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Inhibition of Phosphatidylinositol 3-Kinase/Akt Signaling Suppresses Tumor Cell Proliferation and Neuroendocrine Marker Expression in GI Carcinoid Tumors

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Abstract

Background—Over-activation of PI3K/Akt signaling facilitates tumor proliferation in several cancers. We have shown that various signal transduction pathways promote tumorigenesis in carcinoid tumors, which exhibit endogenously high levels of active, phosphorylated Akt. Therefore, we hypothesized that inhibition of the PI3K/Akt pathway would suppress carcinoid tumor cell growth and neuroendocrine (NE) marker production.

Methods—Human carcinoid BON cells were treated *in vitro* with LY294002, a PI3 kinase inhibitor, or transfected with Akt1 siRNA. Tumor cell proliferation was measured by MTT for six days. The effect of LY294002 or Akt1 siRNA treatment was assessed by western analysis. We examined the levels of phosphorylated Akt, total Akt, Akt1, and the NE markers human achaete-scute homolog1 (ASCL1) and chromogranin A (CgA).

Results—Treatment of BON cells with LY294002 reduced tumor cell proliferation (76%) in a dose-dependent manner. Growth also decreased in Akt1 siRNA transfected cells (29%). Levels of active, phosphorylated Akt and the NE tumor markers, ASCL1 and CgA, were diminished with both LY294002 and Akt1 siRNA treatments proportional to the degree of Akt inhibition. Total Akt, Akt2, and Akt3 levels were unaffected by these experiments.

Conclusions—These data indicate that PI3K/Akt signaling performs a critical role in human carcinoid tumor cell survival and NE hormone generation. Furthermore, the development of novel therapeutics targeting Akt1 or components of the PI3K/Akt pathway may enhance the management of carcinoid disease.

Synopsis—Carcinoid tumor cells were treated with a PI3K inhibitor, LY294002, and Akt1 siRNA to delineate the role of PI3K/Akt signaling in carcinoids. The effects of treatment on cellular proliferation and neuroendocrine marker expression were observed.

Keywords

Carcinoid; PI3K; Akt; LY294002; BON

Carcinoid tumors are neuroendocrine malignancies that primarily develop throughout the gastrointestinal (GI) tract and pulmonary system, but can originate in almost any organ in the body. Carcinoids arise from enterochromaffin cells within the GI epithelium and have a reported incidence of approximately 2–3:100,000.^{1,2} While carcinoid tumors are known for their relatively slow growth and indolent disease course, they are the second most common

cause of isolated hepatic metastases after colorectal carcinoma.³ Patients with widespread metastases often suffer from incapacitating symptoms, including diarrhea, flushing, wheezing, skin rashes, and heart failure, that result from the production of excess biogenic amines, neuropeptides, and hormones. Surgical resection is potentially curative; however, a large number of patients present with unresectable disease.³ Therapeutic alternatives including somatostatin analogs, systemic chemotherapy, ablative procedures, hepatic artery embolization, and liver transplantation, may alleviate symptoms or even prolong survival, but are unable to offer patients a cure.^{4–7} Therefore, novel strategies are needed for the treatment and palliation of patients with advanced carcinoid tumors.

The growth, differentiation, phenotypic expression, and hormone production of neuroendocrine neoplasms is dependent upon a network of signaling cascades within and between cells that remain poorly understood. Our group has investigated several molecules involved in signal transduction, including Raf-1^{2,8,9}, Notch1^{10–12}, and Akt¹³, that may provide targets for the development of new therapies for patients with neuroendocrine tumors. Manipulation of these pathways provides promising approaches for the future.

The PI3K/Akt pathway is hyperactive and genetically selected for during tumorigenesis in a variety of cancers, including ovarian, breast, and colon.¹³ The normal cellular functions that are regulated by PI3K/Akt signaling, such as cell survival, proliferation, growth, and motility, are recruited and exploited by developing malignancies. The phosphatidylinositol 3-kinase (PI3K) phosphorylates and activates Akt, which, in turn, phosphorylates or interacts with a number of downstream substrates including Bad, various caspases, and Forkhead transcription factors among others. One mechanism by which PI3K signaling is turned off is through the actions of PTEN, a well-known tumor suppressor. Loss of function mutations of PTEN lead to activation of Akt.¹⁴ Furthermore, in one study, 54% of poorly differentiated neuroendocrine carcinomas were found to have lost PTEN expression.¹⁵

While PI3K/Akt up-regulation has been established in several cancer types including other NE neoplasms,¹³ the role of this signaling pathway has yet to be elucidated in carcinoid tumors. Moreover, the mechanism by which downstream mediators effect cell growth and survival is still being studied. In addition, active, phosphorylated Akt is known to be present in human carcinoid tumor cells.¹⁶ Therefore, we hypothesized that inhibition of the PI3K/Akt pathways would suppress carcinoid tumor cell growth and NE marker production. To study the effects of PI3K/Akt inhibition, we used the BON cell line, an established human gastrointestinal (GI) carcinoid cell line derived from a pancreatic carcinoid metastasis.¹⁷

We inhibited PI3K/Akt signaling through two different mechanisms. First, we utilized the well known PI3K inhibitor, LY294002,¹⁸ which acts by binding the p110 catalytic subunit of the enzyme and preventing Akt phosphorylation. LY294002 is a flavanoid derivative that works through reversible, competitive ATP inhibition. We also inhibited the PI3K/Akt pathway by transfecting cells with small interfering RNA (siRNA) targeted at Akt1, the predominant isoform in most tissues. In this study, we confirm that BON cells express elevated levels of active, phosphorylated Akt at baseline. To demonstrate the role of PI3K/Akt signaling in tumor cell proliferation, we show that inhibition of this pathway with either LY294002 or Akt1 siRNA leads to suppression of cellular growth. We also determined that these treatments cause reduction in ascl1, a transcription factor that regulates the neuroendocrine phenotype, and chromogranin A, a neuroendocrine tumor markers.

MATERIALS AND METHODS

The experimental protocols utilized were approved by the University of Wisconsin institutional review board and meet the all appropriate guidelines.

Cell Culture

The human GI carcinoid cell line, BON, was obtained from Drs. B. Mark Evers and Courtney M. Townsend, Jr. (University of Texas Medical Branch, Galveston, TX) and maintained in Dulbecco's modified Eagle medium-nutrient mixture Ham's F-12K (DMEM/F12K, 1:1, Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Sigma, St. Louis, MO), 100 IU/ml penicillin and 100 µg/ml streptomycin (Invitrogen) in a humidified atmosphere of 5% CO₂ at 37°C as previously described.^{2,9,10}

LY294002 Treatment

LY294002 (Promega, Madison, WI) was resuspended in dimethyl sulfoxide (DMSO, Sigma) producing a 50 mM stock. BON cells were plated onto 100-cm² dishes. After 24 hours, the cells were washed with phosphate buffered saline and the media changed to phenol-free media containing various concentrations of LY294002 ranging from 0–100 µM. Control cells were treated with solvent (DMSO). The cells were then incubated for 48 hours.

Akt1 siRNA Transfection

Non-specific and Akt1 siRNAs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), while the transfection reagent LipofectamineTM2000 was obtained from the Invitrogen Corporation. BON cells were plated into 6-well plates at a confluence of approximately 30% to optimize transfection conditions. The following day cells were washed with phosphate buffered saline, and the media was changed to 500 µL serum-free RPMI 1640 (Invitrogen). RNA-LipofectamineTM 2000 complexes were created and transfected per the manufacturer's instructions. LipofectamineTM 2000 alone also was used as a control. The concentrations used for both Akt1 and non-specific siRNA were 75 nM based on dose-finding experiments. After 24 hours of incubation, the medium was changed to the standard medium. Cellular extracts were isolated every 2 days for up to 6 days.

Cellular Proliferation Assay

To measure BON cell growth, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) rapid calorimetric assay (Sigma) was utilized. BON cells were plated onto 24-well plates. After 24 hours, the cells were treated with varying concentrations of LY294002 (0–100 µM) in quadruplicate. Every 2 days, the medium was changed with the same concentrations of LY294002 for a total of 6 days. For siRNA transfection, BON cells were initially plated in 100-cm² dishes and transfected with 75nM of either non-specific or Akt1 siRNA. Twenty-four hours after transfection, the medium was changed to phenol-free media and cells were plated in quadruplicate onto 24-well plates. The medium was changed every 2 days without washing the cells. For both the LY294002 and siRNA treatments, the MTT assay was performed every 2 days by removing the standard medium and replacing it with 250 µL of serum-free media containing a 0.5 mg/mL concentration of MTT. After incubation at 37 °C for 4 hours, 750 µL of DMSO (Sigma) was added to each well and gently mixed. Absorbance was then measured at a wavelength of 540 nm using a spectrometer (µQuant; Bio-Tek Instruments, Winooski, VT).

Western Blot Analysis

Whole cell extracts were isolated and prepared as previously described.² A bicinchoninic acid assay kit (Pierce Biotechnology, Rockford, IL) was utilized to quantify the

concentrations of the proteins. Thirty to 40 µg of cell extracts were then separated by gel electrophoresis on NuPAGE® Novex® Bis-Tris 10% Mini Gels (Invitrogen) per manufacturers specifications, transferred onto nitrocellulose membranes (BioRad Laboratories, Hercules, CA), blocked in a milk solution (5% non-fat dry milk and 0.05% Tween 20 in 1x phosphate buffered saline), and then incubated with the primary antibodies overnight at 4°C. The dilutions of primary antibodies used were: 1:1000 for Akt, pAkt, Akt1, Akt2, Akt3, cleaved caspase-3, poly(ADP-ribose)polymerase (PARP) (Cell Signaling Technology, Beverly, MA), mammalian achaete-scute homolog 1 (ASCL1; BD Biosciences, San Diego, CA), chromogranin A (Zymed Laboratories, San Francisco, CA); and 1:10,000 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Trevigen, Gaithersburg, MD). After overnight incubation in the primary antibody, membranes were washed 3 times for either 5 or 10 minutes depending upon the antibody. Next, we incubated the membranes with a 1:2000–3000 dilution of horseradish peroxidase-conjugated goat anti-rabbit (Akt, pAkt, CgA, GAPDH) or a 1:200 dilution of goat anti-mouse secondary (Pierce). Immunstar (Bio-Rad) or SuperSignal West Femto (Pierce) kit were then used according to the manufacturer's instruction for visualization of the protein signal.

Statistical Analysis

A *P* value of <0.05 was considered significant. Student's *t*-test was used for statistical comparisons between treatment groups (SPSS, version 10.0, SPSS Inc, Chicago, IL).

RESULTS

LY294002 inhibited Akt phosphorylation and suppressed tumor marker expression in BON cells

BON cells were treated with LY294002 (0–100 µM) as described in the methods in order to determine the role of PI3K/Akt signaling in GI carcinoid tumors. Preliminary studies showed that the mean inhibitory concentration (IC₅₀) of LY294002 in BON cells was less than 100 µM at 2 days and approximately 75µM at 4 days (data not shown). Based on these data, concentrations of LY294002 were chosen for 2 day treatments (up to 100 µM) to stay below the IC₅₀ for this agent. The IC₅₀ in BON cells was higher than has been previously reported in other cell lines.¹³ Phosphorylation of Akt at serine 473 was measured by western blot analysis as a marker of Akt activation. We observed a dose-dependent decrease in the levels of Akt phosphorylation with LY294002 treatment as illustrated in Figure 1A. This observation demonstrated that PI3K inhibition by LY294002 was successful. In addition, the expression of total Akt was not altered by PI3K inhibition (Figure 1A).

We have previously demonstrated that the neuroendocrine markers ASCL1 and chromogranin A are expressed in BON cells.^{9–12} ASCL1, also referred to as hASH1, is a helix-loop-helix transcription factor that is known to regulate the neuroendocrine phenotype in GI carcinoid cells.^{9,10,12} Chromogranin A is a well-known biomarker secreted by carcinoids that is useful in the diagnosis and monitoring of patients with GI carcinoid tumors. We sought to establish the effects of PI3K inhibition in BON cells on these neuroendocrine markers. Treatment of BON cells with increasing concentrations of LY294002 reduced the detectable protein levels of ASCL1 in a manner directly proportional to the amount of PI3K inhibition (Figure 1B). At a concentration of 100 µM LY294002, the degree of ASCL1 expression was undetectable. Suppression of chromogranin A levels also was seen with LY294002 treatment in a manner similar to ASCL1 (Figure 1B). However, low levels of chromogranin A expression were still observed even at the highest concentrations of LY294002 used.

PI3K inhibition decreased GI carcinoid tumor cell proliferation in vitro

Previous reports of PI3K/Akt pathway inhibition in medullary thyroid cancer, another neuroendocrine tumor, and other cancer types have indicated that this signaling mechanism is important for cell growth.^{13,19} We performed a MTT cellular proliferation assay to examine the effects of LY294002 on cell growth. Figure 2 shows the results of our MTT assay for LY294002 treatment of BON cells over a 6 day time period. BON cell growth was reduced with progressively increasing concentrations of LY294002. At 6 days, significant reductions in cell growth were seen at all time points. We observed 10, 13, and 11% growth reductions at the lowest concentration of LY294002, 10 μ M, at 2, 4, and 6 days, respectively ($p < 0.006$ vs control for all time points). At the highest concentration of LY294002 (100 μ M), cellular proliferation was suppressed by 76% at 6 days ($p < 0.0001$ vs control).

The mechanism of LY294002 growth suppression

After demonstrating that suppression of BON cell growth occurs with LY294002 treatment *in vitro*, we wanted to examine the mechanism of this inhibitory effect. Western blot analysis for various markers of cell cycle arrest and apoptosis was performed. As shown in Figure 3A, we observed increased p27^{Kip1} expression and decreased levels of cyclin D1. p27^{Kip1} is a cyclin-dependent kinase (CDK) inhibitor that regulates progression through the cell cycle and is specifically associated with G1-phase cell cycle arrest.²⁰ Cyclin D1 also is involved in cell cycle progression and is known to undergo degradation when G1-phase arrest occurs.²⁰ We also examined PARP, a well-known marker for apoptosis, and caspase-3 which plays a role in the execution of apoptosis. Treatment of BON cells with LY294002 did not lead to activation of caspase-3 (cleaved form) or PARP (cleaved) (Figure 3B). Taken together, these findings suggested that the observed inhibition of growth was not due to apoptosis, but likely the result of cell cycle arrest.

The role of Akt1 in the expression of the neuroendocrine phenotype in GI carcinoid cells

Inhibition of PI3K by LY294002 resulted in decreased phosphorylation of Akt at serine 473 and reduced ASCL1 and chromogranin A levels in BON cells. However, Akt has 3 different isoforms, Akt1, 2, and 3 or alpha, beta, and gamma, respectively, and the antibody used in our experiments detects all three isoforms. Akt1 is the predominant isoform in the body, while Akt2 is mainly found in tissues that respond to insulin, such as fat, muscle, and liver, and Akt3 is most abundant in the brain. Previous reports have indicated that Akt1 expression is important in the development of several neoplasias and is the most prevalent isoform in the intestine.^{21–23} Therefore, we were interested in determining the role of Akt1 in the expression of neuroendocrine markers in BON cells. RNA interference utilizing Akt1 siRNA allowed us to specifically block mRNA translation of the Akt1 protein. As shown in Figure 4, transient transfection of Akt1 siRNA in BON cells decreased the levels of Akt1 proteins at 2, 4, and 6 days after transfection with little to no effect on Akt2 or Akt3. Thus, our treatment was very specific for Akt1. We were unable to completely knockout Akt1 expression with our transient siRNA treatment; however, significantly reduced levels of Akt1 were observed by western blot compared to the two control groups, lipofectamine alone (Lipo) and non-specific (NS) siRNA. When the degree of Akt1 protein expression at 2 and 6 days was compared grossly, it appeared that the effects of transfection were less prominent at 6 days likely due to our transfection being transient.

Next, we examined the impact of Akt1 RNA interference on the expression of the neuroendocrine markers ASCL1 and chromogranin A. Protein levels of ASCL1 were decreased by the Akt1 siRNA transfection at 2, 4, and 6 days (Figure 4). These levels correlated with those observed for Akt1 protein. As with ASCL1, transfection of BON cells with Akt1 siRNA suppressed expression of chromogranin A (Figure 4). The decreased expression of chromogranin A was not as dramatic as ASCL1, but was similar to that

observed with LY294002 treatment. Taken together, these results indicated that Akt1 is important to the neuroendocrine phenotype of GI carcinoid cells.

Akt1 siRNA inhibited GI carcinoid cancer cell proliferation

In addition to demonstrating that Akt1 plays a role in the expression of neuroendocrine markers, we sought to define the role of Akt1 on tumor cell growth. A MTT cellular proliferation assay was performed over 6 days on BON cells transfected with 75 nM/L Akt1 siRNA. After transfection, BON cells were plated either for MTT or for protein analysis by western blots (discussed above) in order to confirm that the siRNA transfection was successful. Figure 5 shows that inhibition of Akt1 protein production resulted in growth inhibition of BON cells. At 6 days, cellular growth of Akt1 siRNA treated cells was reduced by 26% compared to control (lipofectamine, $p < 0.0001$). Our MTT data indicated the importance of Akt1 in the proliferation of GI carcinoid cells *in vitro*.

DISCUSSION

Signal transduction via the PI3K/Akt pathway has been shown to facilitate tumor cell proliferation in several cancers, including the neuroendocrine cell line, TT, a medullary thyroid cancer line.^{13,19,23} In addition, GI carcinoid tumors have been shown to express activated Akt and have loss of PTEN expression, a negative regulator of PI3K.^{15,16} However, pharmacologic inhibition of PI3K in GI carcinoid tumors has not previously been investigated. The lack of effective treatment options for patients with carcinoid tumors led us to explore the effects of PI3K inhibition in BON cells, a human GI carcinoid cell line. Knowing that suppression of PI3K pathway signaling in medullary thyroid cancer, TT, cells leads to reductions in cellular growth and neuroendocrine marker production, we hypothesized that inhibition of this pathway by LY294002 in GI carcinoid cells would have similar effects to those observed in TT cells.¹³

In this study, we found that treatment of BON cells with LY294002, a PI3K inhibitor, reduces levels of active, phosphorylated Akt in a dose-dependent manner as predicted (Figure 1A). This reduction of phosphorylated Akt directly correlates with suppression of ASCL1 and the neuroendocrine tumor marker chromogranin A (Figure 1B). This result confirms our earlier reports that neuroendocrine markers are reduced in medullary thyroid cancer cells when PI3K/Akt signaling is inhibited.¹³ The mechanism of chromogranin A reduction is thought to be secondary to inhibition of the ASCL1 transcription factor, because knockdown of ASCL1 with siRNA causes a reduction in chromogranin A.¹² Other authors have shown that the neuroendocrine differentiation of prostate cancer cells is dependent on the activation of Akt.²⁵ These findings indicate that PI3K/Akt signal transduction may be important in determining the neuroendocrine phenotype of GI carcinoid and other cancers. Previous reports from our lab have suggested that Raf-1 and Notch1 signaling also are involved in expression of the neuroendocrine phenotype and proliferation.^{8–11} Inhibition of Akt through LY294002 does not activate Raf-1 or inhibit Notch1 (data not shown). Thus, the effects observed in this study appear to be independent of these pathways at this level. Further investigation is needed to examine whether crosstalk occurs up or downstream.

Our results also show that the growth of human GI carcinoid BON cells is significantly inhibited by LY294002 treatment (Figure 2). Furthermore, western blot analysis suggests that cell cycle arrest is the mechanism by which cellular proliferation is suppressed (Figure 3). Previously, von Wichert and colleagues reported that constitutive expression of cyclin D1 in BON cells appears to be dependent on PI3K.²⁵ In addition, inhibition of PI3K in breast cancer cells has been shown to decrease levels of cyclin D1.²⁶ Our data support these findings since we show that PI3K inhibition leads to the downregulation of cyclin D1 protein levels (Figure 3). Our finding of decreased cellular proliferation via PI3K inhibition in BON

cells is consistent with results reported in medullary thyroid cancer TT cells.¹³ However, the mechanism of growth inhibition in TT cells appears to be apoptotic.¹³ In the present study, we did not find evidence that Akt inhibition resulted in apoptosis. Therefore, the method by which cellular proliferation is reduced in neuroendocrine cells, such as BON or TT, appears to be cell type dependent.

An additional finding of our study is that *Akt1*, specifically, appears to be activated in human GI carcinoid cancers. We report here that Akt1 mRNA inhibition by siRNA causes decreased expression of neuroendocrine tumor markers and cell growth suppression in BON cells (Figures 4 and 5). Previously, Liu et al. demonstrated that *Akt1* plays an essential role in increased proliferation and resistance to apoptosis in breast cancer cells.²² In a murine model, *Akt1* knockout also has been implicated in the development of several other tumors.²¹ These observations provide preliminary evidence for therapeutically targeting *Akt1* in patients with GI carcinoid tumors; however, future studies are needed to fully delineate the role of *Akt1*. Furthermore, the roles of *Akt2* and *Akt3* warrant additional investigation.

In several cancers, activation of Akt and its various isoforms has been shown to be critical in tumorigenesis.¹⁴ We show here that Akt signaling is important for neuroendocrine tumor marker expression and cell growth inhibition by cell cycle arrest in GI carcinoid cancer cells. In particular, *Akt1*, a specific isoform of Akt, is involved in these activities—expression of neuroendocrine markers and cellular proliferation. We cannot exclude the possibility, however, that other Akt isoforms may also be active in this process. Nevertheless, we conclude that inhibition of *Akt1* or components the PI3K/Akt pathway may enhance the management of patients with GI carcinoid tumors.

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FIG 1.

Western blot analysis depicting inhibition of PI3K/Akt signaling by LY294002 at 48 hours and resultant dose-dependent reduction in the levels of active, phosphorylated Akt (pAkt) with no effect on total Akt (**A**). PI3K inhibition by LY294002 also led to a reduction in the neuroendocrine tumor markers, ASCL1 and chromogranin A (CgA) that was proportional to the dose of LY294002 (**B**).

FIG 2. MTT growth assay showing dose-dependent inhibition of GI carcinoid cell growth *in vitro*. BON cells were treated with the indicated concentrations of LY294002 treatment over a 6 day time period.

FIG 3.

LY294002-mediated growth inhibition occurs through cell cycle arrest. BON cells were treated with a PI3K inhibitor for 48 hours and Western blot analysis performed on whole cell lysates. LY294002 induced expression of the cyclin-dependent kinase p27 and down regulated cyclin D1, suggesting cell cycle arrest at G1/S phase (**A**). Inhibition of Akt in BON cells did not result in cleavage of poly(ADP-ribose)polymerase or caspase-3 which suggested that apoptosis was not occurring. A positive control was used (**B**). GAPDH was used as a loading control.

FIG 4.

Transfection of BON cells with Akt1 siRNA led to decreased Akt1 protein production as well as reduced levels of the neuroendocrine tumor markers ASCL1 and chromogranin (CgA) by Western blot (A). The transfection reagent (lipofectamine, abbreviated Lipo) alone and non-specific (NS) siRNA were used as controls. Cells were transfected at day 0 and cells isolated every 2 days for 6 days. Akt1 siRNA was very specific for Akt1 and had little to no effect on the other Akt isoforms, Akt2 and Akt3 by Western blot analysis (B).

FIG 5.

Akt1 knockdown suppressed growth of GI carcinoid cancer cells *in vitro*. Transfection of *Akt1* siRNA was performed at day 0 and cell viability measured by MTT growth assay every 2 days for up to 6 days. Lipofectamine, the transfection reagent, and non-specific (NS) siRNA were used for controls.