was added for 48-h incubation. The microscopic observation of the cells was recorded at 0 and 48 h.

The invasiveness of breast cancer cells was investigated with the Invasion Chamber 24-well Plate (Transwell; Costar, Cambridge, MA, USA). Culture inserts were coated with Matrigel and placed into the wells of 24-well culture plates. In the lower chamber, 500 μL of media containing 10% fetal bovine serum was added and MCF-7 cells were seeded to the upper chamber. After 24 h of incubation, the media was removed and the cells fixed with 4% paraformaldehyde for 30 min. The migratory cells, which attached to the bottom of the chamber, were stained with 0.5% crystal violet solution for 15 min. The observation of the migratory cells was recorded with microscopy.

Western blot analysis

For the preparation of whole-cell lysates, cells were washed with ice-cold phosphate-buffered saline (PBS). Lysis buffer was then added to the cells and further incubated on ice for 10 min. For analysis of cytochrome c release, mitochondrial and cytosolic fractions were isolated by the differential centrifugation method previously described and probed by western blotting for cytochrome c. Protein (30 – 100 μg) was fractionated on a 10%
acrylamide denaturing gel and transferred onto a nitrocellulose membrane (Amersham Life Science, Bucks, UK) by electroblotting. Blots were developed using Pierce Pico ECL reagent (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer’s instructions.

Luciferase reporter assays

MCF-7 cells were plated onto 96-well dishes and incubated at 37°C. Twenty-four hours after plating, cell transfections were done using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. All luciferase activities were normalized for transfection efficiency by cotransfection with pRL Renilla luciferase vector. Six hours after transfection, cells were incubated in TMC for an additional 30 h. The cells were then harvested and reporter activity was assayed using the Dual Luciferase Assay System (Promega, Madison, WI, USA).

Apoptosis assay

To assess apoptosis, cells were plated in 6-well plates, and allowed to adhere overnight. After 24 h, cells were treated with 0.5 or 1 μM of TMC or DMSO continuously until harvested. Apoptosis was measured using the Apoptosis Detection kit (BD Biosciences, San Jose, CA, USA). Cell apoptosis analysis was performed by FACSscan (BD Biosciences) using a single laser emitting excitation light at 488 nm. Data were analyzed by CellQuest software (BD Biosciences).

Suppression of PP1 expression with double-stranded RNA (siRNA)

siRNA mediated suppression of PP1 was achieved as described previously (27). The following siRNA sense oligonucleotide sequences were used: Control, 5′-UAAGGCUAUGAAAGAUAACdTdT-3′; PP1α, 5′-CCGCAUCUAUGGUUUCUCdTDdT-3′; PP1λ, 5′-GC AUGAUUUGGAUCUAAdTdT-3′. For treatment, MCF-7 cells were plated at 200,000 per 60 mm dish. Twenty-four hours later, the PP1α and PP1λ annealed double-stranded RNA oligonucleotides were transfected using Lipofectamine 2000.

Data analysis

Data are means and standard deviations of three independent experiments with three to five replicates each. The results were statistically analyzed using a Student’s t-test and considered statistically significant at the level of P < 0.05.

Results

TMC inhibits proliferation and induces apoptosis in breast carcinoma lines

It has previously been shown that TMC is a potential anticancer drug retaining specificity toward colorectal cancer with induction of G1/S phase cell cycle arrest (24). Our studies showed that TMC could inhibit proliferation of different kinds of cancer cells in vitro (Table 1). MCF-7 cells showed growth inhibition with TMC treatment in a dose-dependent manner. In order to detect and quantify the apoptosis induced by TMC, we used annexin V-FITC/PI double staining. The TMC induced effect was remarkable at 24 h of treatment, which showed that at a concentration of 0.5 μM, about 25.6% of the cells were in the early apoptotic stage, whereas about 21.3% of the cells were in the late apoptotic stage (Fig. 1).

Effect of TMC on the migration and invasion of breast cancer cells

To ascertain the inhibitory effect of TMC on breast cancer metastasis, we investigated the effects of TMC on the migration potential of MCF-7 cells using the ‘wound’ healing assay. The vehicle-treated cells had almost completely filled the cleared area. The migration of the cancer cells was reduced with TMC treatment in a dose-dependent manner (Fig. 2A). The ability of TMC to reduce the invasiveness of breast cancer cells was further investigated by invasion assay. We found that TMC significantly inhibited MCF-7 cell invasion in a dose-dependent manner (Fig. 2B).

TMC induced cell death is mediated through activation of caspases

To further examine the mechanism underlying TMC-induced cell death in MCF-7 cells, the possible role of caspases in this process was studied. Western blot analysis of caspases-8, -9, and -7 were carried out after TMC treatment in cells. The expression levels of pro-caspase-9

<table>
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<tr>
<th>Cancer cells</th>
<th>Cell lines</th>
<th>IC_{50} (μM)</th>
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<tr>
<td>Human colorectal cancer cells</td>
<td>HT-29</td>
<td>0.30 ± 0.04</td>
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<tr>
<td>Human breast cancer cells</td>
<td>MCF-7</td>
<td>0.36 ± 0.04</td>
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<tr>
<td>Human cervical cancer cells</td>
<td>Hela</td>
<td>0.74 ± 0.06</td>
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<tr>
<td>Human prostate cancer cells</td>
<td>Du145</td>
<td>0.42 ± 0.04</td>
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<tr>
<td>Human small cell lung cancer cells</td>
<td>NCI-H1688</td>
<td>0.67 ± 0.07</td>
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had gone down significantly with the increase of treatment time; the cleaved active forms remained undetectable. Pro-caspase-8 does not show any significant change in expression levels with TMC exposure. Analysis of caspase-7 showed the presence of active forms of 20 – 23 kDa. Bcl-2, which was known to bind to mitochondria and inhibit the release of cytochrome c, was cleaved as a consequence of TMC treatment in a dose-dependent fashion. Bad was also dephosphorylated in a dose-dependent manner after exposure to TMC. The release of cytochrome c was analyzed in cytosolic fractions of TMC-treated cells. A dose-dependent increase in the cytosolic fraction was observed (Fig. 3A). The involvement of caspases in TMC-induced cell death was confirmed by the use of caspase inhibitors. The growth of cells exposed to 1 μM TMC was inhibited by 90% relative to that of untreated cells. However, pretreatment of MCF-7 cells with Z-VAD-FMK, TMC (1 μM) affected the growth of MCF-7 cells with the inhibition ratio of 42% (Fig. 3B).

The effect of TMC on ERK, β-catenin, and p53 activation

TMC was recently shown to inhibit colorectal cancer cells growth through activation of the ERK pathway (24). We have also observed an increase in levels of phosphorylation of ERK in HT-29 colorectal cancer cells treated with TMC. However, treatment with TMC had no effect on phosphorylation of ERK in MCF-7 breast cancer cells and Hela cervical cancer cells (Fig. 4A). The β-catenin and p53 do not show any significant change in protein expression levels (Fig. 4B) and transcription factor activity (Fig. 4C) with TMC exposure.

TMC inhibits Akt signaling in MCF-7 cells

Because Akt kinase activity can inhibit apoptosis and Akt is expressed prominently in most lung and breast cancer cells, we examined the effect of TMC on the phosphorylation status of Akt. As shown in Fig. 5, TMC inhibited AKT phosphorylation at Ser473 and the phosphorylation of its upstream PDK1. TMC did not inhibit the phosphorylation of HER2 and EGFR (Fig. 5A). We also analyzed the downstream transcription factors, including FKHR and cAMP response element-binding protein (CREB), whose transcription activity is regulated by Akt. Phosphorylation of S256 in FKHR was significantly decreased after TMC treatment. MCF-7 cells treated with 0.4 μM TMC showed a 62% decrease in CREB transcription activity (Fig. 5B).

To assess the role of Akt in TMC-mediated inhibition of cell proliferation, MCF-7 cells were transiently transfected with plasmids encoding myr-Akt or empty vector. Overexpression of Akt significantly restored TMC-mediated inhibition of Akt phosphorylation. Similarly, overexpression of myr-Akt partially but significantly restored pro-caspase-9 level and the proliferation of MCF-7 cells treated with TMC (Fig. 6A).

Suppression of PP1 expression does not affect phosphorylation of Akt

To determine whether endogenous PP1 regulated phosphorylation of Akt levels, siRNA oligonucleotides were used to downregulate PP1 expression in growing MCF-7 cells (Fig. 6C). In contrast with the control oligonucleotide, PP1 siRNA oligonucleotides reduced PP1 expression, but did not affect pSer473 of Akt levels.

Discussion

Breast cancer has one of the highest death rates among different kinds of cancer. Although many different types of cytotoxic drugs have been developed for clinical uses, it is noteworthy that the effectiveness of chemotherapy is limited by the development of drug resistance. As such, there is increasing interest in identifying natural products to complement conventional medicine. In the present study, we report the anticancer activity of TMC, a compound isolated from Streptomyces griseochromogenes,
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Fig. 2. TMC suppresses the migration and invasion of breast cancer cells. A) Microscopic observation of the growth of MCF-7 cells after TMC exposure for 0 h and 48 h. A 'wound' was created with a pipette tip before treatment with TMC. B) The cell invasion assay for evaluating the inhibitory effect of TMC on breast cancer cell invasion. MCF-7 cells were treated with the indicated concentrations of TMC for 24 h. The cells were fixed with 4% paraformaldehyde before staining with 0.5% crystal violet. Scale bar = 20 μm.

Fig. 3. Effect of TMC on Bcl-2, cytochrome c, and caspases in MCF-7 cells. A) Protein expression of Bcl-2, pro-caspase-9, pro-caspase-8, pro-caspase-7, and cytosolic cytochrome c was assessed in MCF-7 cell lines by western blotting. GAPDH is shown as the protein loading control. B) Effect of the broad spectrum caspase inhibitor Z-VAD-FMK on proliferation in TMC-treated cells. Cell proliferation was measured by MTT assay after TMC treatment. **P < 0.01, compared to the control in which cells were not treated with Z-VAD-FMK.
against breast cancer cell lines and its possible mechanisms of action.

TMC has been shown to specifically inhibit the proliferation of colorectal cancer cells through activation of the ERK pathway without inducing apoptosis (24). However, we found that TMC could suppress the growth of many different types of tumor cells independent of the ERK pathway. Interestingly, TMC could also effectively inhibit the growth of Adriamycin-resistant human breast adenocarcinoma MCF-7/ADR. Metastasis has been suggested as one of the characteristics of cancer. It is a complex process involving cell adhesion, cell migration, and cell invasion. The present study is the first to demonstrate the ability of TMC to inhibit the metastatic process of MCF-7 breast cancer cells through the reduction of cell migration and invasion.

In the present study, we demonstrate that TMC was able to induce apoptosis in MCF-7 cells. To examine the role of the pathway in TMC-induced apoptotic death, the expression of p-Bad, Bcl-2, cytochrome c, and caspase in TMC-treated cells were analyzed. Bad promotes cell

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**Fig. 4.** The effect of TMC on ERK, β-catenin, and p53 activation. A) Lysates obtained from cells treated with 1 μM TMC for 24 h were analyzed for p-ERK and GAPDH by western blot analyses. B) MCF-7 cells were harvested at 12 and 24 h after TMC exposure. Extracts were analyzed for β-catenin and p53 by western blot analyses. C) Cells were transiently transfected with the promoter reporter plasmid p53-luc (200 ng), TCF/Lef reporter construct Topflash, and pRL-Tk (20 ng) for normalization. After 6 h, cells were treated with TMC for 30 h. Cell lysates were then assayed for luciferase activity.

**Fig. 5.** TMC affects upstream and downstream Akt pathway proteins in MCF-7 cells. A) Cells were treated with 0, 0.5, and 1 μM of TMC, after which whole cell extracts were prepared, and 50 μg of these extracts were resolved by SDS-PAGE and then probed with specific antibodies. B) MCF-7 cells were transiently transfected with 200 ng CRE-controlled Photinus pyralis luciferase reporter gene construct (pGL4.29; Promega) and 20 ng pRL-Tk. After 6 h, cells were treated with TMC for 30 h. Cell lysates were then assayed for luciferase activity. *P < 0.05, compared to the control in which cells were not treated with TMC.
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death by interacting with antiapoptotic Bcl-2 members such as Bcl-2, which allows the multidomain pro-apoptotic Bcl-2 family members Bax to aggregate and cause release of apoptogenic molecules (e.g., cytochrome c) from mitochondria to the cytosol, culminating into caspase activation and cell death (28). We found that p-Bad and Bcl-2 protein was down-regulated after TMC treatment. Consistent with these results, disruption of mitochondrial membrane potential, release of cytochrome c, and subsequent activation of caspase-9 and caspase-7 were detected in TMC-treated cells. Without caspase-3, MCF-7 cells undergo mitochondrial-dependent apoptosis via caspase-7 activation (29).

Akt is a critical regulator generally involved in cell cycle, proliferation, and apoptosis through regulating gene expression, protein synthesis, and transcription. Akt directly phosphorylates many downstream targets including Bad, GSK-3β, caspase-9, mTOR, FKHR, and other proteins (30). In the present study, we showed that TMC inhibited Akt and its downstream signaling, subsequently resulting in apoptosis of MCF-7 cells. TMC also inhibited the Akt upstream regulators PDK1. However, TMC did not inhibit the phosphorylation of EGFR and HER2, suggesting that they are not the direct targets of TMC. TMC were found to specifically inhibit the protein phosphatase PP1. However, suppression of endogenous PP1 expression does not affect phosphorylation of Akt. Consistent with our data on the structure–activity relationship of TMC (26), these results also suggest that the anticancer activity of TMC is not due to the inhibition of PP1.

In conclusion, this study showed that TMC effectively inhibited breast cancer cell proliferation, migration, and invasion. In addition, TMC was shown to induce the apoptotic response in MCF-7 cells. This apoptotic response was in part mediated by Bcl-2 cleavage, leading to the release of cytochrome c, which facilitates binding of Apaf-1 to caspase-9 in its presence and subsequent activation of caspase-7 in apoptosis induction signaling pathways. Furthermore, for the first time, we identified that TMC induced apoptosis by suppressing the Akt signaling pathway activation, which is independent of PP1 inhibition. Our study provides new mechanisms for the anticancer effects of TMC.

References


