Tautomycetin and tautomycin suppress the growth of medullary thyroid cancer cells via inhibition of glycogen synthase kinase-3β

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Abstract

Medullary thyroid cancer (MTC) is a relatively uncommon neuroendocrine tumor that arises from the calcitonin-secreting parafollicular cells of the thyroid gland. Unfortunately, MTC frequently metastasizes, precluding curative surgical resection and causing significant morbidity. Thus, there is an urgent need for new treatment modalities. Tautomycin and tautomycetin are antifungal antibiotics isolated from *Streptomyces spiroverticillatus* and *Streptomyces griseochromogens*, respectively. Glycogen synthase kinase-3β is a serine/threonine protein kinase that regulates multiple cellular processes and is important in various cancers, including MTC. Treatment with tautomycin and tautomycetin decreased neuroendocrine markers, suppressed hormonal secretion, and inhibited growth through apoptosis in MTC cells. Importantly, we describe a novel action of these compounds: inhibition of glycogen synthase kinase-3β. [Mol Cancer Ther 2009;8(4):914–20]

Introduction

Medullary thyroid cancer (MTC) is a neuroendocrine tumor that arises from the calcitonin-secreting parafollicular cells of the thyroid gland. MTC is relatively uncommon, representing only 3% of all thyroid malignancies; nevertheless, it accounts for 14% of all thyroid cancer–related deaths (1, 2). Unfortunately, MTC is difficult to treat, metastasizing in >50% of cases, which precludes surgical resection and causes significant morbidity (2). Surgery is the only curative therapy for MTC, and chemotherapeutic regimens are ineffective. Thus, there is an urgent need for new, targeted treatment modalities.

Glycogen synthase kinase-3β (GSK-3β) is a serine/threonine protein kinase that regulates multiple cellular processes, including differentiation, metabolism, proliferation, and survival, through targets such as β-catenin, c-myc, and c-Jun (3). GSK-3β is highly active and nonphosphorylated in unstimulated cells, and its activity is inhibited by phosphorylation of a single serine residue at position 9 (Ser[9]). There is a wide range of pharmacologic inhibitors of GSK-3β, such as lithium, SB216763, and SB415286 (4). With such diverse effects, the GSK-3β pathway has been shown to limit growth of various cancers, including pancreatic (5), prostate (6), and MTC (7).

The antifungal antibiotics tautomycin (TTY) and tautomycetin (TMC) are isolated from *Streptomyces spiroverticillatus* and *Streptomyces griseochromogens*, respectively (8, 9). These two compounds are structurally similar, differing only in the presence of a spiroketal group on TTY. In addition, TTY and TMC are potent inhibitors of protein phosphatases 1 (PP1) and 2A (PP2A), a family of dephosphorylating enzymes (10). TMC was recently shown to inhibit the growth of colorectal cancer cells (11). However, the role of TTY and TMC in other cancers remains unclear. We present here the use of TTY and TMC for *in vitro* treatment of MTC. TTY and TMC decreased neuroendocrine markers, suppressed hormonal secretion, and inhibited growth through apoptosis. Moreover, these effects were mediated through inhibition of GSK-3β, a novel action of these compounds.

Materials and Methods

Cell Culture

Human MTC cells (TT) were obtained from the American Type Culture Collection and maintained in RPMI 1640 (Life Technologies) supplemented with 18% fetal bovine serum (Sigma-Aldrich), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Life Technologies) in a humidified atmosphere of 5% CO_2 in air at 37°C as previously described (7, 12).

Cellular Proliferation Assay

To do the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) growth assay with TTY and TMC, 100,000 MTC or 20,000 NIH 3T3 (murine fibroblast) cells were seeded in quadruplicate on 24-well plates and incubated

overnight. After incubation, MTC cells were treated with DMSO as control and increasing concentrations of TTY and TMC. Cells were incubated for up to 6 d. Every 2 d, treatment medium was changed, and the MTT assay was done by replacing the treatment medium with 250 μL of serum-free RPMI 1640 containing MTT (0.5 mg/mL) and incubating at 37°C for 4 h. Next, 750 μL DMSO was added to each well and mixed thoroughly. The plates were then measured at 540 nm using a spectrophotometer (μQuant, Bio-Tek Instruments).

Western Blot Analysis

TTY and TMC were synthesized and dissolved in DMSO (Sigma-Aldrich). MTC cells were treated with concentrations of TTY and TMC of up to 1,000 nmol/L, and an equal volume of DMSO was used as a control. After 2 d of treatment, whole-cell lysates were prepared as previously described (13). Total protein concentrations were quantified with a bicinchoninic acid assay kit (Pierce Biotechnology). Denatured cellular extracts were resolved by SDS-PAGE, transferred onto nitrocellulose membranes (Schleicher and Schuell), blocked in milk, and incubated with appropriate antibodies.

The antibody dilutions were as follows: 1:500 for chromogranin A (CgA; Zymed Laboratories); 1:1,000 for achaete-scute complex-like 1 (ASCL1; BD Biosciences), phosphorylated GSK-3β (pGSK-3β), GSK-3β, phosphorylated PP1, PP1, Akt phosphorylated at Ser^{473} (pAkt), total Akt, poly(ADP-ribose) polymerase (PARP), cleaved caspase-3, and β-actin (Cell Signaling Technology); and 1:10,000 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Trevigen).

Horseradish peroxidase–conjugated goat anti-rabbit IgG (1:2,000; Cell Signaling Technology) secondary antibody was used for CgA, pGSK-3β, GSK-3β, phosphorylated PP1, PP1, pAkt, total Akt, PARP, cleaved caspase-3, and GAPDH, whereas goat anti-mouse IgG (1:200; Pierce Biotechnology) secondary antibody was used for ASCL1. For visualization of the protein signal, SuperSignal West Femto (Pierce Biotechnology) was used for ASCL1 and cleaved caspase-3. Immunstar (Bio-Rad Laboratories) was used for CgA, pGSK-3β, GSK-3β, PARP, and GAPDH per the manufacturer's instructions.

GSK-3β Knockdown Experiments

To measure cellular effects after gene knockdown, MTC cells were plated onto six-well plates and allowed to adhere overnight. The next day, cells were treated with Lipofectamine (Invitrogen), 75 nmol/L nonspecific small interfering RNA (siRNA; Santa Cruz Biotechnology), and 75 nmol/L GSK-3β siRNA (Santa Cruz Biotechnology) according to the manufacturer's instructions. After 2 d, cells were harvested and lysed as described above and analyzed for expression of ASCL1, GSK-3β, and CgA. Relative calcitonin levels were measured as described below.

Calcitonin ELISA

An ELISA kit (Invitrogen) was used to quantify the amount of calcitonin in MTC cells as previously described (7, 14). MTC cells were treated with increasing concentrations of both TTY and TMC for 48 h. Cell lysates were then used as the antigen source in a standard sandwich ELISA per the manufacturer's instructions. Samples were analyzed in triplicate.

Apoptosis ELISA

Apoptosis was measured by the quantitation of cytosolic mononucleosome- and oligonucleosome-bound fragmented DNA by using an ELISA kit (Roche Applied Biosciences) as previously described (14). Briefly, MTC cells were treated with TTY and TMC (up to 1,000 nmol/L). After 48 h, cell lysates were harvested and the cytosolic fraction was prepared. The lysate was used as an antigen source in an ELISA that consisted of a primary anti-histone antibody and a secondary anti-DNA antibody coupled to peroxidase. Absorbance values were used to calculate the induction of DNA fragmentation in comparison with control, and all samples were measured in quadruplicate.

Statistical Analysis

One-way ANOVA and the independent sample *t* test were done using Statistical Package for the Social Sciences (version 11; SPSS, Inc.) as appropriate to presented data. A *P* value of ≤0.05 was considered to be significant. All points represent the average readings ± SE.

Results

TTY and TMC Inhibit the Growth of MTC Cells

To begin, we first wanted to see if TTY and TMC could affect cellular proliferation. We used an MTT growth assay to determine the effect of TTY and TMC on MTC cell growth. Growth was inhibited in a dose-dependent manner by both compounds in doses of up to 1,000 nmol/L (Fig. 1A and B). Importantly, growth was suppressed significantly (18% by TTY and 10% by TMC; *P* < 0.01, one-way ANOVA) after only 2 days of treatment. This was even more statistically significant (*P* < 0.001) after 4 days. Additionally, TTY and TMC did not significantly affect the growth of murine fibroblasts until higher doses were used (Fig. 1C and D).

TTY and TMC Decrease Neuroendocrine Markers in MTC Cells

After observing that both TTY and TMC could inhibit the growth of MTC cells, we wanted to explore the effects of TTY and TMC on the neuroendocrine markers ASCL1 and CgA (Fig. 2A). Previously, we have shown that pharmacologic inhibition of GSK-3β in MTC cells leads to growth inhibition and a decrease of the neuroendocrine markers ASCL1 and CgA (7). ASCL1 is a basic helix-loop-helix transcription factor that promotes neuronal differentiation and is a protein marker for neuroendocrine hormone production (15). CgA is an acidic glycoprotein cosecreted with hormones by MTC, and the reduction of CgA is correlated with decreases in hormonal secretion (16).

Treatment for 48 hours with up to 1,000 nmol/L of TTY and TMC led to a dose-dependent decrease in the neuroendocrine markers ASCL1 and CgA. Previous results from our studies in MTC showed that these decreases become more significant with both longer treatments and increased
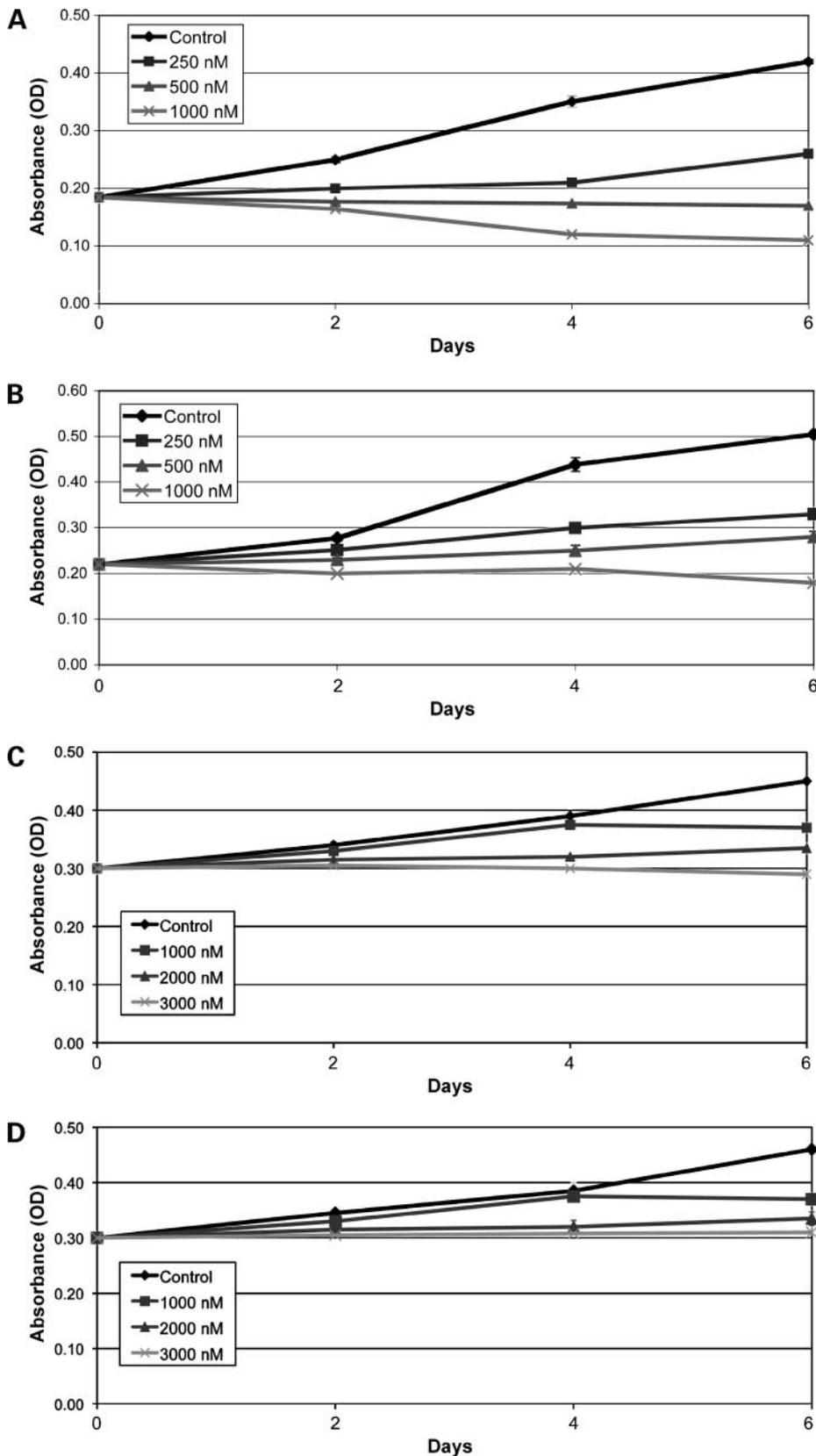
Figure 1. TTY and TMC inhibit the growth of MTC cells. MTC cells were treated with the indicated concentrations of TTY (**A**) and TMC (**B**) for up to 6 d, whereas murine fibroblast cells were treated with the indicated concentrations of TMC (**C**) and TTY (**D**). Cell viability was determined by an MTT colorimetric growth assay. *Points,* mean; *bars,* SE. All treatments of MTC cells were significantly different from control after 2 d of treatment ($P < 0.01$, one-way ANOVA), whereas treatment was significantly different after 6 d of treatment of murine fibroblast cells.
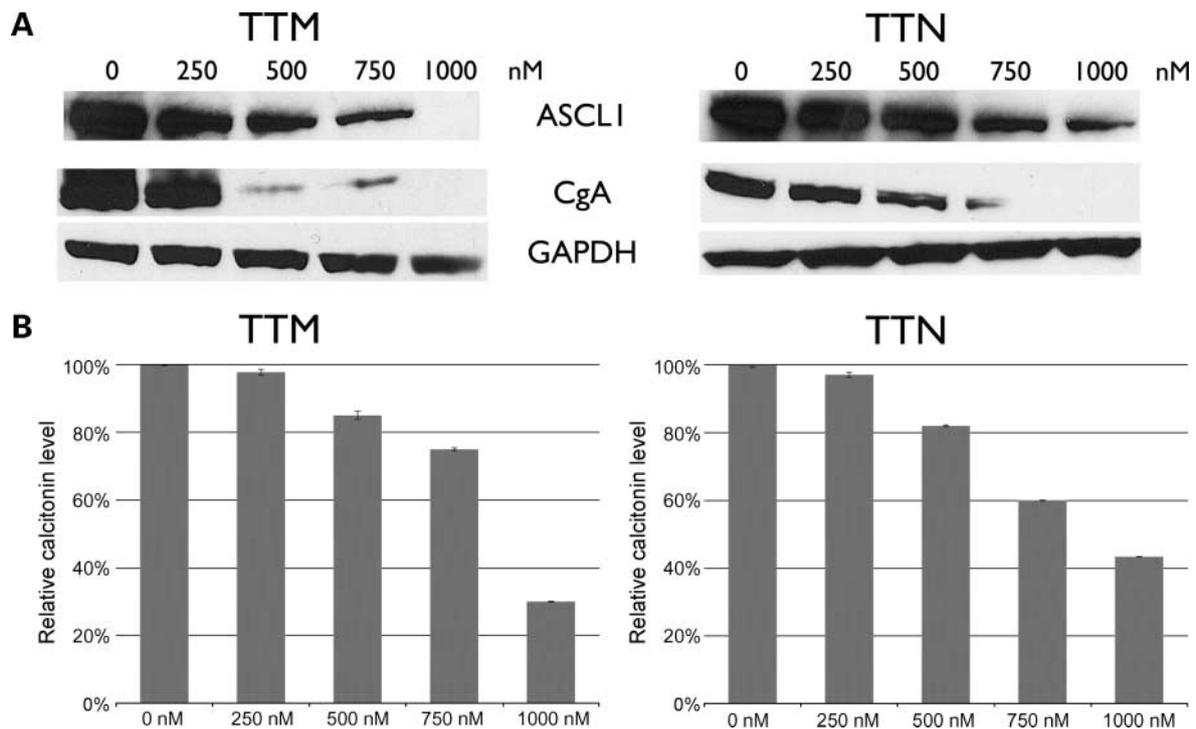
Figure 2. TTY and TMC decrease neuroendocrine markers in MTC cells. **A,** treatment for 2 d with TTY or TMC decreased the neuroendocrine markers *ASCL1*, a pro-neuroendocrine gene, and CgA, a marker of hormonal secretion, in a dose-dependent manner. GAPDH is shown as a loading control. **B,** to confirm that treatment with TTY and TMC suppressed hormonal secretion, a calcitonin ELISA was done. TTY and TMC caused a significant dose-dependent hormonal suppression, with a decrease of 70% and 67%, respectively, at the highest doses used (P < 0.001 for both, one-way ANOVA).

concentrations (7, 17). To confirm that TTY and TMC affect hormonal secretion, we carried out an ELISA for calcitonin, a secretory product of MTC cells (Fig. 2B). TTY and TMC decreased calcitonin secretion in a dose-dependent manner, with a maximum decrease of 70% and 67% with 1,000 nmol/L of TTY and TMC, respectively. This decrease was statistically significant (P < 0.001 for both, one-way ANOVA). Thus, TTY and TMC decrease neuroendocrine markers and suppress hormonal secretion of MTC cells.

TTY and TMC Inhibit GSK-3β and Decrease Neuroendocrine Markers in MTC Cells

After observing that TTY and TMC affected both growth and neuroendocrine markers in MTC cells, we wanted to understand the mechanism of action of these compounds. Given the known role of TTY and TMC as protein phospha-

tase inhibitors (18), we explored the effects of TTY and TMC on GSK-3β. GSK-3β, in contrast to other kinases, becomes inactivated by phosphorylation in response to signaling cascades. Moreover, GSK-3β is an important target in MTC cells: both nonspecific (lithium chloride) and specific (SB216763 and SB415286) GSK-3β inhibitors lead to decreased hormonal secretion and inhibition of growth (7). Treatment for 48 hours with up to 1,000 nmol/L of TTY and TMC led to a dose-dependent phosphorylation of GSK-3β, showing inhibition of the protein (Fig. 3).

Inhibition of GSK-3β Decreases Neuroendocrine Markers in MTC Cells

To determine that these effects were due to direct inhibition of the GSK-3β protein, we used siRNA against GSK-3β in MTC cells. Treatment for 48 hours with 75 nmol/L

Figure 3. TTY and TMC inhibit GSK-3β in MTC cells. GSK-3β, in contrast to other kinases, becomes inactivated by phosphorylation in response to signaling cascades. Treatment for 2 d with TTY or TMC pGSK-3β in a dose-dependent fashion, showing inhibition of the pathway.
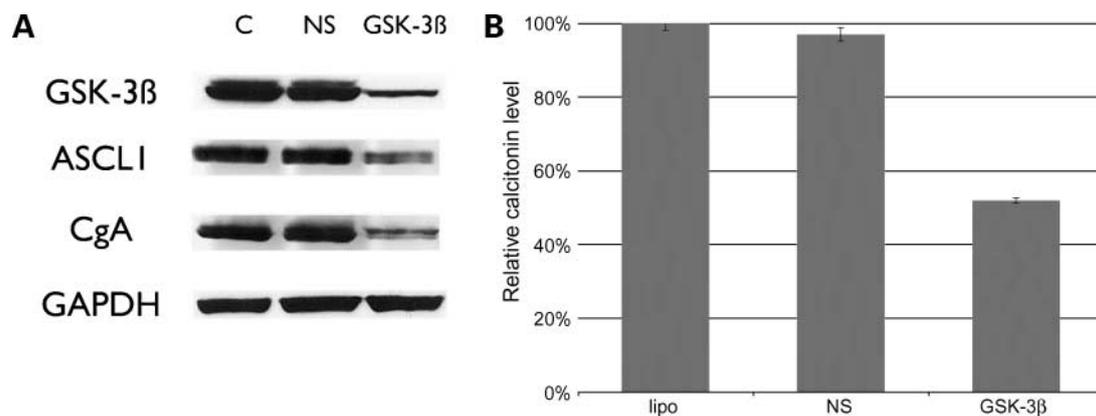
Figure 4. Knockdown of GSK-3β decreases neuroendocrine markers in MTC cells. MTC cells were transfected with Lipofectamine as control, 75 nmol/L nonspecific siRNA, or 75 nmol/L GSK-3β siRNA for 2 d. **A,** GSK-3β siRNA decreased the amount of GSK-3β protein and decreased the neuroendocrine markers ASCL1 and CgA. **B,** a calcitonin ELISA confirmed that GSK-3β inhibition significantly limited hormonal secretion ($P = 0.02$, independent sample t test).

GSK-3β siRNA caused a significant decrease in total GSK-3β protein, ASCL1, and CgA compared with both control cells and cells transfected with 75 nmol/L nonspecific siRNA (Fig. 4A). A calcitonin ELISA showed a 48% decrease in GSK-3β siRNA-treated cells, showing a statistically significant inhibition of hormonal secretion ($P = 0.02$, independent sample t test; Fig. 4B). Thus, direct inhibition of GSK-3β results in a decrease in neuroendocrine markers, similar to the results obtained by pharmacologic inhibition of the protein by TTY and TMC.

TTY and TMC Act on GSK-3β via PP1

As previously noted, TTY and TMC are known protein phosphatase inhibitors (18). As such, we explored the effects of TTY and TMC on PP1. Increasing doses of TMC led to an increase in phosphorylated PP1 with a concomitant decrease in total PP1 protein, whereas increasing doses of TTY only decreased the total amount of PP1. Both of these actions suggest an inhibition of PP1. Thus, the known action of TTY and TMC as inhibitors of PP1 also occurs in MTC cells, which leads to inactivation of the GSK-3β pathway (Fig. 5A). In addition, we measured the effects of TTY and TMC on a known upstream protein of GSK-3β, Akt. Treatment with both TTY and TMC had no effect on phosphorylation of Akt at the Ser[473] sites and total Akt (Fig. 5B). In total, these data suggest that TTY and TMC act in a similar way as described in other cell lines by inhibiting PP1, which then inhibits GSK-3β.
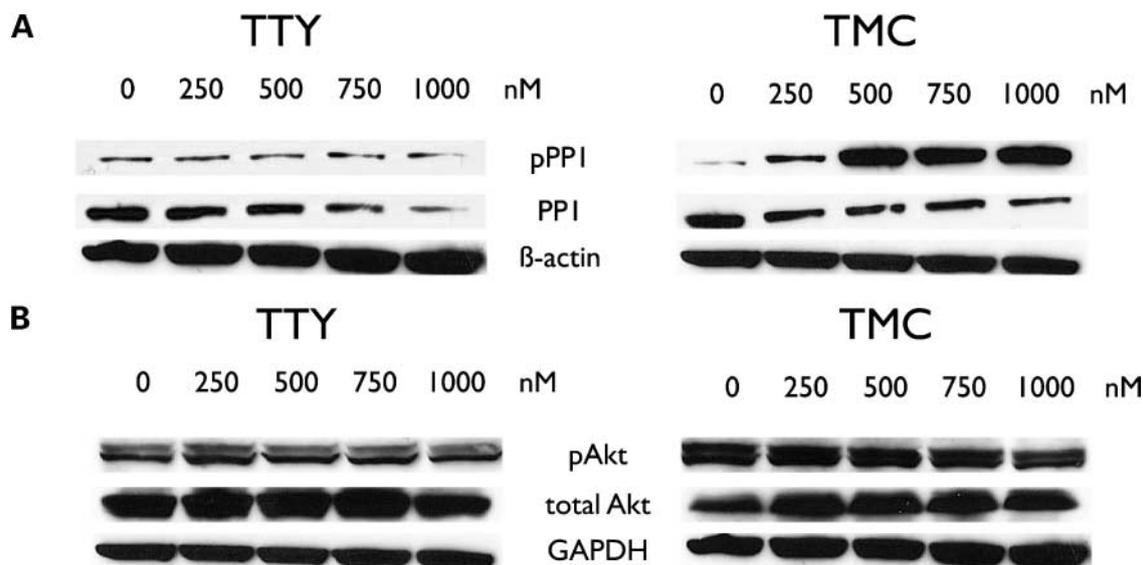

Figure 5. TTY and TMC affect GSK-3β via PP1. MTC cells were treated with the indicated concentrations of TTY and TMC for 2 d, total cell lysates were prepared, and levels of PP1 were measured. **A,** an increase in the phosphorylation of PP1 was observed with TMC, and total levels of the PP1 protein decreased, suggesting decreased activity. These results suggest that TTY and TMC function through PP1 to inhibit GSK-3β, as reported in other cell lines. **B,** moreover, TTY and TMC did not affect pAkt or total amount of Akt, an upstream protein of GSK-3β. Therefore, TTY and TMC act through PP1 to inhibit GSK-3β via phosphorylation.
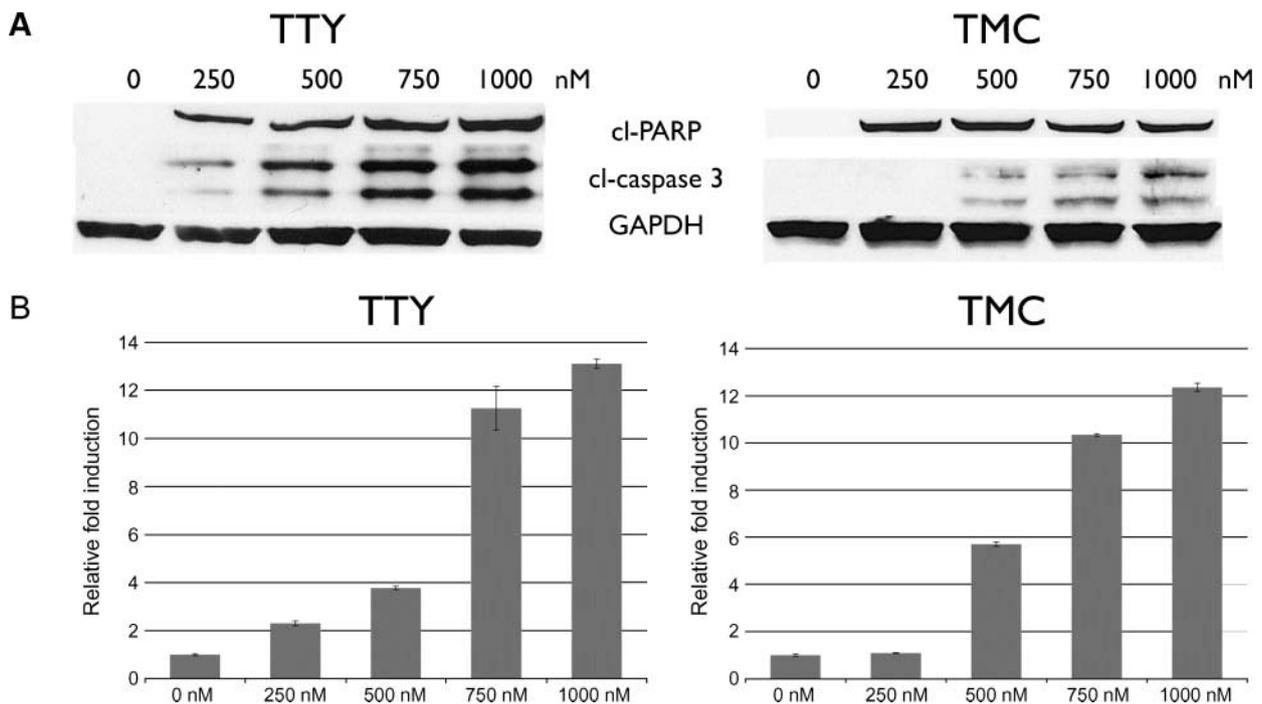
Figure 6. TTY and TMC cause growth inhibition via apoptosis. MTC cells were treated with the indicated concentrations of TTY and TMC for 2 d, and total cell lysates were prepared. **A,** an increase in the cleavage of PARP and caspase-3 suggests that the mechanism of growth inhibition is apoptosis. GAPDH was used as a loading control. **B,** a DNA fragmentation cell death ELISA was done to confirm apoptosis. Treatment with TTY and TMC caused a dose-dependent increase in DNA fragmentation, confirming that apoptosis occurred in MTC cells treated with TTY and TMC.

TTY and TMC Inhibit the Growth of MTC Cells through Apoptosis

We wanted to explore the mechanism of growth inhibition. After 48 hours of treatment with TTY and TMC, cellular lysates were prepared. A Western blot was carried out for PARP and caspase-3, two well-characterized markers of the apoptotic pathway whose cleavage is indicative of apoptosis. After 48 hours of treatment, a dose-dependent cleavage was observed in both PARP and caspase-3 (Fig. 6A).

To confirm that the compounds caused a significant amount of apoptosis, a DNA fragmentation cell death ELISA was done. This showed a dose-dependent increase in fragmented DNA for both TTY and TMC (Fig. 6B). There was an ~13-fold increase in DNA fragmentation at the highest doses of TTY and TMC used. These results indicate that TTY and TMC cause growth inhibition via apoptosis in MTC cells.

Discussion

MTC is a rare tumor that accounts for 3% of thyroid malignancies and 14% of thyroid cancer deaths (1, 2, 19). Derived from the parafollicular calcitonin-secreting cells of the thyroid gland, these neuroendocrine tumors frequently metastasize and cause a poor quality of life. Unfortunately, there are limited therapeutic options for patients with these tumors. As surgical resection is the only potentially curative treatment, there is an urgent need for new treatment modalities. We present here the use of two antifungal compounds,

TTY and TMC, and their novel role as inhibitors of GSK-3β in MTC.

TTY and TMC are naturally occurring antifungal compounds. TMC is isolated from *S. griseochromogens*, whereas TTY is isolated from *S. spiroverticillatus*, respectively (8, 9). Both TTY and TMC are potent inhibitors of PP1 and PP2A (10). Additionally, TMC inhibits the growth of colorectal cancer cells, showing that these agents may be useful as anticancer drugs (11). Having shown that pharmacologic inhibition of GSK-3β has antiproliferative effects in MTC (7), we wanted to know if TTY and TMC may be able to inhibit GSK-3β, limit neuroendocrine markers, and suppress hormonal production of MTC cells.

In this study, we show conclusively that GSK-3β plays a key regulatory role in cellular proliferation and neuroendocrine markers in MTC cells. Gene knockdown experiments with siRNA for GSK-3β led to a decrease in the neuroendocrine markers ASCL1 and CgA. We have previously shown that these proteins are markers for decreased hormonal secretion, which does not seem to be due to cell death (7, 20). Transfection of GSK-3β siRNA also caused significant hormonal suppression, an important component of targeted therapies for neuroendocrine tumors. Although we have previously shown pharmacologic inhibition of GSK-3β in MTC (7), we now show the key role that the protein plays via specific gene knockdown experiments, emphasizing the role of GSK-3β as a therapeutic target in MTC.

Most importantly, we show that TTY and TMC are novel, naturally occurring inhibitors of GSK-3β in MTC. Compared

with other inhibitors used in MTC cells, TTY and TMC are significantly more potent than lithium chloride (millimolar) and of similar potency to SB216763 (micromolar) ranges. Moreover, the inhibition caused by TTY and TMC significantly decreases the neuroendocrine markers ASCL1 and CgA and secretion of the neuroendocrine hormone calcitonin. These effects seem to be due to direct GSK-3β inhibition through inhibition of PP1 rather than less specific effects of TTY and TMC. These drugs also significantly inhibited growth of MTC cells via apoptosis. These data conclusively show that TTY and TMC inhibit GSK-3β, affect neuroendocrine markers, decrease hormonal production, and inhibit growth of MTC cells. Moreover, the growth effects of these compounds are not as pronounced in murine fibroblast cells, suggesting the specificity of these treatments for cancerous cells.

Overall, the present study is consistent with our earlier results in the targeting of GSK-3β in MTC and pheochromocytoma cells (21). MTC, similar to other neuroendocrine tumors, seems to have a wide range of molecular targets that regulate the neuroendocrine phenotype and hormonal secretion, including Notch1, phosphatidylinositol 3-kinase/Akt, and raf-1 (22). These results suggest that targeted molecular therapy of MTC is possible. Clearly, both TTY and TMC are molecular inhibitors of GSK-3β in MTC cells.

In conclusion, TTY and TMC are novel, naturally occurring inhibitors of GSK-3β in MTC cells. Treatment with TTY and TMC led to a decrease in neuroendocrine markers and suppression of calcitonin secretion. Most importantly, TTY and TMC also led to growth inhibition via apoptosis *in vitro*. Crucially, this study further extends our understanding of the importance of GSK-3β in MTC cells. We show here the use of two naturally occurring, potent compounds that inhibit GSK-3β and may serve as viable compounds for future therapeutic use.

Disclosure of Potential Conflicts of Interest

H Chen: honoraria from Novartis. No other potential conflicts of interest were disclosed.

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Correction: Tautomycetin and Tautomycin Suppress the Growth of Medullary Thyroid Cancer Cells via Inhibition of Glycogen Synthase Kinase-3β

In this article (Mol Cancer Ther 2009;8:914–20), which was published in the April 1, 2009 issue of *Molecular Cancer Therapeutics* (1), Figs. 2 and 3 contained incorrect abbreviations for tautomycin and tautomycetin. The correct abbreviation for tautomycin is TTY, and the correct abbreviation for tautomycetin is TMC. The correct Figs. 2 and 3 appear here.
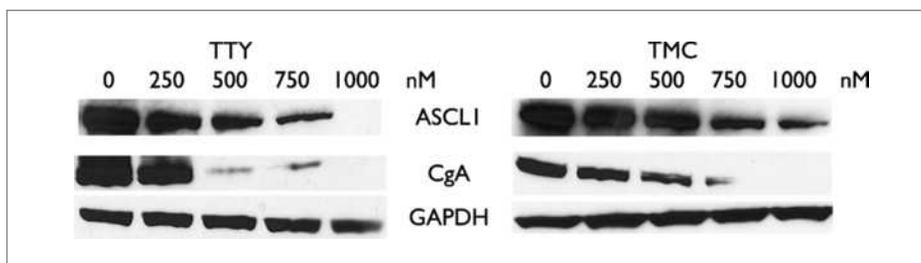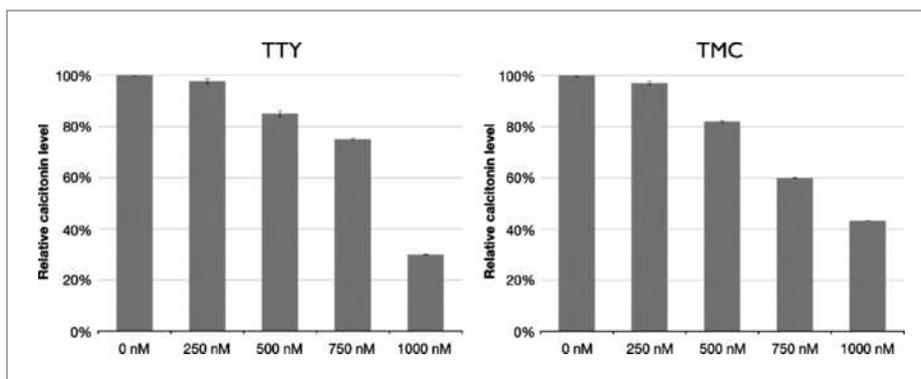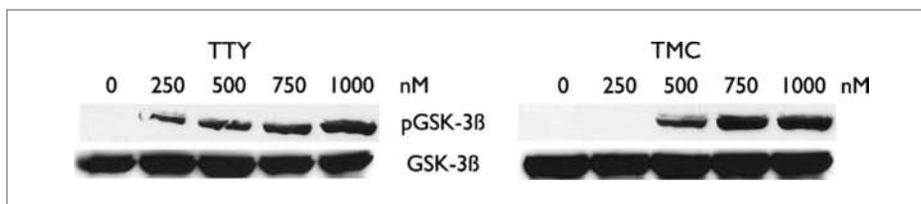

Figure 2A.

Figure 2B.

Figure 3.

Reference

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