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Tautomycin suppresses growth and neuroendocrine hormone markers in carcinoid cells through activation of the Raf-1 pathway

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Abstract

Background—Carcinoids are neuroendocrine (NE) tumors with limited treatment options. Raf-1 pathway activation has been shown to suppress hormone production in carcinoid cells. Here, we investigated a novel treatment for carcinoid cell growth based on pharmacologic Raf-1 activation using the compound tautomycin (TTM).

Methods—Human carcinoid cells were treated with TTM for 48 hours. Western analysis was used to demonstrate Raf-1 pathway activation by phosphorylation of ERK1/2 and to determine the effect on NE tumor markers. Cellular growth was measured by MTT assay.

Results—Treatment with TTM resulted in a dose-dependent activation of the Raf-1 pathway. Furthermore, a significant reduction in NE tumor markers was seen. Importantly, TTM inhibited carcinoid cellular growth and induced the cell cycle inhibitors p21 and p27.

Conclusions—TTM activates the Raf-1 pathway, limits carcinoid cell growth, and suppresses NE marker production *in vitro*. This new compound warrants further investigation in animal models of carcinoid cancer.

Keywords

tautomycin; extracellular-regulated kinase pathway; Raf-1; carcinoid tumor; targeted therapies

Introduction

Gastrointestinal (GI) carcinoids are neuroendocrine (NE) tumors characterized by the secretion of amines and polypeptide hormones like serotonin (5-HT) and histamine. As a result of the production of these vasoactive substances, patients with advanced disease often have painful and debilitating symptoms of the carcinoid syndrome, which include diarrhea, bronchospasm, cutaneous flushing, and fibrous thickening of the right-sided heart valves [1–3]. Though

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carcinoid tumors are relatively rare, occurring in approximately 2/100,000 people [4–5], they frequently metastasize to the liver. Besides surgical resection, combinations of radiation and complex chemotherapeutic regimens have been attempted to control metastatic tumors; however, by the time many of these tumors are diagnosed, widespread metastases render conventional treatment palliative at best. Therefore, there is a great need to develop novel targeted therapeutic strategies both to reduce tumor burden and control symptoms of the carcinoid syndrome.

The Raf-1/mitogen-regulated extracellular kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway has long been recognized for its role in cellular growth and transformation. In this pathway, ras signaling is thought to involve activation of Raf-1, a cytosolic serine/threonine kinase. Once activated, Raf-1 phosphorylates MEK1/2, which then activates the downstream effector ERK1/2. While activation of this signaling pathway is commonly considered to be growth promoting in several cancers, in certain cell-specific subtypes, Raf-1 activation results in growth suppression [6]; specifically, activation of the Raf-1 signaling pathway in small cell lung cancer (SCLC), medullary thyroid cancer (MTC), and carcinoid cancer has been shown to result in phenotypic change and reduction in cellular proliferation [7–10]. Indeed, Raf-1 activation may be a novel approach in treating certain cancers such as GI NE tumors. Utilizing an estrogen-inducible Raf-1 construct in human GI carcinoid tumor cells, our laboratory has previously shown that Raf-1 activation results in carcinoid cell morphological change accompanied by a marked decrease in NE secretory granules by electron microscopy; likewise, Raf-1 induction in carcinoid tumor cells leads to significant reductions in 5-HT, chromogranin A (CgA), and synaptophysin (SYP) levels *in vitro* [11].

Tautomycin (TTM), a dialkylmaleic anhydride antibiotic (Fig. 1) originally isolated from the common soil microorganism *Streptomyces spiroverticillatus*, belongs to the okadaic acid class of compounds. Compounds within this class of molecules—specifically tautomycin (TTN)—have been identified as potent T cell immunosuppressors which are superior to cyclosporine A both *in vitro* and *in vivo* [12,13]. This activity is thought to be related to the ability of TTN to inhibit tyrosine phosphorylation of intracellular signaling molecules involved in various cellular responses such as T-cell receptor-proximal signaling [13]. More recently, Lee *et al.* demonstrated that TTN inhibits growth of colorectal cancer *in vitro*, and this growth inhibition is acquired by regulation of Raf-1 activity through inhibition of protein phosphatase type 1 (PP1) and protein phosphatase type 2A (PP2A) with high preference toward PP1 [14]. In this article, we detail the response of carcinoid tumor cell lines to TTM, a structurally similar compound to TTN, showing that TTM results in progressive phosphorylation of Raf-1 pathway mediators in these cells. Furthermore, CgA, a surrogate marker for bioactive hormone production in carcinoid tumors, and human achaete-scute complex-like 1 (ASCL1), a neuroendocrine transcription factor, are significantly reduced in response to this compound. Likewise, carcinoid tumor growth is efficiently inhibited by TTM and is likely dependent on p21^{Waf1/Cip1} and p27 induction via the ERK pathway. This represents the first characterization of a soluble Raf-1 activating drug which is capable of suppressing cellular proliferation and inducing the cell cycle inhibitors p21^{Waf1/Cip1} and p27.

Materials and Methods

Cell Culture

Human GI carcinoid tumor (BON) cells, graciously provided by Drs. B. Mark Evers and Courtney M. Townsend, Jr. (University of Texas Medical Branch, Galveston, TX), and NCI-H727 human bronchopulmonary carcinoid tumor (H727) cells (American Type Culture Collection, Manassas, VA) were maintained as previously described [11,15].

Cell Proliferation Assay

Carcinoid tumor cell proliferation was measured by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) rapid colorimetric assay as previously described [15]. Briefly, cells were seeded in quadruplicate on 24-well plates and incubated overnight under standard conditions to allow cell attachment. The cells were then treated with TTM in concentrations of 0–3 μ M and incubated for up to 6 days. The MTT assay was performed by replacing the standard medium with 250 μ l of serum-free medium containing 0.5 mg/ml MTT and incubating at 37°C for 4 hours. After incubation, 750 μ l of dimethyl sulfoxide (Fischer Scientific, Pittsburgh, PA) was added to each well and mixed thoroughly. The multiwell plates were then measured at 540 nm using a spectrophotometer (μ Quant; Bio-Tek Instruments, Winooski, VT).

Immunoblot Analysis

Human carcinoid tumor cells treated with TTM were harvested after 2 days of treatment and lysed. Total protein concentration in the prepared cell lysates was quantified with a bicinchoninic acid assay kit (Pierce Protein Research Products, Rockford, IL). Denatured cellular extracts (20–40 μ g) were resolved by 10%–12% SDS-PAGE (Invitrogen Life Technologies, Carlsbad, CA), transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA), blocked in milk, and incubated overnight in the appropriate primary antibody. The antibody dilutions were as follows: 1:1,000 for ERK1/2, pERK1/2, p27, (Cell Signaling Technology, Beverly, MA), and mammalian achaete scute homologue-1 (MASH1) for ASCL1 (BD PharMingen, San Diego, CA); 1:2,000 for p21^{Waf1/Cip1} (Cell Signaling Technology) and CgA (Zymed Laboratories, San Francisco, CA); and 1:10,000 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Trevigen, Gaithersburg, MD). Horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Pierce) were used depending on the source of the primary antibody. Immunstair (Bio-Rad) or SuperSignal West Femto (Pierce) kits were used per the manufacturer's instructions for detection.

Statistical Analysis

Statistical analyses were performed utilizing analysis of variance testing (SPSS software version 10.0, SPSS; Chicago, IL). A *P* value of <0.05 was considered significant. Unless specifically noted, all data are represented as mean $\pm$ SE.

Results

Tautomycin Activates the Raf-1 Pathway in a Dose-Dependent Manner

Recent data have demonstrated the involvement of the ERK-mediated pathway in okadaic acid class-mediated growth regulation, as indicated by phosphorylation and subsequent activation of ERK1/2, as well as the upstream kinases, Raf-1 and MEK, in colorectal cells [14]. To determine whether TTM is capable of activating the Raf-1 pathway, Western analysis was used to demonstrate phosphorylation of the Raf-1 pathway mediators, ERK1/2. In control pulmonary (H727) and GI (BON) carcinoid tumor cells, there is no phosphorylation of ERK1/2 consistent with our earlier reports [11]. As shown in figure 2, however, the phosphorylation of ERK1/2 was significantly increased in cultures treated with up to 3 μ M TTM for 48 h. In contrast to this, total ERK1/2 was unchanged in both control and treated cells (Fig. 2A and 2B).

Tautomycin Suppresses Growth of Carcinoid Tumor Cells *In Vitro*

We have previously shown that activation of the Raf-1 pathway by a pharmacologic compound, ZM336372, inhibits carcinoid cell growth [15]; unfortunately, the dose of this synthetic drug required to effect these changes *in vitro* has precluded further *in vivo* studies, as the compound

is nearly water insoluble at treatment doses and readily precipitates in media. While compounds within the okadaic acid class have recently been shown to inhibit the growth of HCT-15, HT-29, and DLD-1 human colorectal cancer cells *in vitro* [14], the effects of TTM on carcinoid tumor cell growth have not been characterized to date. We utilized the MTT rapid colorimetric assay to measure cell viability after TTM treatment of BON GI and H727 pulmonary human carcinoid tumor cells. Both pulmonary and GI carcinoid cells treated with TTM had a profound dose-dependent inhibition of growth (Fig. 3A and 3B). After 6 days of treatment, growth of cells exposed to as little as 1 μ M of TTM was inhibited in excess of 50% relative to untreated cells.

The Mechanism of Tautomycin-Induced Carcinoid Growth Suppression is Cell Cycle Arrest

After determining that TTM inhibits carcinoid tumor cell growth *in vitro*, we were interested in the mechanism of action for this effect. Raf-1 activation has been shown to induce expression of multiple families of cell cycle inhibitors, including p21^{Waf1/Cip1} and members of the INK family, such as p18 [15–19]. We performed Western blot analysis using BON and H727 whole cell lysates after 2-day treatments with TTM to assess the effect of the drug on cell cycle regulators. As shown in figure 4, carcinoid tumor cells have minimal levels of p21^{Waf1/Cip1} and p27 at baseline. However, Western analysis of H727 and BON cells treated with 1 μ M to 3 μ M TTM for 2 days resulted in significant induction of p27 in BON cells and p21^{Waf1/Cip1} and p27 in H727 cells in a dose-dependent manner (Fig. 4A and 4B). This ability of TTM to induce p21^{Waf1/Cip1} and p27 and suppress cellular proliferation through Raf-1 activation appears to be specific to NE tumor cells, as treatment of human pancreatic cancer cells (MiaPaCa2) with the known Raf-1 activator ZM336372 shows no up-regulation of p21^{Waf1/Cip1} despite natural activation of the Ras/Raf-1 pathway in these cells [15].

Tautomycin Inhibits the Production of Neuroendocrine Tumor Markers in Carcinoid Cells

Carcinoids are NE tumors characterized by the excess secretion of various bioactive amines and polypeptides. CgA is the major member of the granin family of acidic secretory glycoproteins that are expressed in NE cells and are cosecreted with bioactive hormones. We have previously demonstrated that Raf-1 activation in carcinoid tumor cells results in reduced levels of CgA. Furthermore, we have previously shown that changes in CgA levels correspond with alterations in other NE hormones like 5-HT and histamine [11]. We were therefore interested in whether TTM had the ability to alter levels of CgA in carcinoid tumor cells *in vitro*. Western blot analysis of total cellular extracts from BON (Fig. 5A) and H727 (Fig. 5B) cells treated with 1 μ M to 3 μ M TTM for 48 h were analyzed to determine the effect upon CgA. In control cells, there are very high levels of CgA present; however, with addition of TTM, a dose-dependent decrease in CgA is noted in both pulmonary and GI carcinoid tumor cells.

Previous studies in our laboratory have also shown that activation of the Raf-1 signaling pathway in carcinoid cells results in marked suppression of human achaete-scute complex-like 1 (ASCL1), a gene that encodes a member of the basic helix-loop-helix (BHLH) family of transcription factors [20]. It is thought that this protein plays a role in neuronal commitment and differentiation, and it has been shown to be highly expressed in NE tumors like carcinoids, medullary thyroid cancer (MTC) and small cell lung cancer (SCLC). If TTM is able to activate the Raf-1 pathway, we would expect to see a significant reduction in ASCL1 protein levels. Western blot analysis of TTM-treated GI (Fig. 5A) and pulmonary (Fig. 5B) carcinoid tumor cells demonstrated very high levels of ASCL1 in DMSO (control) treated cells; however, exposure of H727 and BON cells to 1 μ M to 3 μ M TTM for 48 hours reduced ASCL1 levels to undetectable levels.

Comments

Though relatively rare, carcinoid tumors represent the most common GI NE tumors and are second only to colorectal adenocarcinoma as the most common cause of isolated hepatic metastases [4,21–23]. Currently, surgery is the only potentially curative treatment modality, yet complete resection is often impossible due to the presence of widespread disease at the time of initial presentation. As other conventional oncologic treatments such as chemotherapy and radiotherapy are largely ineffective against carcinoids [24], a significant need exists for the development of new target therapeutics for advanced carcinoid cancer.

There is a growing body of literature suggesting that methods to deliver activated Raf-1 to NE cancer cells may be a novel approach in treating these specific tumors. The Raf-1/MEK/ERK pathway has long been recognized for its role in cancer biology. We have previously shown that exogenous activation of the Raf-1 pathway using a retroviral construct with a gene encoding a fusion protein consisting of the active portion of Raf-1 and the hormone-binding domain of the estrogen receptor results in CgA depletion and ASCL1 reduction [11]. While these results suggest that activation of Raf-1 signaling might be a possible strategy to treat advanced carcinoid tumors, feasible methods to deliver activated Raf-1 to tumor cells are extremely limited. We have previously detailed the response of carcinoid tumor cell lines to ZM336372, a novel Raf-1 activating agent originally designed to inhibit Raf-1 [15]; however, *in vitro* studies of the drug showed a paradoxical response of >100 fold Raf-1 activation when used in insect cell culture systems [25]. Further studies with this drug have demonstrated that ZM336372 activates the Raf-1 pathway, reduces NE hormone production, suppresses cell proliferation and induces cell cycle inhibitors in human carcinoid tumor cells *in vitro*. Unfortunately, the dose of ZM336372 required to effect these changes *in vitro* has precluded our laboratory from performing *in vivo* studies, as the compound is nearly water insoluble at these doses and readily precipitates in media.

Here we show that 1 μ M TTM potently inhibits proliferation of pulmonary and GI carcinoid tumor cells. The mechanism of this cellular inhibition appears to depend on the induction of cell cycle arrest, as demonstrated by the dose-dependent up-regulation of the cell cycle regulators p21^{Cip/Waf} and p27. Accordingly, we observed that TTM activates Raf-1 in a dose-dependent manner based on the presence of the phosphorylated Raf-1 pathway mediators, ERK1/2, at micromolar doses of the drug. Likewise, activation of Raf-1 with TTM markedly suppressed CgA, the acidic secretory glycoprotein which is expressed in NE cells and is cosecreted with bioactive hormones, and ASCL1, a basic helix-loop-helix transcription factor that regulates the NE phenotype. We believe this represents the first characterization of a soluble, naturally occurring, Raf-1 activating compound which is capable of inhibiting GI NE tumor growth and suppressing markers of the NE phenotype *in vitro*.

It is clear that the regulation of numerous cellular functions and signal transduction pathways is dependent upon the interplay between protein phosphatases and kinases. Previous studies have demonstrated that TTM is the most potent and specific PP1 inhibitor out of over 40 species of natural phosphatase inhibitors reported [26]. Furthermore, in a study of the effects of the structurally-related compound TTN on human colorectal cells *in vitro*, Lee *et al.* showed that TTN activates the ERK pathway by suppressing the activity of the phosphatase inhibitors, PP1 and PP2A, enzymes thought to be important regulators of Raf-1, phospho-MEK and –ERK [14]. In these studies, the site of action for TTN in the Raf-1 pathway was examined by detailing the effect of TTN on levels of GTP-bound Ras (Ras-GTP) and the activities of Raf-1, MEK, and ERK. *In vitro*, the level of Ras-GTP was not altered in response to TTN; however, the activities of Raf-1, MEK, and ERK were significantly increased based on the presence of phosphorylated bands by Western analysis [14]. While not specifically examined in our carcinoid tumor cell lines, it may be hypothesized that TTM utilizes a similar mechanism of

action, which may be responsible for Raf-1 mediated growth inhibition *in vitro*. As such, further studies examining the role of PP1 and PP2A-mediated Raf-1 activation in GI and pulmonary carcinoid cell lines are planned.

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Figure 1.
The tautomycin biosynthetic gene cluster from *Streptomyces spiroverticillus* [27].

Figure 2. Western analysis for Raf-1 pathway activation in response to TTM treatment
Total cellular extracts from (A) BON and (B) H727 carcinoid cells treated with DMSO control or 1–3 μM TTM for 48h. In control BON and H727 cells, there is little activation of the Raf-1/MEK/ERK system by protein phosphorylation; however, with treatment, there are dose-dependent increases in phosphorylated ERK1/2 indicating Raf-1 pathway activation. All samples were loaded equally as shown by GAPDH.

Figure 3. Cell proliferation analysis of TTM-treated carcinoid cells

3,4-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) bromide growth assay of BON (A) and H727 (B) treated with DMSO (*Control*) or 1–3 μ M TTM for up to 6 days. Both BON and H727 cell proliferation was significantly inhibited in response to drug treatment compared with controls.

Figure 4. Western analysis of cell cycle inhibitors p21^{waf1/cip1} and p27

BON (A) and H727 (B) carcinoid cells were treated with DMSO control or 1–3 μM TTM for 48h. BON cells treated with TTM had little or no detectable p27 present in control treated cells; however, some detectable p21^{waf1/cip1} was noted. Increasing levels of TTM resulted in a dose-dependent induction of p27 with minimal effect on p21^{waf1/cip1}. Interestingly, H727 cells treated with increasing doses of TTM demonstrated increased levels of both p21^{waf1/cip1} and p27, indicating the mechanism of growth inhibition is likely related to arrest of the cell cycle. All samples were loaded equally as shown by GAPDH.

Figure 5. Western analysis of TTM effect on NE tumor markers

Total cellular extracts from BON (A) and H727 (B) cells treated with DMSO control or 1–3 μ M TTM for 48h. In control BON and H727 cells, there are high levels of the NE tumor markers CgA and ASCL1. However, addition of increasing doses of TTM results in a dose-dependent decrease in both CgA and ASCL1.