ZM336372, a Raf-1 activator, suppresses growth and neuroendocrine hormone levels in carcinoid tumor cells

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Abstract

Neuroendocrine tumors, such as carcinoids, are highly metastatic neoplasms that secrete bioactive hormones resulting in carcinoid syndrome. Few curative treatments exist outside of surgical resection. We have previously shown that activation of the Raf-1 signaling pathway can suppress hormone production in carcinoid tumor cells. In this study, we investigated a novel treatment for carcinoid tumor cell growth based on pharmacologic Raf-1 activation using the compound ZM336372. Treatment of carcinoid tumor cells with ZM336372 resulted in progressive phosphorylation of Raf-1, mitogen-activated protein kinase 1/2, and extracellular signal–regulated kinase 1/2. Importantly, exposure to ZM336372 resulted in a significant reduction of bioactive hormone levels as well as the transcription factor, human acheate-scute homologue-1 in carcinoid tumor cells. Furthermore, treatment with ZM336372 led to a marked suppression of cellular proliferation and induction of the cell cycle inhibitors p21 and p18. In summary, ZM336372 targets both proliferation and palliative issues associated with carcinoid tumor cells, and therefore, warrants further investigation as a possible therapeutic strategy for patients with carcinoid tumors.


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Introduction

Ninety-five percent of carcinoid tumors arise from the gastrointestinal tract or lung (1–3). Surgical resection for localized carcinoid neoplasms is usually curative; however, these tumors are highly metastatic and often unresectable (1). Besides surgical resection, combinations of radiation and multiple chemotherapeutic regiments have been attempted to control metastatic carcinoid tumors; however, no protocol has been proven effective (4). Moreover, patients with advanced disease often have painful and debilitating symptoms of the malignant carcinoid syndrome, which include cutaneous flushing, diarrhea, right-sided heart disease, and bronchospasm. Although, somatostatin analogues are effective therapy for this syndrome, patients often become resistant to somatostatin and are left with no palliative option for symptomatic control of the carcinoid syndrome. Therefore, there is a great need to develop novel therapeutic strategies both to reduce tumor burden as well as to control symptoms in patients with carcinoid neoplasms.

The ras/Raf-1 signaling pathway has long been recognized for its importance in cancer biology. In this pathway, ras signaling often involves activation of Raf-1, a cytosolic serine/threonine kinase. Activated Raf-1 then phosphorylates mitogen-activated protein kinase kinase 1 and 2 (MEK1/2), which then activates downstream extracellular signal–regulated kinase 1 and 2 (ERK1/2). Activating mutations in this pathway are common among adenocarcinomas of the colon, pancreas, and lung as well as squamous cell cancers (5). However, in neuroendocrine tumors such as small cell lung (SCLC), medullary thyroid, and carcinoid cancer, mutations causing elevated Raf-1 signaling are rarely detected in pathologic specimens (5–7). In SCLC, Raf-1 pathway activation results in phenotypic change and reduction in cellular proliferation (8, 9). We and others have previously reported that Raf-1 activation in medullary thyroid results in growth suppression as well as reduction in neuroendocrine hormones (such as calcitonin and serotonin), human acheate-scute homologue-1 (hASH1), and levels of the RET proto-oncogene (10, 11). Furthermore, in human carcinoid tumor cells, there is significant reduction in levels of neuroendocrine hormones with Raf-1 activation (12, 13). These results suggest that Raf-1 activation may be a possible therapeutic target in certain cancers such as neuroendocrine tumors. Other than gene therapy, applications to deliver activated Raf-1 to tumor cells are limited. Therefore, we explored the possibility of pharmacologically activating Raf-1.

In this article, we detail the response of carcinoid tumor cell lines to ZM336372, a novel Raf-1-activating agent. ZM336372 was initially formulated as a small molecular
inhibitor of Raf-1 (14). However, the drug showed a paradoxical response of >100-fold Raf-1 activation when used in insect cell culture systems (14). We show that ZM336372 results in progressive phosphorylation of Raf-1 pathway mediators in carcinoid tumor cells. Furthermore, chromogranin A, a surrogate marker for bioactive hormones in carcinoid tumors, is significantly reduced in response to this compound. Additionally, the neuroendocrine transcription factor, hASH1, is suppressed in response to treatment with ZM336372. Thus, we report the first characterization of the compound, ZM336372, which is capable of suppression of cellular proliferation and induction of cell cycle inhibitors in carcinoid tumor cells.

Materials and Methods

Cell Culture

Human pulmonary carcinoid cells (NCI-H727) were obtained from American Type Culture Collection (Manassas, VA) and human pancreatic carcinoid tumor cells, BON, a generous gift of Drs. Evers and Townsend (Department of Surgery, University of Texas, Galveston, TX; ref. 15), were maintained in RPMI 1640 and DMEM/F12 (Life Technologies, Rockville, MD), respectively, supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), 100 IU/mL penicillin and 100 µg/mL streptomycin (Life Technologies) in a humidified atmosphere of 5% CO₂ in air at 37°C.

BON-raf Cells Maintenance and Raf-1 Activation

BON-raf cells were created and maintained and Raf-1 was activated essentially as described (12, 13, 16). Briefly, BON cells were stably transduced with the retroviral vector pLNC.raf:ER to create BON-raf cells. This construct contains estrogen receptor fused with c-raf kinase domain. BON-raf cells were maintained in DMEM/F12 supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, and 400 µg/mL G418 (Life Technologies) in a humidified atmosphere of 5% CO₂ in air at 37°C. To activate Raf-1 in BON-raf cells, 1 µmol/L β-estradiol (Sigma) was added to the medium. An equivalent amount of ethanol, solvent for the β-estradiol, was used to treat control cells.

ZM336372 Treatment

H727 and BON cells were plated at 50% to 60% confluence in 100-mm cell culture dishes and incubated overnight. Cells were treated with ZM336372 (Fig. 1; BioMol, Plymouth Meeting, PA) in different concentrations for up to 6 days. Of note, treatments were done in serum-containing medium. Furthermore, the DMSO concentration never exceeded 2% in all treatment groups. ZM336372 at 500 µmol/L concentrations were used only in the toxicity experiments. In this case, we made stock ZM336372 at higher concentration to reduce DMSO toxicity.

Western Blot Analysis

Cellular extracts were prepared and quantified by bicinechonic acid protein assay kit (Pierce, Rockford, IL) as previously described (13). Denatured proteins (30-50 µg) from each sample underwent electrophoresis on a SDS-polyacrylamide gel and transferred to a nitrocellulose membranes (Schleicher and Schuell, Keene, NH). Membranes were blocked for 1 hour in milk solution (1 × PBS, 5% nonfat dry milk, 0.05% Tween 20) and incubated at 4°C overnight with primary antibodies. The following primary antibody dilutions were used: phospho-ERK1/2 (1:1,000), phospho-MEK (1:1,000), phospho-Raf-1 (Ser338; 1:1,000), p21 and p18, (1:1,000), (Cell Signaling Technology, Beverly, MA), Chromogranin A (1:2,000; Zymed Laboratories, San Francisco, CA), MASH-1 (1:1000, BD Pharmingen, San Diego, CA), and G3PDH (1:10,000; Trevigen, Gaithersburg, MD). After primary antibody incubation, membranes were washed 3 × 5 minutes in PBS-T wash buffer (1 × PBS and 0.05% Tween 20). Then the membranes were incubated with either 1:2,000 dilution of goat anti-rabbit or goat anti-mouse secondary antibody (Cell Signaling Technology) depending on the source of the primary antibody for 1 hour at room temperature. Membranes were washed 3 × 5 minutes in PBS-T wash buffer and developed by Immunstar horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s directions. For the detection of hASH1 protein, membranes were developed with SuperSignal West Femto chemiluminescence reagent (Pierce).

Drug Toxicity Assay

Briefly, cells were trypsinized and plated in a 6-well plate at 1 to 2 × 10⁵ in triplicate and allowed to adhere overnight. Then the media was replaced with fresh media containing various concentrations (31.25, 62.5, 125, 250, and 500 µmol/L) of ZM336372 and incubated for up to 3 days. As a control, DMSO was added. After incubation, the medium was removed and cells were trypsinized and added to removed medium. Cells were incubated on ice and 2.5 µg/mL propidium iodide (Sigma) was added 5 minutes before flow cytometry (17). Data was acquired using a FACSCalibur benchtop flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest acquisition and analysis software.

Figure 1. Chemical structure of ZM336372. ZM336372’s chemical name is N-[(5-(dimethylaminobensamido)-2-methylphenyl)-4-hydroxybenzamide.
Results

Raf-1 Activation Results in Reduction of hASH1 in Carcinoid Tumor Cells

We have previously described that Raf-1 activation in carcinoid tumor cells results in activation of the Raf-1 pathway mediators MEK1/2 and ERK1/2 and reduction in chromogranin A. Untreated, native carcinoid tumor cells (BON C) have little to no phosphorylation of MEK1/2 or ERK1/2 at baseline (Fig. 2). Furthermore, native carcinoid cells have high levels of chromogranin A and hASH1. Activation of Raf-1 in gastrointestinal carcinoid BON-raf cells by estradiol treatment leads to activation of MEK1/2 and ERK1/2 as well as reduction in chromogranin A compared with controls (Fig. 2). Because hASH1 is highly expressed in neuroendocrine tumor cells and has been shown to mediate the neuroendocrine phenotype, we hypothesized that the decrease in neuroendocrine markers induced by Raf-1 pathway activation could be due to a decrease in hASH1. Estradiol (E2)–induced activation of Raf-1 in BON-raf tumor cells resulted in significant reductions in hASH1 by Western analysis at days 2, 4, and 6 (Fig. 2), suggesting that Raf-1 may play a role in regulation of this transcription factor in carcinoid tumors.

**ZM336372 Activates the Raf-1/MEK/ERK System in a Dose-Dependent Manner**

Whereas ZM336372 has been shown to activate Raf-1 *ex vivo*, there are little data to illustrate that this leads to phosphorylation of downstream mediators such as ERK1/2 and MEK1/2 *in vitro*. Therefore, Western analysis was used to show that Raf-1/MEK/ERK pathway activation occurs in response to treatment with ZM336372 in carcinoid tumor cells. In control pulmonary (H727) and gastrointestinal (BON) carcinoid tumor cells, there is little phosphorylation of Raf-1, MEK1/2, or ERK1/2. At 2 days, treatment with 20 and 100 μmol/L ZM336372 led to activation of MEK1/2 and ERK1/2 in native H727 cells compared with control (DMSO) treatments as evidenced by protein phosphorylation (Fig. 3A). Furthermore, Raf-1 is also phosphorylated at Ser338 by addition of ZM336372, indicating that this system is activated at least at the level of Raf-1. Similarly, in BON tumor cells, there is strong activation of ERK1/2 and MEK1/2 with increasing doses of ZM336372 (Fig. 3B). Moreover, activation of the Raf-1/MEK/ERK system is sustained, as exemplified by MEK1/2 phosphorylation in H727 cells at days 4 and 6 with the addition of 100 μmol/L ZM336372 (Fig. 3C), showing prolonged action of this compound.

**ZM336372 Reduces Neuroendocrine Hormone Production in Carcinoid Tumor Cells**

We and others have previously shown that changes in chromogranin A levels are concordant with alterations in other neuroendocrine hormones such as histamine and serotonin (13). Additionally, we have shown that hASH1 is expressed in most neuroendocrine tumors and correlates with neuroendocrine hormone levels as previously described. Therefore, to determine if ZM336372 can reduce chromogranin A and hASH1 proteins significantly in a time-dependent manner. There is no reduction of these proteins in control treatments.
and BON with ZM336372 caused reduction in both chromogranin A and hASH1 as shown by Western analysis (Fig. 4A and B). Furthermore, to determine when chromogranin A depletion occurs, we treated H727 cells with ZM336372 for 2 d. In control H727 and BON 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 Raf-1 at Ser338 (pRaf-1), MEK 1/2 (pMEK 1/2), and ERK 1/2 (pERK 1/2) indicating Raf-1 pathway activation. C, Western analysis of H727 cells treated at 2, 4, and 6 d with control and 100 μmol/L ZM336372. There is phosphorylation of MEK 1/2 (pMEK 1/2) greater than controls out to 6 d. Samples are loaded equally as shown by G3PDH.

Figure 3. Western analysis for Raf-1 pathway activation in response to ZM336372 treatment. Total cellular extracts from (A) H727 and (B) BON cells treated with DMSO (Control) and 20 and 100 μmol/L ZM336372 for 2 d. In control H727 and BON 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 Raf-1 at Ser338 (pRaf-1), MEK 1/2 (pMEK 1/2), and ERK 1/2 (pERK 1/2) indicating Raf-1 pathway activation. C, Western analysis of H727 cells treated at 2, 4, and 6 d with control and 100 μmol/L ZM336372. There is phosphorylation of MEK 1/2 (pMEK 1/2) greater than controls out to 6 d. Samples are loaded equally as shown by G3PDH.
hormone depletion, we carried out Western analysis of H727 cells treated with the drug or carrier control at days 2, 4, and 6. Treatment with ZM336372 had a lasting effect as chromogranin A depletion was maintained to 6 days with treatment in carcinoid cells (Fig. 4D).

ZM336372 Suppresses Cell Proliferation and Induces Cell Cycle Inhibitors in Carcinoid Tumor Cells

Raf-1 activation results in growth inhibition in many cell types, as well as cell senescence in others (8, 10, 17). Initially, when treating H727 cells plated at 50% confluence with ZM336372, it was noticed that the cells never reached confluence as nontreatment cells would at days 2, 4, or 6 (data not shown). This observation was substantiated by 3,4-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide growth assay. H727 cells treated with ZM336372 were growth suppressed, whereas control treatments had significantly more growth by day 6, continuing up to 16 days (Fig. 5A). A similar response was also seen in BON cells as growth suppression occurred as early as day 4 and was maintained out to day 10 (Fig. 5B). All our experiments were done in serum-containing medium including control and ZM336372 treatments. However, growth arrest was only seen in ZM336372-treated cells. We have also showed that ZM336372 inhibits growth of other neuroendocrine tumor cell lines including medullary thyroid cancer cells (data not shown). It is also possible ZM336372 may have more pronounced growth inhibition in the absence of serum.

We then explored the possible mechanism of ZM336372-induced growth inhibition. Raf-1 activation has been shown to induce expression of cell cycle inhibitors of separate families, including p21 and those of the INK family, such as p18 (17). As shown in Fig. 5C, carcinoid tumor cells have minimal levels of p21 and p18 at baseline. Western analysis of cellular extracts revealed that treatment with ZM336372 induced p21 and p18 at 2 days compared with treatment controls. The ability of ZM336372 to induce p21 and suppress cellular proliferation through Raf-1 activation seems specific to neuroendocrine tumor cells.

Figure 5. Cell proliferation analysis of ZM336372. 3,4-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide growth assay of H727 (A) and BON (B) treated as control, DMSO, and 100 μmol/L ZM336372 to days 16 and 10, respectively. Both H727 and BON cell proliferation was inhibited in the presence of drug compared with controls. C, Western analysis of cell cycle inhibitors p21 and p18. H727 cells treated with DMSO or 20 and 100 μmol/L ZM336372 for 2 d. H727 cells without treatment had little or no detectable expression of p21 or p18; however, with treatment, there was significant induction of p21 and p18. G3PDH shows equal loading. D, expression of cell cycle inhibitor p21 in ZM336372-treated carcinoid tumor cells. H727 cells treated with DMSO or 100 μmol/L ZM336372 for 2 d. Pancreatic cancer cells, MiaPaCa2 in that Raf-1 pathway is naturally activated and was used as control without ZM336372 treatment. Cell lysates were obtained and analyzed for Raf-1 pathway activation (pERK1/2) and p21 expression by western blot. H727 cells without treatment had little or no detectable expression of pERK1/2 and p21 proteins whereas treatment by ZM336372 on H727 cells showed high levels of pERK1/2 and p21 proteins. Importantly, MiaPaCa2 cells had no detectable expression of p21 but high levels of pERK1/2 proteins. G3PDH shows equal loading.
We subsequently analyzed human pancreatic cancer cell lines that have constitutive activation of the ras/Raf-1/MEK/ERK1/2 pathway. In the human pancreatic cancer cell line (MiaPaCa2), there was no up-regulation of p21, although the Ras/Raf-1 pathway is naturally activated as evidenced by the presence of high levels of phosphorylated, active ERK1/2 (Fig. 5D).

We then did propidium iodide exclusion to assess direct cytotoxicity of ZM336372. As seen in Fig. 6, at high concentrations of the drug (500 μmol/L), 40% to 50% of H727 cells remain viable at 2 days with treatment compared with native H727 cells. Furthermore, at concentrations used in this article (20–100 μmol/L), cytolyis is <20%. Moreover, in BON cells, ~70% of cells remain viable by propidium iodide exclusion at 2 days from 63 to 500 μmol/L ZM336372. As a control, H727 and BON cells were treated with doxorubicin, an agent known to cause significant cytotoxicity to tumor cells, to validate our assay. IC50 values were obtained with doxorubicin of 1.1 and 1.4 μmol/L in H727 and BON tumor cells, respectively.

**Discussion**

Neuroendocrine tumors, such as carcinoid, are second only to colorectal cancer as the most common source of isolated liver metastasis and if left untreated, have less than a 30% 5-year survival for patients with metastatic carcinoid tumors (18). Available alternatives to surgery including ablative therapies and chemotherapy have limited efficacy (19). Current chemotherapeutic regiments have been disappointing, where there is <6% volume response rate with a combination of streptozocin and doxorubicin, the best available treatment (19). In addition, patients receiving therapies directed at symptom control, such as octreotide, often become recalcitrant to this palliative regimen and are left without treatment alternatives (20). Besides obvious problems with symptoms associated with the carcinoid syndrome, high levels of serum chromogranin A are also associated with poor clinical prognosis for patients with carcinoid tumors (21–23). Clearly, new therapies are needed for improvement of quality of life as well as prolongation of survival in carcinoid tumors and disease. One possible strategy is the manipulation of various signaling pathways to control the growth of carcinoid tumor cells. We have previously shown that activation of the Raf-1 pathway by exogenous Raf-1 results in chromogranin A depletion in carcinoids (13) and medullary thyroid cancer (11) cells. These results suggested that activation of Raf-1 might be a possible strategy to treat carcinoid tumor cells. However, applications to deliver activated Raf-1 to tumor cells are limited.

Therefore, in this article, we identify ZM336372, which is capable of reducing tumor cell growth as well as neuroendocrine hormones production in carcinoid cells, addressing both proliferation and palliative issues associated with carcinoid tumors. Furthermore, we have reported here that treatment with ZM336372 significantly reduces chromogranin A in carcinoid tumor cells quickly and with prolonged effect.

In addition to hormone production, carcinoid cells express high levels of hASH1, a basic helix-loop-helix transcriptional factor. In human tumor cell lines, hASH1 expression is seemingly limited to neuroendocrine tumors and neuroblastoma (24). Mammalian achaete-scute homologue-1 (MASH-1), the mouse equivalent of hASH1, is essential for development of a subset of neurons, pulmonary neuroendocrine cells, thyroid C cells, and adrenal chromaffin cells in fetal mice, although its expression is lost in mature forms of these cells (25–29). Furthermore, in SCLC, hASH1 expression correlates independently and strongly with shortened survival times in SCLC patients (30). Most importantly, in SCLC, depletion of hASH1 results in significant reductions of neuroendocrine...
markers, such as synaptophysin and neuron-specific enolase (26). Here, we report for the first time that Raf-1 activation in carcinoid tumors results in decreased hASH1 levels. This finding, as well as research in other tumor cell lines, suggests that hASH1 may be involved in the control of the production of neuroendocrine hormones such as chromogranin A, NSE, and synaptophysin. Furthermore, the addition of ZM336372 to native carcinoid cells also prominently reduces intracellular hASH1, and importantly, more pronounced than with induction of Raf-1 gene expression (Figs. 2 and 4).

In the initial description of ZM336372, they noted that by a standardized ex vivo Raf-1 assay, the compound was as an efficient inhibitor of Raf-1. However, when the drug was added to cell culture and Raf-1 activity was measured by a coupled assay, there was a 100-fold activation, although they noted no evidence of phosphorylation of Raf-1 pathway mediators, specifically, ERK1/2 (14). We have shown here that it is possible to achieve strong activation of the Raf-1/MEK/ERK pathway with ZM336372 treatment as evidenced by phosphorylation of Raf-1 and its downstream pathway mediators. Therefore, this is the first report describing a new class of drug in part as Raf-1 activators that warrants further investigation as possible therapeutic strategies for patients with carcinoid tumors. Treatment of neuroendocrine tumor cells such as pancreatic carcinoid, pulmonary carcinoid and medullary thyroid carcinoid cells by ZM336372 led reduction in cellular proliferation (present study).4 Although ZM336372 affects cellular proliferation in many cell types nevertheless the mechanism may not be general, because ZM336372 has been shown to phosphorylate other proteins (14). Therefore, at present it is difficult to speculate the exact mechanism by which ZM336372 inhibits cellular proliferation. However, we and others have previously shown that Raf-1 activation inhibits neuroendocrine tumor cellular proliferation and neuroendocrine marker reduction (8–10). Similarly, we have shown here that ectopic Raf-1 activation in BON-raf cells led to neuroendocrine marker reduction. Similar effects such as Raf-1 activation and growth inhibition were also observed by ZM336372 in carcinoid tumor cells, suggested that the growth inhibition at least in part is due to the Raf-1 activation. On the other hand, we have not excluded the possibility that other pathways might also be affected by ZM336372.

Clinically available chemotherapy and radiotherapy have shown little efficacy in halting progression of solid disease or reduction of tumor burden in patients with carcinoid tumors (1, 4, 31). ZM336372, as reported in this article, shows impressive inhibition of carcinoid tumor cell proliferation. Although the mechanism of this action is unclear, cell cycle inhibitors such as p21 and p18 are induced with treatment. Furthermore, we have shown this growth inhibition is not due to toxicity elicited from ZM336372 or its carrier DMSO as cell viability is minimally reduced with treatment. ZM336372 causes reduction in hormone production and further has a growth inhibitory effect, the first agent to address both in carcinoid tumor cells. In summary, we have presented a potentially new category of chemotherapeutic agents, Raf-1 pathway activators, which inhibit cell proliferation, induce cell cycle inhibitors, and reduce production of bioactive hormones in carcinoid tumor cells in vitro.

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References


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