Identification of a Novel Raf-1 Pathway Activator that Inhibits Gastrointestinal Carcinoid Cell Growth

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Abstract
Carcinoids are neuroendocrine tumors (NET) that secrete hormones, including serotonin, resulting in the malignant carcinoid syndrome. In addition to the significant morbidity associated with the syndrome, carcinoids are frequently metastatic at diagnosis, and untreated mortality at 5 years exceeds 70%. Surgery is the only curative option, and the need for other therapies is clear. We have previously shown that activation of Raf-1 inhibits carcinoid cell proliferation. We investigated the ability of leflunomide (LFN), a Food and Drug Administration–approved medication for the treatment of rheumatoid arthritis, and its active metabolite teriflunomide (TFN) as a potential anti-NET treatment. LFN and TFN inhibit the in vitro proliferation of gastrointestinal carcinoid cells and induce G2-M phase arrest. Daily oral gavage of nude mice with subcutaneous xenografted carcinoid tumors confirms that LFN can inhibit NET growth in vivo. Treatment with TFN suppresses the cellular levels of serotonin and chromogranin A, a glycopeptide co-secreted with bioactive hormones. Additionally, TFN reduces the level of achaete-scute complex-like 1 (ASCL1), a NET marker correlated with survival. These effects are associated with the activation of the Raf-1/mitogen-activated protein kinase kinase/extracellular signal-regulated kinase-1/2 pathway, and blockade of mitogen-activated protein kinase kinase signaling reversed the effects of TFN on markers of the cell cycle and ASCL1 expression. In summary, LFN and TFN inhibit carcinoid cell proliferation in vitro and in vivo and alter the expression of NET markers. This compound thus represents an attractive target for further clinical investigation. Mol Cancer Ther; 9(2); 429–37. ©2010 AACR.

Introduction
A compound originally reported to be effective in rheumatoid arthritis, leflunomide (LFN; Arava, SU-101) has proved to be remarkably safe in human patients (1, 2). Approved by the Food and Drug Administration in 1998, LFN is nearly completely converted to its main active metabolite, teriflunomide (TFN), in first-pass metabolism (1–3). Although its current indications are limited to rheumatoid arthritis, have shown limited success and patients become rapidly resistant to octreotide (15, 17, 19, 20). The severity of metastatic disease and optimal resection still results in significant mortality at 5 years (16). Adjuvant therapies, including chemotherapy and radiation, have shown limited success and patients become rapidly resistant to octreotide (15, 17, 19, 20). The severity...
of carcinoid disease and the lack of effective therapies highlight the need for targeted treatment options.

The Raf-1 pathway has been described as having antineuroendocrine effects and validated as a potential target in the treatment of carcinoid disease. We and others have shown that human NETs lack phosphorylated extracellular signal–regulated kinase 1/2 (ERK1/2), a marker of Raf-1 pathway activation. Activation of the Raf-1/mitogen-activated protein kinase kinase (MEK)/ERK1/2 pathway suppresses levels of ASCL1 as well as CgA and serotonin. In a model of medullary thyroid cancer, a related NET, the ability of Raf-1 pathway activation to suppress in vivo NET growth was confirmed (21–25). Whereas several excellent reviews of the Raf-1 pathway exist, we will briefly summarize the important points. Active, phosphorylated Raf-1 phosphorylates MEK1/2 and subsequently ERK1/2, which then targets multiple key cellular pathways (26).

In this article, we describe, for the first time, the activity of LFN and TFN in NET cells. We show that LFN and its active metabolite, TFN, are capable of suppressing the growth of human carcinoid cells in vitro by inducing cell cycle arrest, and that this finding can be replicated in vivo. We also show that these compounds can inhibit the cellular expression of CgA and serotonin, compounds linked to the poor prognosis of NETs. Furthermore, we show that these compounds suppress ASCL1, a well-characterized marker of neuroendocrine malignancy, at the protein and mRNA levels through a mechanism that is predominately dependent on the Raf-1/MEK/ERK1/2 pathway.

Materials and Methods

Cell Culture and Treatment

Human GI carcinoid cancer cells (BON), graciously provided by Drs. B. Mark Evers and Courtney M. Townsend, Jr. (University of Texas Medical Branch, Galveston, TX), and NCI-H272 human bronchopulmonary carcinoid tumor cells (American Type Culture Collection) were maintained in DMEM/F-12 and RPMI 1640 (Life Technologies), respectively, supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Life Technologies) in a humidified atmosphere of 5% CO2 in air at 37°C. LFN and TFN (Calbiochem) were dissolved in DMSO and treated in quadruplicate at up to 125 μmol/L and subsequently ERK1/2, which then targets multiple cell cycle pathways (26).

Cell Proliferation Assay

Carcinoid tumor cell proliferation was measured by the MTT rapid colorimetric assay as previously described (27). Briefly, BON cells were seeded in 24-well plates and incubated overnight under standard conditions to allow cell attachment. Cells were then treated with LFN or TFN (Calbiochem) in quadruplicate at up to 125 μmol/L and incubated for up to 6 d.

The MTT assay was done by replacing the standard medium with 250 μL of serum-free medium containing 0.5 mg/mL MTT and incubating at 37°C for 4 h. After incubation, 750 μL of DMSO (Fischer Scientific) were added to each well and mixed thoroughly. The multiwell plates were then measured at 540 nm using a spectrophotometer (μQuant, Bio-Tek Instruments).

Cell Cycle Analysis by Flow Cytometry

BON cells were treated with TFN (0, 50, and 100 μmol/L) for 48 h. After treatment, the cells were harvested and washed with ice-cold 0.9% saline buffered with phosphate to a pH of 7.4 (1× PBS), and viability was determined using trypan blue exclusion (Mediatech, Inc.). For DNA content analysis, cells (1 × 10⁶) were fixed with ice-cold 70% ethanol, washed with 1× PBS, incubated with 0.2 mg/mL RNase-A, and stained with 10 μg/mL propidium iodide staining solution. Fluorescence-activated cell sorting analysis was done on a flow cytometer at 488 nm (FACS Calibur flow cytometer, BD Biosciences); results were analyzed with ModFit LT 3.2 software (Verity).

Tumor Xenograft Studies

Male nude (Nu/Nu) mice (Charles River Laboratories) were injected s.c. with 1 × 10⁶ GI carcinoid cells suspended in 100 μL of HBSS (Mediatech). Palpable tumors were allowed to develop, and stratified randomization was used to assign mice to either the control or the treatment group. Mice were treated with either 35 mg/kg LFN suspended in 1.5% carboxymethyl cellulose alone by daily oral gavage. This dose was chosen based on previously published doses in a mouse model of colon cancer (5). Tumor size was measured with vernier calipers every 4 d, and tumor volume calculated using the following formula: 0.52 × [(length) × (width)²], where width was defined as the shorter tumor dimension. After 28 d of treatment, animals were sacrificed. All animal care and treatment was done in compliance with our animal care protocol approved by the University of Wisconsin-Madison animal care and use committee.

Immunoblot Analysis

After treatment as described, cells were washed with ice-cold 1× PBS, and total protein lysates prepared. Total protein concentration was measured with a bicinchoninic acid assay kit (Pierce Protein Research Products). Denatured cellular extracts were resolved by 10% to 12% SDS-PAGE (Invitrogen), transferred onto a nitrocellulose
membrane (Bio-Rad Laboratories), and incubated overnight in the appropriate primary antibody. The antibody dilutions were as follows: 1:1,000 for phosphorylated ERK1/2\textsuperscript{Thr202/Tyr204}, total ERK1/2, phosphorylated MEK1/2\textsuperscript{Ser217/221}, phosphorylated Raf-1\textsuperscript{Ser338}, phosphorylated glycogen synthase kinase-3β\textsuperscript{Ser9}, phosphorylated AKT\textsuperscript{Ser473}, and cyclin B1 (Cell Signaling Technology); 1:2,000 for mammalian achaete-scute homologue-1 for detection of ASCL1 (BD PharMingen) and CgA (Zymed Laboratories); 1:1,000 (Cell Signaling) and 1:10,000 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Trevigen). Horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Pierce) were used depending on the source of the primary antibody. Immunstar (Bio-Rad) or SuperSignal West Pico or Femto (Pierce) kits were used per manufacturer's instructions for detection.

**Serotonin ELISA**

To determine serotonin levels in cellular extracts of carcinoid cells treated with TFN for 48 h, we used a serotonin ELISA kit as per manufacturer's instructions (Fitzgerald). The multwell plates were then measured at 405 nm using a spectrophotometer (μQuant, Bio-Tek Instruments). Serotonin concentrations were calculated based on the manufacturer's standard curve.

**Quantitative Real-time Reverse Transcriptase-PCR**

BON cells were treated with TFN (0, 50, and 100 μmol/L) for 48 h. Total RNA was harvested using a Qiagen RNeasy minikit (Qiagen) as per manufacturer's directions. Integrity was ensured and concentration determined using a Nanodrop spectrophotometer (Nanodrop). Exactly 2 μg of total RNA were then converted to cDNA using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's directions. The quantitative real-time reverse transcriptase-PCR (qPCR) reactions were done on the Bio-Rad iCycler; conditions were 3 min at 95°C, 35 cycles of 30 s at 95°C, 25 s at 60°C, and 30 s at 72°C, followed by 1 min at 95°C and 1 min at 55°C. Primers were obtained from Integrated DNA Technologies and sequences used were ASCL1, F: 5′-TCCCCCAACTACTCCAAACGCAC-3′ and R: 5′-CCCTCCCAACCCGACTGTG-3′, and GAPDH, F: 5′-ACCTGCCAATTAGTACGAC-3′ and R: 5′-ACCTGGTGCTCAGTGAT-3′. Results were normalized to GAPDH from the same sample. Expression of ASCL1 was calculated for each treatment using the formula 2^\text{ΔCt (GAPDH)} / 2^\text{ΔCt (ASCL1)} as outlined in the iCycler Applications Guide (Bio-Rad). Expression was then plotted as average ± SEM.

**Statistical Analysis**

Densitometric analysis of Western blotting results was done using Quantity One software v. 4.6.3 (Bio-Rad). Analysis of MTT growth curves was done using one-way ANOVA test and Bonferroni post hoc test. In the analysis of the nude mouse xenograft experiment, we used Pearson’s χ² test. Student's t test was used to compare the ELISA and qPCR results. In this work, P < 0.05 was considered statistically significant, and analyses were done with SPSS software v. 10.0. Unless specifically noted, all data are represented as mean ± SEM.

**Results**

**TFN and TFN Inhibit In vitro Proliferation of GI Carcinoid Cells**

As recent studies have described attributed antiproliferative effects to LFN and TFN, we first sought to examine the ability of LFN and TFN to inhibit carcinoid proliferation in vitro (4, 6). BON cells were treated with increasing doses of either LFN or TFN, and the MTT assay was done at 2, 4 and 6 days. Dose-dependent, statistically significant growth inhibition was observed after treatment with both LFN (Fig. 1A) and TFN (Fig. 1B) at 4 and 6 days (P ≤ 0.05 versus control). These data suggest that LFN and its major metabolite can inhibit the in vitro proliferation of human GI carcinoid cells.

Treatment with LFN and TFN additionally resulted in the suppression of in vitro proliferation after 2 days of treatment, although this did not reach statistical significance in all treatment groups (Data not shown). In total, these data suggest that LFN and TFN can inhibit in vitro proliferation of GI carcinoid cells.

**Teriflunomide Induces Cell Cycle Arrest and Suppresses In vitro Carcinoid Growth**

TFN has been published to induce cell cycle arrest and apoptosis; thus, we performed Western blot analysis for markers of cell cycle progression and apoptosis as well as propidium iodide exclusion flow cytometry to determine the mechanism of in vitro growth suppression (4, 6).

Treatment with increasing concentrations of TFN resulted in a dose-dependent induction of cyclin B1, a marker of G2-M phase transition (Fig. 2A; refs. 28, 29). We did not observe any cleavage of poly(ADP-ribose) polymerase, a marker of apoptosis (data not shown). These protein results suggest that the mechanism of growth inhibition is cell cycle arrest. We next sought to confirm this finding using propidium iodide exclusion flow cytometry. Treatment with increasing doses of TFN for 48 hours resulted in the progressive accumulation of S-phase products (Fig. 2B). These data, in total, suggest that arrest before the G2-M transition is the mechanism of TFN-induced in vitro growth suppression.

Approved by the Food and Drug Administration in 1998, LFN is currently in clinical use and has proved to be remarkably safe in human patients (2, 3). With this in mind, we wanted to investigate the ability of LFN, converted to TFN in first-pass metabolism, to suppress the in vivo growth of a NET xenograft (1, 30). Treatment of subcutaneous GI carcinoid xenografts with daily oral
gavage of LFN resulted in the statistically significant suppression of tumor growth after 24 days of treatment \((P < 0.02; \text{Fig. } 2C)\). These data suggest that the growth inhibitory effects observed in vitro can be replicated in vivo with oral LFN.

**LFN and TFN Can Alter the Expression of Neuroendocrine Markers**

We then looked to investigate the effect of LFN and TFN on CgA, ASCL1, and serotonin. These markers are of particular interest as they have been associated with poor prognosis and the malignant carcinoid syndrome \((10–12, 14, 22, 31, 32)\). To determine if LFN and TFN were able to modulate the expression of neuroendocrine markers, we treated BON cells for 48 hours with increasing doses of LFN or TFN. Treatment with 125 \(\mu\)mol/L LFN resulted in 61% suppression of CgA and 42% suppression of ASCL1 protein (Fig. 3A). Treatment with 125 \(\mu\)mol/L TFN resulted in 65% suppression of CgA and 84% suppression of ASCL1 protein (Fig. 3B). Densitometric analysis was used to compare the 125 \(\mu\)mol/L dose of LFN and TFN to their respective controls.

The ability of TFN to alter the expression of cellular serotonin, a hormone important in the development of the carcinoid syndrome, was next investigated using an ELISA. Treatment with TFN reduced the cellular expression of serotonin by 77% after 48 hours of treatment \((P < 0.005; \text{Fig. } 3C)\).

As the expression of ASCL1 could potentially be modulated at several levels, we next investigated the levels of ASCL1 mRNA after treatment with TFN \((33, 34)\). Using qPCR, we observed that treatment with 50 and 100 \(\mu\)mol/L TFN for 48 hours resulted in the suppression of ASCL1 levels by 54% and 62%, respectively \((P < 0.05; \text{Fig. } 3D)\).

We next sought to confirm that the above results could be generalized. Treatment of NCI-H727 bronchopulmonary carcinoid cells with increasing doses of either LFN (Fig. 3E) or TFN (Fig. 3F) resulted in a dose-dependent inhibition of ASCL1 protein expression by Western blot analysis. Treatment with 100 \(\mu\)mol/L LFN and TFN resulted in 43% and 71% reductions in ASCL1 protein expression, respectively. These data suggest that the ability of LFN and TFN to alter the expression of a NET marker associated with poor prognosis is not limited to a single cell line.

These data, in total, suggest that LFN and TFN can suppress the expression of NET markers that are associated with the carcinoid syndrome as well as poor survival in NETs \((14, 31)\).

**Teriflunomide Activates the Raf-1/MEK/ERK Pathway**

The Raf-1/MEK/ERK1/2 pathway has been described by our group as being an important regulator of NET cellular proliferation and hormone production \((13, 23–25)\). A relationship between phosphorylated ERK1/2 and the regulation of the G2-M checkpoint has additionally been described \((35, 36)\). Given these known associations and the effects described above, we hypothesized that TFN was inducing Raf-1 pathway activation. To examine the status of Raf-1 signaling, BON cells were treated with increasing doses of TFN and Western blotting was done for key components of the pathway.

A dose-dependent induction of phosphorylated Raf-1 as well as phosphorylated MEK1/2 and phosphorylated ERK1/2 was observed in BON cells treated for 48 hours with TFN (Fig. 4A). These results suggest that the Raf-1/MEK/ERK1/2 pathway is intact and that TFN is capable of efficiently inducing pathway activation. The levels of total ERK1/2 remained unchanged, suggesting that pathway activation, and not translation of additional protein, was responsible for the observed results.

**Suppression of ASCL1 and Induction of Cell Cycle Arrest Are a Direct Result of Raf-1 Pathway Activation**

To show that the effects on NET cells are directly due to Raf-1/MEK/ERK pathway activation, we used U0126, an inhibitor of phosphorylated MEK1/2, to disrupt activation of this pathway. The induction of phosphorylated...
Figure 2. TFN induces cell cycle arrest in vitro and suppresses in vivo proliferation of GI carcinoid cells. BON GI carcinoid cells were treated with the indicated doses of TFN and incubated for 48 h. A, a dose-dependent accumulation of cyclin B1 was observed by Western blot, with GAPDH serving as a loading control. B, propidium iodide exclusion flow cytometry showed a dose-dependent accumulation of S-phase products. Together, these data suggest that TFN can induce in vitro cell cycle arrest before the G2-M transition. C, daily oral gavage with 35 mg/kg LFN of nude mice with subcutaneous xenografted NETs resulted in the progressive inhibition of growth, statistically significant after 24 d ($P < 0.02$). This result suggests that the observed in vitro growth suppression can be replicated in vivo.
ERK1/2 seen with 100 μmol/L TFN can be totally blocked by pretreatment with 10 μmol/L U0126. This suggests that TFN induces the phosphorylation of ERK1/2 via MEK1/2. Importantly, the reduction of ASCL1 and the induction of cyclin B1 are similarly reversed by pretreatment with U0126 (Fig. 4B).

As the regulation of ASCL1 was shown to be at the level of mRNA, we next performed qPCR on BON cells treated with either TFN alone or TFN after pretreatment with U0126. Whereas 100 μmol/L TFN potently suppresses the level of ASCL1 mRNA, pretreatment with U0126 is able to block this suppression (Fig. 4C). These data suggest that TFN-mediated suppression of ASCL1 is dependent on the Raf-1/MEK/ERK pathway at both the protein and mRNA levels. Additionally, these results suggest that TFN-induced cell cycle arrest may also be modulated by this pathway.

To show that the effects on protein phosphorylation were pathway specific, we next investigated the phosphorylation state of two other anti-carcinoid pathways: glycogen synthase kinase-3β (Fig. 4D) and Akt (Fig. 4E; refs. 37–39). No change in the phosphorylation state of these proteins was noted after treatment with TFN. These data suggest that the effects on protein phosphorylation may be specific to the Raf-1/MEK/ERK1/2 pathway.

**Discussion**

Carcinoid tumors are the second most common source of isolated hepatic metastases, after colorectal cancer, with an untreated 5-year mortality in excess of 70% (16). The most effective chemotherapeutic combination tried to date has resulted in a less than 6% volume response rate (40). This lack of efficacy extends to other
adjuvant therapies as well as the palliative option, octreotide, to which patients rapidly become resistant (10). The need for novel targeted therapies is therefore clear.

We present data that suggest that LFN and TFN are novel potential therapeutic options for the treatment of carcinoid disease. LFN and TFN are capable of suppressing in vitro cell proliferation, and TFN is shown to induce cell cycle arrest before the G2-M phase transition. Additionally, in animal studies, we show that the in vitro growth inhibitory effects of LFN can be replicated in vivo with daily oral dosing of LFN. These drugs can additionally suppress the levels of CgA, ASCL1, and serotonin, all markers of neuroendocrine malignancy. The suppression of these three markers is an important point because serotonin is a mediator of the malignant carcinoid syndrome and the expressions of CgA and ASCL1 have been correlated with poor prognosis (10–12, 14, 31, 32). These effects on carcinoid cell markers seem to be mediated predominately through the Raf-1/MEK/ERK1/2 pathway, a pathway that has been extensively studied as a potential anti-carcinoid target (13, 23–25).

The Raf-1 pathway has been traditionally thought of as a tumorigenic pathway and has been noted to be either mutated or overexpressed in hepatocellular carcinoma, non–small-cell lung cancer, melanoma, and papillary thyroid carcinoma (41, 42). In tumors of neuroendocrine origin, however, there is minimal phosphorylation of ERK1/2 at baseline, suggesting that the Raf-1 pathway does not play an essential oncogenic role. Activation of Raf-1 signaling in a GI carcinoid cell, with an estrogen inducible construct, results in the suppression of ASCL1, CgA, and serotonin (13, 23). Additionally, in a medullary thyroid cancer xenograft model, activation of the Raf-1 pathway resulted in the suppression of in vivo tumor growth (24).

This work suggests that Raf-1 pathway activation, if accomplished pharmacologically, could be a potent strategy for the inhibition of carcinoid cancer and other NETs.

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**Figure 4.** TFN suppresses ASCL1 via the Raf-1/MEK/ERK1/2 pathway. 
A, treatment of BON cells for 48 h with TFN resulted in a dose-dependent increase in the level of phosphorylated Raf-1, MEK, and ERK1/2 with no overall change in the level of total ERK. B, pretreatment with the MEK1/2 inhibitor U0126 resulted in a reversal of TFN-induced ASCL1 suppression at the protein level as well as a reversal of the effects on cyclin B1, a marker of G2-M arrest. GAPDH is presented as a loading control. C, similar pretreatment reversed the effects of TFN on the level of ASCL1 mRNA, as measured by qPCR normalized to the GAPDH for the respective sample. These data together suggest that TFN activates the Raf-1/MEK/ERK pathway and that the suppression of ASCL1 protein and mRNA previously observed is regulated via this pathway. Additionally, the observed cell cycle arrest may be mediated by the same pathway. D and E, no change in the phosphorylation state of glycogen synthase kinase-3β (GSK-3β; D) or Akt (E) was observed after carcinoid cells were treated with TFN. This suggests that the modulation of protein phosphorylation may be specific to the Raf-1/MEK/ERK pathway.
Our group has described ZM336372 and tautomycetin as two compounds that seem to activate the Raf-1 pathway in vitro and inhibit the growth of carcinoid cancer and medullary thyroid cancer (25, 43). Significant obstacles, however, exist between these compounds and clinical applicability. Tautomycetin is a natural compound that must be isolated from Streptomyces spirioverticillatus, limiting the quantity that can be produced at any one time. The exceptionally poor solubility of ZM336372 limits its clinical utility, and attempts to produce more soluble sister compounds have met with limited success. In addition to these limitations, neither compound has been described in vivo, and thus the ability to achieve necessary concentrations with acceptable toxicity is unknown.

In contrast, we present LFN as a Raf-1 pathway activator in NETs that is Food and Drug Administration approved and known to be safe in humans. Serum concentrations of TFN in human rheumatoid arthritis patients treated with daily oral LFN are greater than 200 μmol/L, making the doses used in vitro comparable to those attainable in humans (1, 2). Additionally, peak serum TFN concentrations in an in vivo model of oral LFN administration approach 500 μmol/L (44). Our animal data suggest that it is possible, even with oral dosing, to achieve blood concentrations of TFN sufficient to slow the rate of GI carcinoid cell proliferation. It is possible that higher doses would result in more significant tumor inhibition. It is interesting that tumor size in the treatment groups did not seem to diverge until after 14 days of treatment, perhaps suggesting a role for the antiangiogenic effects of LFN described by others (5). A larger upcoming study designed to confirm and extend the in vivo data presented here will examine higher doses and allow for histologic examination of the xenografted tumors. LFN, therefore, more than ZM336372 and tautomycetin, represents a potential therapy for NETs targeting the Raf-1 pathway. We conclude that LFN is worthy of additional study, including a larger animal study and potentially an initial human trial.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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