

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

Muthusamy Kunnimalaiyaan, PhD,^{a,b,c} Mary Ndiaye, BS,^{a,b,c} and Herbert Chen, MD,^{a,b,c}
Madison, Wisc

Background. We have shown previously that activation of the Raf-1/mitogen-activated protein kinase (MEK)/extracellular signal-regulated kinase (ERK)1/2 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 neoplasms (NENs).

Methods. NEN cells were treated with ZM336372 (20 to 100 $\mu\text{mol/L}$) 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 NEN cells resulted in increasing raf-1 activation and inactivation of glycogen synthase kinase-3 beta (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 the phosphatidyl inositol 3 kinase 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 chromogranin A and achaete-scute complex like-1 reductions were persistent even after blocking the raf-1 pathway. Treatment with ZM336372 in the presence of small interfering RNA against raf-1 resulted in an increase in Raf-1 production, suggesting that ZM336372 upregulates raf-1 at the transcriptional level.

Conclusion. This is the first description of a novel compound ZM336372 that regulates multiple pathways in NEN cells. (Surgery 2007;142:959-64.)

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

NEUROENDOCRINE NEOPLASMS (NENs), such as carcinoids and medullary thyroid cancer (MTC), produce excess amounts of various bioactive hormones that cause important symptoms. Although operative resection is currently the only potential curative treatment for NENs, 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/achaete-scute complex like-1 (ASCL1), and glycogen synthase kinase-3 beta (GSK-3 β) signaling pathways, play important roles in regulating the growth of NENs.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

Presented at the Annual meeting of the American Association of Endocrine Surgeons, Tucson, AZ, April 29-May 1, 2007.

Supported in part by a Research Scholars Grant from the American Cancer Society, National Institutes of Health grants DK064735, DK066169 and CA109053; the George H.A. Clowes, Jr., Memorial Research Career Development Award of the American College of Surgeons, the Carcinoid Cancer Foundation Award (HC) the University of Wisconsin Medical School Grant (MK); and the Robert Draper Technology Innovation award (MK).

Accepted for publication September 11, 2007.

Reprint requests: Muthusamy Kunnimalaiyaan, PhD, K4/623 Clinical Science Center, 600 Highland Avenue, The University of Wisconsin School of Medicine and Public Health, Madison, WI, 53792. E-mail: kunni@surgery.wisc.edu.

0039-6060/\$ - see front matter

© 2007 Mosby, Inc. All rights reserved.

doi:10.1016/j.surg.2007.09.020

and human MTC (TT) cells, resulted in phosphorylation of GSK-3 β , which is associated with growth inhibition.¹ We have also shown previously that activation of the raf-1 pathway by a pharmacologic compound, ZM336372, inhibits carcinoid cell growth.¹³ 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 increasing the phosphorylation of ERK1/2 in a dose-dependent manner similar to our earlier reports on carcinoid cells.¹³ Treatment of NEN 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 ZM336372 regulates multiple pathways. Importantly, the reduction in the neuroendocrine markers, such as chromogranin A (CgA) and ASCL1, did not change with U0126 treatment. We also demonstrated the progressive upregulation of Raf-1 protein in carcinoid cells treated with ZM336372 in the presence of small interfering RNA (siRNA) against *raf-1*.

MATERIALS AND METHODS

Cell culture. TT and human bronchopulmonary carcinoid (H727) cells were obtained from ATCC and maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 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 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 to 200 μ mol/L) and incubated for different time points as indicated. Control cells were incubated with DMSO, because this was used as a solvent for ZM336372. In the case of the MEK inhibitor study, cells were treated with U0126 at 15 μ mol/L concentrations for 45 minutes before adding ZM336372.

Immunoblot analysis. Total cellular proteins were isolated and the protein concentrations were determined with a bicinchoninic acid assay kit

(Pierce, Rockford, IL) as previously described.³ Denatured cellular extracts (30 μ g) were separated on a 10% SDS-PAGE and Western blot was carried out as previously described.³ Proteins transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH) were blocked in milk (5% nonfat dry milk, 0.05% Tween 20; in 1 \times phosphate-buffered saline) and incubated with primary and secondary antibodies as previously described.³ The following primary antibody dilutions were used: MASH1 for human ASCL1 (1:1,000; BD Pharmingen, San Diego, CA), G3PDH (1:10,000, Trevigen, Gaithersburg, MD), p-ERK1/2 (1:1,000), p-GSK3 β ^{Ser9} (1:1,000), raf-1 (1:1,000, Cell Signaling Technology, Beverly, MA) and CgA (1:1,000, Invitrogen). Primary antibody incubations were kept overnight at 4°C and then, depending on the antibodies, membranes were washed 3 times for 5 minutes or 3 times for 10 minutes. Then the membranes were incubated with a 1:2,000 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 Immun-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 mol/L concentrations of ZM336372 for 6 and 8 days. At each time point, cell viability for the treated cells was determined by thiazolyl blue tetrazolium bromide (MTT; Sigma) assay as previously described.^{11,14} Experiments were performed at least twice.

siRNA assay. To determine the mechanism by which ZM336372 reduces neuroendocrine 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 with Bonferroni post hoc testing was performed using a statistical analysis software package (SPSS version 10.0, SPSS, Chicago, IL). $P < .05$ was considered significant.

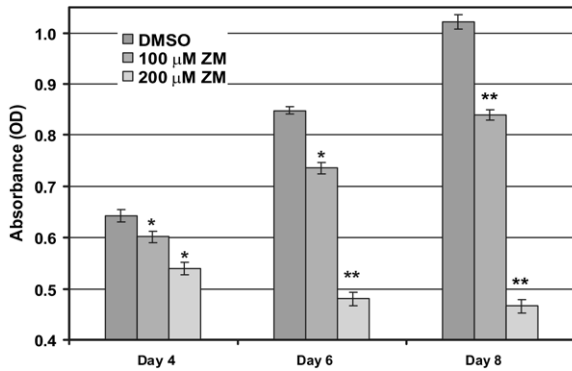


Fig 1. Effect of ZM336372 on viability of TT cells. Human MTC-TT cells were treated with indicated concentrations of ZM336372 (ZM) for 4, 6, and 8 days, and cell viability was determined by MTT assay. There was a dramatic reduction in growth seen at 200 $\mu\text{mol/L}$ concentrations compared with DMSO-treated control cells. * $P > .05$; ** $P > .001$.

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 that was associated with growth inhibition.¹³ Furthermore, we reported recently 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, in 300- $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$), and the results of the cell viability assay are shown in Figure 1. Control experiments contains equal amount of 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 activity.¹³ Cells treated with DMSO showed more growth than cells treated with 100 $\mu\text{mol/L}$ of ZM336372. At the 200 $\mu\text{mol/L}$ concentration of ZM336372, treated cells had more significant growth reduction compared with the 100 $\mu\text{mol/L}$ 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 the 100 $\mu\text{mol/L}$ concentra-

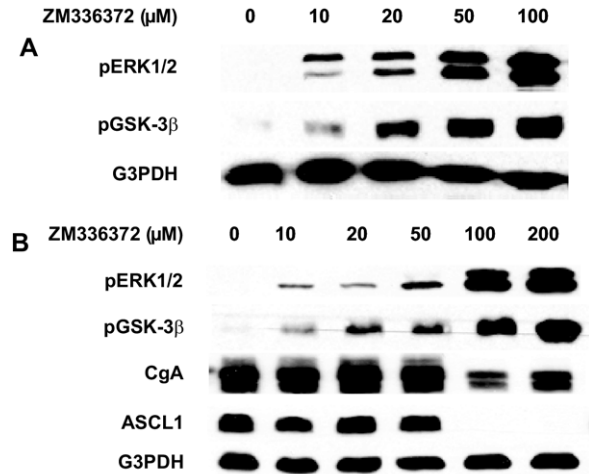


Fig 2. ZM336372 regulates multiple pathways in NEN cells. Immunoblot to identify the activation of raf-1 pathway and inhibition of the GSK-3 β pathway by ZM336372 in NEN cells. A, Increasing concentrations of ZM336372 treatment of TT cells (A) and H727 cells (B) lead to the activation of the raf-1 pathway as evidenced by increased levels of phosphorylated ERK1/2 protein. There was also an increase in the levels of phosphorylated GSK-3 β protein at Ser 9th position, indicating the inactivation of GSK-3 β protein. Neuroendocrine markers such as CgA and ASCL1 decreased with treatment. G3PDH was used as loading control.

tion.¹³ Statistical analysis showed that the growth inhibition is significant at all time points.

ZM336372 activates multiple pathways in NEN 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. Phosphorylation of ERK1/2 is increased progressively with increasing concentrations of ZM336372 (Fig 2, A). These data provide evidence that the raf-1 pathway was activated successfully by ZM336372 in TT cells. Recently, we observed the inactivation of GSK-3 β by phosphorylation at Ser 9th position in raf-1 activated TT cells.¹ Furthermore, we have shown that inactivation of GSK-3 β by pharmacologic inhibitors alone is sufficient to reduce neuroendocrine markers and growth of TT cells.¹ 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 Ser 9th

position was observed with treatment (Fig 2, A); 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 NENs, we treated H727 cells with increasing concentrations of ZM336372 for 2 days and Western analysis was carried out as described in Methods. Treatment with ZM336372 resulted in an increase in both phosphorylated ERK1/2 and GSK-3 β proteins similar to TT cells (Fig 2, B). 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 NEN cells express significant levels of neuroendocrine 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 a reduction in neuroendocrine 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 NEN cells. Therefore, we were interested in determining if both pathways are required to see a decrease in neuroendocrine markers in ZM336372-treated NEN cells. 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, an effect associated with a reduction in neuroendocrine markers such as CgA and ASCL1 (Fig 3, A). Blocking raf-1 activation by U0126, a well-known MEK inhibitor, before ZM336372 treatment led to the absence of phosphorylated ERK1/2 (lane 4). Importantly, there was 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. The levels of CgA and ASCL1 were persistent even after blocking the raf-1 pathway in ZM336372-treated cells. To determine if this effect was limited to H727 cells or applicable to other NENs, we treated TT cells with same combinations of ZM336372 and U0126 and similar results were observed (Fig 3, B). Taken together, these results suggest that the reduction in neuroendocrine markers in ZM336372-treated NEN cells was independent of ERK1/2 phosphorylation.

ZM336372 induces raf-1 protein in H727 cells. We have shown that carcinoid cells treated with ZM336372 resulted in the phosphorylation of raf-1

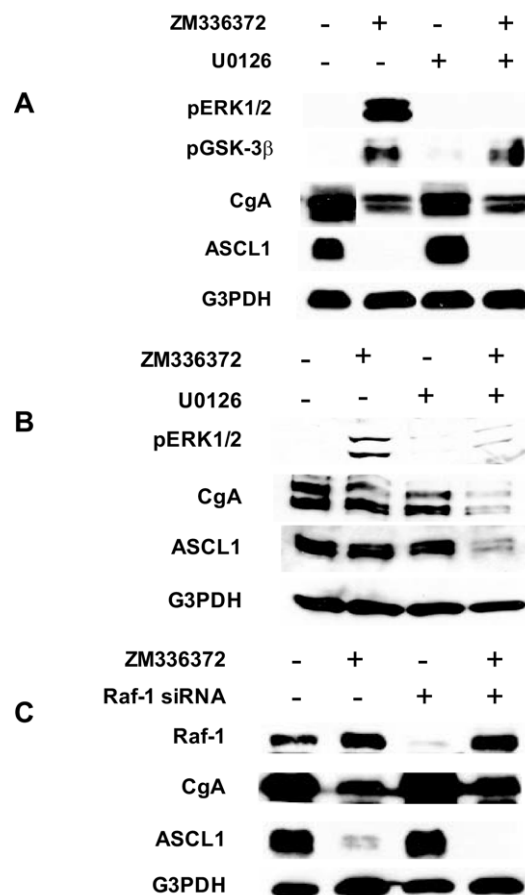


Fig 3. Reduction in neuroendocrine markers was independent of ERK1/2 activation by ZM336372. H727 (A) and TT cells (B) were pretreated with 15 μ mol/L U0126 for 45 minutes and then with ZM336372 at 100 μ mol/L concentrations. During the treatment period, U0126 was present in the treatment groups. Total 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 neuroendocrine 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 blocked successfully by U0126. In cells treated with ZM336372 alone, there was a reduction in neuroendocrine markers such as ASCL1 and CgA. 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. siRNA against *raf-1* reduced the protein level of Raf-1 significantly, 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.

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 siRNA against *raf-1* and then treated them with ZM336372. Cells treated with siRNA against *raf-1* did not have Raf-1 protein, and there was no change in the levels of neuroendocrine markers (ASCL1 and CgA; lane 3; Fig 3, C). Cells treated with ZM336372 alone and combination of both ZM336372 and *raf-1* siRNA, however, 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 neuroendocrine markers were decreased under both ZM336372 alone or with siRNA treatment.

DISCUSSION

NENs such as MTC and carcinoid neoplasms represent a diverse group of neoplasms with unique clinical presentations. NENs metastasize frequently to distant organs. Endocrinopathies related to the production of various amines and peptides from these neoplasms can result in debilitating symptoms. Operative resection is the only curative therapy for patients with NENs, but complete resection is often not possible owing to the metastatic nature of the disease. Therefore, there is a great need for the development of novel treatment strategies for patients with NENs. In NEN 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 overexpression of estradiol-inducible raf-1 leads to CgA and ASCL1 reduction.^{5,7,8,13}

Although ZM336372 was identified originally 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, pharmacologic activation of raf-1 by ZM336372 led to neuroendocrine marker reductions in carcinoid cells.^{13,15} The molecular mechanism by which ZM336372 inhibits growth and reduces neuroendocrine markers, however, is not known. We showed in the present study that ZM336372 is capable of reducing tumor growth in TT cells, another NEN, suggesting that ZM336372 inhibits growth of several types of NENs. Furthermore, we demonstrated that ZM336372 regulates the GSK-3 β pathway independent of the raf-1 pathway. Importantly, reduction in neuroendocrine 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.

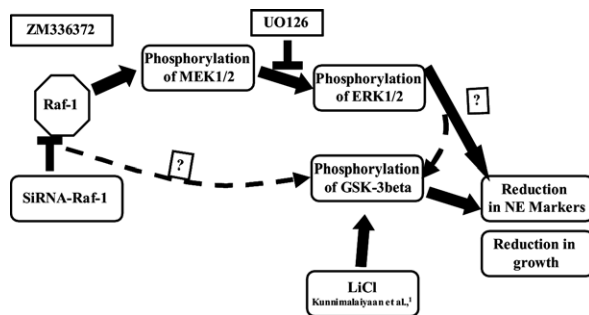


Fig 4. Schematic representation of the targets evaluated after ZM336372 treatment.

In summary, the novel compound, ZM336372, inhibits growth of several cancer cell lines that are representative of a wide range of NEN 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 NENs. In its current formulation, ZM336372 would need to be administered systemically. 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 NENs.

REFERENCES

1. Kunnimalaiyaan M, Vaccaro A, Ndiaye M, Herbert Chen. Inactivation of glycogen synthase kinase-3beta, a downstream target of the raf-1 pathway, is associated with growth suppression in medullary thyroid cancer cells. *Mol Cancer Ther* 2007;6:1151-8.
2. Chen H, Kunnimalaiyaan M, Van Gompel JJ. Medullary thyroid cancer: the functions of raf-1 and human achaete-scute homologue-1. *Thyroid* 2005;15:511-521.
3. Kunnimalaiyaan M, Traeger K, Chen H. Conservation of the Notch1 signaling pathway in gastrointestinal carcinoid cells. *Am J Physiol Gastrointest Liver Physiol* 2005;289:G636-42.
4. Kunnimalaiyaan M, Yan S, Wong F, Zhang YW, Chen H. Hairly Enhancer of Split-1 (HES-1), a Notch1 effector, inhibits the growth of carcinoid tumor cells. *Surgery* 2005;138:1137-42.
5. Kunnimalaiyaan M, Chen H. The Raf-1 pathway: a molecular target for treatment of select neuroendocrine tumors? *Anticancer Drugs* 2006;17:139-42.
6. Nakakura EK, Sriuranpong VR, Kunnimalaiyaan M, Hsiao EC, Schuebel KE, Borges MW, et al. Regulation of neuroendocrine differentiation in gastrointestinal carcinoid tumor cells by notch signaling. *J Clin Endocrinol Metab* 2005;90:4350-6.

7. Sippel RS, Carpenter JE, Kunnimalaiyaan M, Chen H. The role of human achaete-scute homolog-1 in medullary thyroid cancer cells. *Surgery* 2003;134:866-71.
8. Sippel RS, Carpenter JE, Kunnimalaiyaan M, Lagerholm S, Chen H. Raf-1 activation suppresses neuroendocrine marker and hormone levels in human gastrointestinal carcinoid cells. *Am J Physiol Gastrointest Liver Physiol* 2003;285:G245-54.
9. Sippel RS, Chen H. Carcinoid tumors. *Surg Oncol Clin North Am* 2006;15:463-78.
10. Lal A, Chen H. Treatment of advanced carcinoid tumors. *Curr Opin Oncol* 2006;18:9-15.
11. Kunnimalaiyaan M, Ndiaye MA, Chen H. Apoptosis mediated medullary thyroid cancer growth suppression by the PI3K inhibitor LY294002. *Surgery* 2006;140:1009-15.
12. Sriuranpong V, Borges MW, Ravi RK, Arnold DR, Nelkin BD, Baylin SB, et al. Notch signaling induces cell cycle arrest in small cell lung cancer cells. *Cancer Res* 2001;61:3200-5.
13. Van Gompel JJ, Kunnimalaiyaan M, Holen K, Chen H. ZM336372, a Raf-1 activator, suppresses growth and neuroendocrine hormone levels in carcinoid tumor cells. *Mol Cancer Ther* 2005;4:910-7.
14. Kunnimalaiyaan M, Vaccaro AM, Ndiaye MA, Chen H. Over expression of the Notch1 intracellular domain (NICD) inhibits cellular proliferation and alters the neuroendocrine phenotype of medullary thyroid cancer cells. *J Biol Chem* 2006;281:39819-30.
15. Kappes A, Vaccaro A, Kunnimalaiyaan M, Chen H. ZM336372, a Raf-1 activator, inhibits growth of pheochromocytoma cells. *J Surg Res* 2006;133:42-5.

DISCUSSION

Dr Michael J. Demeure (Tucson, Arizona): You mentioned the formulation IP. And I was going to ask you if you did something with xenografts. But can you tell us about the toxicities you see with the agent to know whether it would be possibly used in humans? What toxicities do you see with ZM336372?

Dr Muthusamy Kunnimalaiyaan (Madison, Wisconsin): In a toxicity experiment, we used concentrations up to 300 $\mu\text{mol/L}$ of ZM336372 in TT cells, and at this concentration there was 50% cell death at 2 days. Therefore, we used <300 $\mu\text{mol/L}$ concentration of ZM336372 in our in vitro experiments.

Dr Michael J. Demeure (Tucson, Arizona): So you have not seen the toxicity in your nude mice?

Dr Muthusamy Kunnimalaiyaan (Madison, Wisconsin): No, we have not tested ZM336372 toxicity in xenograft model. However, in our preliminary experiments we treated mice with 1, 2 and 3 molar concentrations, just to see whether the mice had any side effects and we did not find any change in the mice activity.

Dr Sonia L. Sugg (Milwaukee, Wisconsin): I think it is very exciting that you are seeing the pathways being interrupted at multiple places, because, as we know, cancer cells have multiple ways of overcoming growth inhibition. Do you have any idea of the mechanism of how this compound works? Are these pathways that you have been looking at active in other neuroendocrine tumor cells, as well as pheochromocytoma or insulinoma cells?

Dr Muthusamy Kunnimalaiyaan (Madison, Wisconsin): When the NET cells were treated with ZM336372, we observed an increase in the phosphorylation of ERK1/2, suggesting that the Raf-1 pathway is activated by ZM336372 in these cells. Interestingly, we showed here that in addition to the raf-1 pathway activation, inactivation of the GSK3 pathway by phosphorylation of GSK-3 β at Ser 9th position also was seen. However, when the cells were pretreated with a known MEK inhibitor, which blocks the phosphorylation of ERK1/2, inactivation of GSK-3 β was persistent. Moreover, siRNA assay against raf-1 in the presence or absence of ZM336372 indicated that ZM336372 increases the levels of raf-1 protein. These experiments indicated that ZM336372 activates multiple pathways.

Regarding the second question, we have recently shown that treatment of pheochromocytoma cells with ZM336372 resulted in raf-1 activation by an increase in the phosphorylation of ERK1/2 proteins. We have previously shown that neuroendocrine cells so far tested did not show endogenous activation of the raf-1 signaling.

Dr Cord Sturgeon (Chicago, Illinois): You showed that there was growth inhibition with this agent. Do you know anything more about whether this is leading toward apoptosis, perhaps, or necrosis, or some other way that the cells are not proliferating as much?

Dr Muthusamy Kunnimalaiyaan (Madison, Wisconsin): Raf-1 gene activation in TT cells resulted in growth inhibition mediated by cell cycle arrest. In addition, treatment of carcinoid cells with ZM336372 also showed growth reduction mediated through cell cycle arrest by an increase in p21 protein. Therefore, we assume that growth inhibition by ZM336372 in other NET cell line, might also be due to cell cycle arrest.