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Neuroendocrine tumor cell growth inhibition by ZM336372 through alterations in multiple signaling pathways

Muthusamy Kunnimalaiyaan, Ph.D., Mary Ndiaye, B.S, and Herbert Chen, M.D

Endocrine Surgery Research Laboratories, Section of Endocrine Surgery, Department of Surgery, The University of Wisconsin School of Medicine and Public Health and the University of Wisconsin Paul P. Carbone Comprehensive Cancer Center, Madison, WI.

Abstract

Background— We have previously shown that activation of the Raf-1/MEK/ERK1/2 signaling pathway by ZM336372 inhibits carcinoid cells growth. In the present study, we further characterize the molecular details of the growth inhibition by the signaling-based compound ZM336372 in neuroendocrine tumors (NETs).

Methods—NET cells were treated with ZM336372 (20–100 μ M) or carrier (DMSO). Western Blot was used to determine the activation of the Raf-1/MEK/ERK, other pathways activation, and cellular bioactive hormone production.

Results— ZM336372 in NET cells resulted in increasing raf-1 activation and inactivation of GSK-3 β as measured by phosphorylation of ERK1/2 and GSK-3 β respectively. There was no alteration in the levels of phosphorylated AKT an important mediator of PI3K pathway. Importantly, blocking of raf-1 pathway by U0126, a potent inhibitor, in the presence of ZM336372 did not reduce the levels of p-GSK-3 β indicating that GSK-3 β inactivation is independent of raf-1 pathway activation. Moreover, the levels of CgA and ASCL1 reductions were persistent even after blocking the raf-1 pathway. Treatment with ZM336372 in the presence of siRNA against *raf-1* resulted in an increase in Raf-1 production, suggesting that ZM336372 up regulates raf-1 at the transcriptional level.

Conclusion— This is the first description of a novel compound ZM336372 that regulates multiple pathways in NET cells.

INTRODUCTION

Neuroendocrine Tumors (NETs), such as carcinoids and medullary thyroid cancer (MTC), produce excess amounts of various bioactive hormones that cause significant symptoms. Although surgery is currently the only potential curative treatment for NETs, most patients present with metastatic disease. Therefore, other forms of therapy are needed. Several signaling pathways, such as the phosphatidyl-inositol 3-kinase (PI3K)/Akt, Raf-1/mitogen activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK), Notch1/Hairy Enhancer of Split-1 (HES-1)/achaete-scute complex like-1 (ASCL1), and glycogen synthase kinase-3 beta (GSK-3 β) signaling pathways, have been shown to play important roles in regulating the

Reprint requests: Muthusamy Kunnimalaiyaan, Ph.D., K4/623 Clinical Science Center, 600 Highland Avenue, The University of Wisconsin School of Medicine and Public Health, Madison, WI, 53792 Telephone:(608) 265-3749; Fax: (608) 263-8613; E-Mail: kunni@surgery.wisc.edu.

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growth of NETs^{1–12}. Thus, a potential therapeutic target could be manipulation of these various cellular signaling pathways. Recently, we have shown that activation of the raf-1 pathway in MTC, TT cells, resulted in phosphorylation of GSK-3 β , which is associated with growth inhibition¹. We have also previously shown that activation of the raf-1 pathway by a pharmacological compound, ZM336372, inhibits carcinoid cell growth¹³. However, other than raf-1 activation, the molecular mechanism(s) by which carcinoid cells growth inhibition occurs by ZM336372 is unknown.

Given the background information on ZM336372 and our recent results, we hypothesized that ZM336372 might alter other signaling pathways. In this study, we demonstrated that the treatment of MTC cells resulted in activation of raf-1 pathway by increase in the phosphorylation of ERK1/2 in a dose-dependent manner similar to our earlier reports on carcinoid cells. Treatment of NET cells with ZM336372 resulted in inactivation of GSK-3 β by phosphorylation. To determine the importance of the GSK-3 β pathway in cellular proliferation, we treated MTC and bronchopulmonary carcinoid cells with a known MEK inhibitor, U0126, to block the raf-1 pathway and showed that the inhibition of the raf-1 pathway did not alter the levels of phosphorylated GSK-3 β protein suggesting that the ZM336372 regulates multiple pathways. Importantly, the reduction in the NE markers such as chromogranin A (CgA) and ASCL1 did not change with U0126 treatment. We also demonstrated for the first time the progressive up regulation of Raf-1 protein in carcinoid cells treated with ZM336372 in the presence of siRNA against *raf-1*.

MATERIAL AND METHODS

Cell culture

Human medullary thyroid cancer cells (TT) and human bronchopulmonary carcinoid (H727) cells were obtained from ATCC and maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 18% Fetal Bovine Serum (Sigma, St. Louis, MO), 100 IU/ml penicillin and 100 μ g/ml streptomycin (Invitrogen) in a humidified atmosphere of 5% CO₂ in air at 37°C as previously described^{4,11,14}.

ZM336372 treatment

TT or H727 cells were plated onto 100 mm dishes. The following day media was changed to fresh media containing varying concentrations of ZM336372 (0–200 μ M) and incubated for different time points as indicated. Control cells were incubated with DMSO, since this was used as a solvent for ZM336372. In the case of the MEK inhibitor study, cells were treated with U0126 at 15 μ M concentrations for 45 minutes before adding ZM336372.

Immunoblot analysis

Total cellular proteins were isolated and the protein concentrations were determined with a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL) as previously described³. Denatured cellular extracts (30 μ g) were separated on a 10% SDS-PAGE and western blot was carried out as previously described³. Proteins transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH) were blocked in milk (5% nonfat dry milk, 0.05% Tween 20; in 1X PBS) and incubated with primary and secondary antibodies as previously described³. The following primary antibody dilutions were used: MASH1 for human ASCL1 (1:1000; BD Pharmingen, San Diego, CA), G3PDH (1:10000, Trevigen, Gaithersburg, MD), p-ERK1/2 (1:1000), p-GSK3 β ^{Ser9} (1:1000), raf-1 (1:1000, Cell Signaling Technology, Beverly, MA) and CgA (1:1000, Invitrogen). Primary antibody incubations were kept overnight at 4°C and then depending upon the antibodies, membranes were washed 3 $\times$ 5 min or 3 $\times$ 10 min. Then the membranes were incubated with a 1:2000 dilution of HRP-linked anti-rabbit or anti-mouse secondary antibody (Cell Signaling Technology) depending on the source of the antibody used.

Membranes were developed by Immuno-Star (Bio-Rad Laboratories, Hercules, CA) for p-ERK1/2, p-GSK3 β ^{Ser9}, raf-1, CgA and G3PDH or Super West Femto chemiluminescence's substrate (Pierce) for ASCL1 according to the manufacturer's directions.

MTT cellular proliferation assay

To measure proliferation rate, cells were plated in triplicate in 24 well plates. Then the cells were treated with 100 and 200 μ M concentrations of ZM336372 for 6 and 8 days. At each time point, cell viability for the treated cells was determined by MTT (thiazolyl blue tetrazolium bromide; Sigma) assay as previously described^{11,14}. Experiments were performed at least twice.

Small interfering RNA assay

To determine the mechanism by which ZM336372 reduces NE markers, siRNA against raf-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was transfected into bronchopulmonary carcinoid cells using Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions. Next day, the media containing the transfection complexes were replaced with fresh medium containing either ZM336372 or DMSO and the cells were incubated for another 2 days. Then the cell lysates were prepared and analyzed for the levels of raf-1, ASCL1 and CgA proteins by Western analysis.

Statistical Analysis

Analysis of variance (ANOVA) with Bonferroni post hoc testing was performed using a statistical analysis software package (SPSS version 10.0, SPSS, Chicago, IL). A P-value of < 0.05 was considered significant.

RESULTS

ZM336372 inhibits cellular growth in TT cells

We have reported that treatment of carcinoid cells (BON and H727) with ZM336372 resulted in activation of raf-1 pathway and this was associated with growth inhibition¹³. Furthermore, recently we reported that inhibition of pheochromocytoma (PC12) cell growth *in vitro* by ZM336372 was shown to be associated with raf-1 pathway activation¹⁵. However, the effect of ZM336372 on the growth of TT cells is not known. We have done cytotoxic experiment with different cell lines including TT and observed that 300- μ M concentrations there was 30% cell death at day 2 (data not shown). Therefore, we treated TT cells with below the cytotoxic concentrations of ZM336372 (100 and 200 μ M) and the results of the cell viability assay are shown in Fig 1. Control experiments contains equal amount of dimethyl sulfoxide (DMSO; solvent for ZM336372), which did not exceed 0.2% (v/v) in any of our experiments. We have earlier shown that this concentration did not affect the cell growth or any activity¹³. Cells treated with DMSO, showed more growth than cells treated with 100 μ M ZM336372. At the 200 μ M concentration of ZM336372 treated cells had more significant growth reduction compared to 100 μ M treatment. Even at the 4-day time point, the growth reduction can be seen with different doses of ZM336372, indicating that the growth inhibition is concentration dependent rather than time dependent. Interestingly, carcinoid cells showed dramatic growth reduction at 100 μ M concentration¹³. Statistical analysis showed that the growth inhibition is significant at all time points.

ZM336372 activates multiple pathways in NET cells

It has been shown that ZM336372 activates the raf-1 pathway indicated by phosphorylation of ERK1/2 in carcinoids and pheochromocytoma cell lines. To determine that similar results were obtainable in TT cells, ZM336372 treated cell lysates were analyzed for the activation of the

raf-1 pathway by Western blot analysis for phosphorylation of ERK1/2 as a surrogate measure. As shown in Fig 2A, phosphorylation of ERK1/2 is progressively increased with increasing concentrations of ZM336372. This provides evidence that the raf-1 pathway was successfully activated by ZM336372 in TT cells. Recently we have observed the inactivation of GSK-3 β by phosphorylation at ser⁹th position in raf-1 activated TT cells¹. Furthermore, we have shown that inactivation of GSK-3 β by pharmacological inhibitors alone is sufficient to reduce NE markers and growth of TT cells¹. Therefore, to determine if other pathways such as PI3K/Akt and GSK-3 β , are activated by ZM336372 in TT cells, western analyses were carried out for the ZM336372-treated cells. Interestingly, a dose-dependent increase in the phosphorylation of GSK-3 β at ser⁹th position was observed with treatment (Fig 2A). However, no increase in the phosphorylation of Akt was observed (data not shown) indicating that perhaps raf-1 activation by ZM336372 is also associated with phosphorylation of GSK-3 β in TT cells. To determine if this effect is limited to TT cells or applicable to other NE tumors, we treated H727 cells, with increasing concentrations of ZM336372 for two days and western analysis was carried out as described in methods. As shown Fig 2B treatment with ZM336372 resulted in an increase in both phosphorylated ERK1/2 and GSK-3 β proteins similar to TT cells (Fig 2B). As expected, ZM336372-treated cells also resulted in a reduction in the levels of CgA and ASCL1 proteins.

Reduction in Neuroendocrine markers by ZM336372 is independent of raf-1 activation

We have reported that NET cells express significant levels of NE markers such as ASCL1 and CgA and that activation of raf-1 lead to significant reductions in these markers^{7,8,13}. Furthermore, we have shown that inactivation of GSK-3 β alone is sufficient for reduction in NE markers. In the present study, we showed both raf-1 activation and inactivation of GSK-3 β as evidenced by phosphorylation of ERK1/2 and GSK-3 β , respectively in ZM336372-treated NET cells. Therefore, we were interested in determining if both pathways are required to see a decrease in NE markers in ZM336372-treated NET cells. As shown in Fig 3A, treatment of H727 cells with ZM336372 in the absence of U0126 treatment showed an increase in the levels of both phosphorylated ERK1/2 and GSK-3 β proteins. This was associated with a reduction in NE markers such as CgA and ASCL1. However, blocking raf-1 activation by U0126, a well-known MEK inhibitor, prior to ZM336372 treatment led to the absence of phosphorylated ERK1/2 (lane 4). Importantly, there is no change in the levels of phosphorylation of GSK-3 β in both U0126 treated and untreated cells, suggesting that inactivation of or phosphorylation of GSK-3 β is independent of raf-1 activation. Interestingly, the levels of CgA and ASCL1 are persistent even after blocking raf-1 pathway in ZM336372 treated cells. To determine if this effect is limited to H727 cells or applicable to other NE tumors, we treated TT cells with same combinations of ZM336372 and U0126 and similar results were observed (Fig 3B). Taken together these results suggest that the reduction in NE markers in ZM336372-treated NET cells is independent of ERK1/2 phosphorylation.

ZM336372 induces raf-1 protein in H727 cells

Earlier we have shown that carcinoid cells treated with ZM336372 resulted in the phosphorylation of raf-1 at Ser³³⁸, which indicated that this system is activated at least at the level of raf-1. To further characterize the effect of ZM336372 on raf-1, we treated H727 cells with small interfering RNA (siRNA) against *raf-1* and then treated with ZM336372. As shown in Fig 3C, cells treated with siRNA against *raf-1* do not have Raf-1 protein and there is no change in the levels of NE markers (ASCL1 and CgA) (lane 3). However, cells treated with ZM336372 alone and combination of both ZM336372 and *raf-1* siRNA showed an increase in the levels of Raf-1 protein suggesting that ZM336372 activates raf-1 at transcriptional level (lanes 2 and 4 respectively). As expected, the levels of NE markers were reduced under both ZM336372 alone or with siRNA treatment.

DISCUSSION

NETs such as MTC and carcinoid tumors represent a diverse group of neoplasms with unique clinical presentations. NE tumors frequently metastasize to distant organs. Endocrinopathies related to the production of various amines and peptides from these neoplasms can result in debilitating symptoms. Surgical resection is the only curative therapy for patients with NETs. However, complete resection is often not possible due to the metastatic nature of the disease. Therefore, there is a great need for the development of novel treatment strategies for patients with NETs. In NET cells, the raf-1 pathway is not expressed or expressed at a very minimal level that could not be detected by western analysis. We have shown that activation of the raf-1 signaling pathway in these cells by over expression of estradiol inducible raf-1 leads to CgA and ASCL1 reduction^{5,7,8,13}.

Though ZM336372, N-[5-(dimethyl-aminobensamido)-2-methylphenyl]-4- hydroxyl benzamide, was originally identified as an inhibitor of raf-1 protein, we and others showed a paradoxical response as raf-1 activator in an in vitro cell culture systems. Furthermore, pharmacological activation of raf-1 by ZM336372, led to NE marker reductions in carcinoid cells^{13,15}. However, the molecular mechanism by which ZM336372 inhibits growth and reduces NE markers is not known. We showed, in the present study, that ZM336372 is capable of reducing tumor growth in TT cells, another NET, suggesting that ZM336372 inhibits growth of several types of NETs. Furthermore, we demonstrated that ZM336372 regulates the GSK-3 β pathway independent of the raf-1 pathway. Importantly, reduction in NE markers is independent of the ERK1/2 or raf-1 pathway. Interestingly, western analysis of raf-1 siRNA treated cells suggested that ZM336372 activates raf-1 at transcriptional level. Figure 4 summarizes the downstream targets of ZM336372.

In summary, this is the first description of a novel compound, ZM336372, that significantly inhibits growth of several cancer cell lines that are representative of wide range of NET phenotypes and does so by regulating multiple pathways. Furthermore, this compound pharmacologically blocks hormone production. In addition, the present study extends our understanding and appreciation of this drug in cancer treatment, particularly its potential role against NETs. In its current formulation ZM336372 would need to be administered systematically. In proposed animal studies, the drug would be given either intravenously or intraperitoneally. Thus, ZM336372 may display significant potential in an *in vivo* setting, but a fundamental question is whether the concentrations of ZM336372 required to regulate these pathways in this study are achievable in the human. Therefore, these results warrant future studies examining the therapeutic and palliative potential of this drug alone or in combination with other drugs for patients with metastatic NETs.

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Figure 1. Effect of ZM336372 on viability of TT cells

The human MTC-TT cells were treated with indicated concentrations of ZM336372 (ZM) for 4, 6 and 8 days and cell viability was determined by MTT assay. There is a dramatic reduction in growth seen at 200 μ M concentrations compared to DMSO treated control cells that are statistically significant at all time points. * $p < 0.05$; ** $p < 0.001$.

Figure 2. ZM336372 regulates multiple pathways in NET cells

Immunoblot to identify the activation of raf-1 pathway and inhibition of the GSK-3β pathway by ZM336372 in NET cells. **A.** Increasing concentrations of ZM336372 treatment of TT cells (**A**) and H727 cells (**B**) lead to the activation of the raf-1 pathway as evidenced by increased levels of phosphorylated ERK1/2 protein. Notably, there is also an increase in the levels of phosphorylated GSK-3β protein at ser 9th position indicating the inactivation of GSK-3β protein. As expected NE markers such as chromogranin A (CgA) and achaete-scute complex like-1 (ASCL1) reduced with treatment. G3PDH was used as loading control.

Figure 3. Reduction in NE markers is independent of ERK1/2 activation by ZM336372
 H727 (**A**) and TT cells (**B**) were pre-treated with 15 μ M U0126 for 45 minutes and then with ZM336372 at 100 μ M concentrations. During the entire treatment period, U0126 was present in the treatment groups. Total cellular extracts were isolated and analyzed by Western blot using antibodies against phospho-ERK1/2, phospho-GSK-3 β , ASCL1, and CgA proteins to determine if Raf-1 pathway activation is required for NE marker reduction. ZM336372 treated cells showed an increase in phosphorylated ERK1/2 whereas similar treatment in the presence of U0126 showed the absence of phosphorylated ERK1/2, indicating that the Raf-1 pathway was successfully blocked by U0126. As expected, in cells treated with ZM336372 alone there was a reduction in NE markers such as ASCL1 and CgA. Interestingly, and perhaps

surprisingly, inhibition of the ERK1/2 pathway did not increase ASCL1 and CgA to normal levels in ZM336372 and U0126 treated cells. (C). To determine the effect of raf-1, H727 cells were treated with siRNA against *raf-1* for 2 days and then measured for the levels of Raf-1, CgA, and ASCL1. As shown in the figure, siRNA against *raf-1* significantly reduced the protein level of Raf-1 whereas an increase in the levels of Raf-1 protein was seen in ZM336372-treated cells. Importantly, both ZM336372 alone or with siRNA against *raf-1* treated cells showed the reduction in the levels of CgA and ASCL1. G3PDH was used as a loading control.

Figure 4.

A schematic representation of the targets evaluated after ZM336372 treatment.