Antitumor activity of rapamycin and octreotide as single agents or in combination in neuroendocrine tumors

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Abstract

The mammalian target of rapamycin (mTOR) signaling pathway has emerged as a promising target for cancer therapy. Rapamycin inhibits mTOR activity but induces upstream signaling, leading to Akt activation, potentially limiting antitumor activity. Octreotide, a somatostatin analog, decreases phosphatidylinositol-3-kinase/Akt signaling in some models, and thus theoretically may enhance rapamycin's antitumor activity. The aim of this study was to determine the antitumor activity of rapamycin and octreotide as single agents and in combination in neuroendocrine tumors. In carcinoid cell lines BON-1 and NCI-H727, cell proliferation was significantly inhibited by rapamycin *in vitro*, although rapamycin treatment did lead to Akt phosphorylation. Octreotide had limited antiproliferative effects alone, and did not demonstrate synergistic or additive interactions with rapamycin. Furthermore, octreotide did not overcome rapamycin-induced Akt phosphorylation. *In vivo*, rapamycin alone caused significant tumor suppression. Octreotide alone did not inhibit *in vivo* tumor growth and did not enhance rapamycin-mediated growth inhibition. In conclusion, rapamycin causes significant growth inhibition in carcinoid tumor cell lines *in vitro* and *in vivo*, thus mTOR is a promising therapeutic target for neuroendocrine tumors. Octreotide does not enhance the efficacy of rapamycin's antiproliferative effects in the models tested, and does not inhibit rapamycin-mediated feedback activation of Akt. Further study is needed in order to determine whether octreotide or other somatostatin analogs enhance the efficacy of mTOR inhibitors in other models.

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Introduction

Carcinoid tumors are rare neuroendocrine tumors, often characterized by symptoms due to hypersecretion of various bioamines and neuropeptides. Systemic therapies for carcinoid tumors have had limited antitumor efficacy to date. Since most carcinoid tumors highly express somatostatin receptors, treatment of gastrointestinal carcinoid tumors has largely involved somatostatin analogs or the combination of various chemotherapeutic agents. Although somatostatin analogs such as octreotide are generally well tolerated and effectively ameliorate many symptoms caused by neuroendocrine tumors, their antitumor efficacy is limited (Wymenga *et al*. 1999, Ricci *et al*. 2000, Tomassetti *et al*. 2000, Dogliotti *et al*. 2001).

The phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway has recently emerged as a potential target for cancer therapy. mTOR signaling is activated in many tumor types. The activated mTOR kinase in a complex with raptor (mTORC1) leads to the phosphorylation of ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), two key proteins that regulate protein translation of several proteins necessary for cellular proliferation, and growth (Dancey 2006). Rapamycin and its analogs bind immunophilin FK506-binding protein 12, and inhibit mTOR signaling.

The antitumor efficacy of rapamycin and its analogs are being actively studied in many tumor types. mTOR

has already been validated as a therapeutic target in advanced renal cell carcinoma. Rapamycin analog temsirolimus (CCI-779 (Torisel), Wyeth, Collegeville, PA, USA) has been shown to improve overall survival among patients with metastatic renal cell carcinoma (Hudes *et al.* 2007), leading to the FDA approval of temsirolimus. However, mTOR inhibitors have shown only modest clinical activity for most tumor types in single agent therapy. Sequential biopsies in patients treated in the phase I trial of mTOR inhibitors have determined that mTOR inhibition induces upstream receptor tyrosine kinase signaling, and phosphorylation (activation) of Akt, a mechanism that may potentially limit its antitumor activity (O'Reilly *et al.* 2006). Octreotide has been reported to alter PI3K signaling and decrease Akt phosphorylation (Charland *et al.* 2001, Theodoropoulou *et al.* 2006). Thus, it may be rational to use octreotide in combination with mTOR inhibitors to overcome the feedback loop activation observed with single agent mTOR inhibitor therapy. Indeed, rapamycin analogs in combination with octreotide are undergoing clinical trials in neuroendocrine tumors with preliminary evidence of clinical activity (Yao 2007, Yao *et al.* 2007).

The objective of this study was to determine whether rapamycin has antitumor activity in carcinoid cancer cells and whether octreotide enhances rapamycin's effects. Here, we report that *in vitro*, rapamycin treatment as a single agent leads to a significant reduction in cell proliferation and that rapamycin alters the PI3/Akt signaling pathway by inhibiting S6K1 and 4E-BP1 phosphorylation with feedback loop Akt activation in both carcinoid cell lines. *In vitro*, octreotide showed little or no growth inhibitory activity in NCI-H727 and BON cells respectively. Octreotide also did not significantly enhance rapamycin's antiproliferative effects. Furthermore, rapamycin's effect on cell signaling, primarily the treatment-induced Akt phosphorylation in both BON and H727 cells, was not affected by the presence of octreotide. *In vivo*, rapamycin alone caused significant tumor suppression while octreotide produced no significant tumor remission alone in combination with rapamycin. However, the evidence of clinical activity in a phase II study of octreotide and rapamycin analog everolimus (RAD001, Novartis, Basel, Switzerland) call for further studies to determine whether octreotide or other somatostatin analogs enhance the efficacy of various mTOR inhibitors in other models. Overall, these results may provide an insight on patient eligibility for combination treatments and in predicting treatment sensitivity.

Materials and methods

Cell culture

The human pancreatic carcinoid-derived cell line, BON-1, was a kind gift from Dr Courtney M Townsend, Jr (University of Texas Medical Branch, Galveston). NCI-H727, a human bronchial carcinoid tumor cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). BON and H727 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Mediatech Inc., Herndon, VA, USA) supplemented with 10% fetal bovine serum (SAFC Biosciences, Lenexa, KA, USA) in a humidified atmosphere of 5% CO_2 at 37 °C.

Reagents

Rapamycin was purchased from LC Labs (Woburn, MA, USA). Octreotide acetate (Bedford Laboratories, Bedford, OH, USA) was provided as a stock solution of 1 mg/ml in acetate buffer (pH 4.2). Sandostatin long acting release (LAR) Depot (octreotide acetate for injectable suspension) was purchased from Novartis (East Hanover, NJ, USA) and used for *in vivo* experiments. Antibodies against Akt, phospho-Akt (Ser473), S6K, phospho-S6K (Thr389), 4E-BP1, and phospho-4E-BP1 (Cell Signaling Technologies, Beverly, MA, USA) were used at a 1:1000 dilution and β-actin (Sigma Chemical Co) at a 1:5000 dilution. All other chemicals were purchased from Sigma Chemical Co. and Fisher Scientific Company LLC (Pittsburg, PA, USA).

Western blot analysis

Cells were washed with cold PBS and lysed in lysis buffer as described elsewhere (Mondesire *et al.* 2004). Protein (50 μg) was separated by 10% SDS-PAGE and was transferred to a 0.2 μm polyvinylidene difluoride membrane (Bio-Rad Laboratories) for 1 h at 100 V. The membranes were blocked with 5% nonfat dry milk or with 0.1% casein in Tris-buffered saline with Tween 20 (TBS-T), and immunoblotted with primary antibodies overnight at 4 °C. After primary antibody incubation, membranes were washed 3×5 min in TBS-T wash buffer and incubated for 1 h at room temperature with secondary antibodies. Membranes were visualized by the Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE, USA).

Cell proliferation assays

To test the effect of rapamycin and octreotide on cell proliferation as single or combined agents, cells were

plated into 96-well flat-bottomed plates. After overnight incubation, triplicate or quadriplicate wells were treated with varying concentrations of rapamycin and octreotide alone or in combination for 4 days. Cell survival was determined by either of two methods: by the relative percentages of metabolically active cells compared with untreated controls on the basis of mitochondrial conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazine or alternately, by the protein content of treated and untreated cells through the sulforhodamine-B (SRB) assay. Results were assessed in a 96-well format plate reader by measuring the absorbance at a wavelength of 570 nm ($A_{570\,nm}$) for MTT plates and at 490 nm ($A_{490\,nm}$) for SRB plates.

Direct cell counts

Cells were plated into four six-well flat-bottomed plates at 2×10^5 cells/3 ml per well. After overnight incubation, triplicate wells in each plate were treated with dimethyl sulfoxide (DMSO) or 100 nM rapamycin. Upon treatment, one plate was harvested every 24 h by collecting the media and washing the cells with PBS, followed by 5-min incubation in 0.5 ml trypsin. The harvested cells were added to collected media and centrifuged at 1000 g for 5 min at 4 °C. The supernatant was removed and pellet suspended into DMEM/F12. Samples of cell suspension were diluted 1:2 into 0.4% Trypan blue solution, and the number of viable cells was determined.

Animal studies

Male BALB/c mice at 4 weeks of age were obtained from the Department of Experimental Oncology at The University of Texas MD Anderson Cancer Center. A total of 69 mice were each injected s.c. in the upper flank with 2×10^7 BON cells suspended in 200 µl PBS. After 8 days, when average tumor size was 120 mm³, the mice were separated into six groups (12 mice/group for treatment groups and 11 mice/group for control groups) and were randomly assigned to treatment with: Sandostatin LAR Depot (octreotide), s.c. at 30 mg/kg; rapamycin, i.p. at 15 mg/kg; octreotide and rapamycin; octreotide diluent (octreotide control), s.c.; DMSO (rapamycin control), i.p; or DMSO and octreotide diluent. Octreotide and octreotide control injections were given only once on the first day of treatment (day 8), and rapamycin and DMSO injections were given once a week starting the same day, i.p. to the corresponding groups. Tumor growth was followed every 2 days by caliper measurements, and tumor volumes were calculated as previously described

(Mondesire *et al*. 2004). Mice were killed on day 30. Whole blood samples were collected on day 30, and blood samples were then transferred into BD Microtainer tubes containing EDTA (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and centrifuged at 6000 g for 10 min at room temperature. After separation, plasma samples were obtained from a total of 18 mice. Samples of three mice each were pooled for assessment of octreotide levels. Octreotide levels were determined by the Inter Science Institute (Inglewood, CA, USA).

Statistical analysis

Results were presented as mean $\pm$ s.d. for cell proliferation experiments. Differences between treatment groups for *in vitro* data were assessed by ANOVA with appropriate linear contrasts. Differences between treatment groups for *in vivo* data on day 30 were assessed by the nonparametric Kruskal–Wallis test. A nonparametric multiple comparisons test was used with a Bonferroni correction to the significance level for multiple testing. The Wilcoxon signed-rank test was used to determine whether there were differences in tumor size distribution between each experimental treatment and its control group. Statistical analysis was performed using SAS version 9.1 statistical software (Copyright © 2002–2003 by SAS Institute Inc., Cary, NC, USA) and STATA/SE version 9.0 statistical software (Copyright © 1984–2005 by Stata. Corp., College Station, TX, USA).

Results

Rapamycin significantly inhibits cell proliferation in BON and H727 cells

In order to test rapamycin's growth inhibitory effect in neuroendocrine tumors, the human-derived neuroendocrine tumor cell line BON which exhibits a constitutively active Akt pathway and expresses somatostatin receptors 2 and 5, along with insulin-like growth factor-I (IGF-I) receptors, was treated with vehicle DMSO or with serial dilutions of rapamycin. After 4 days of exposure, cell growth was measured by MTT assay, a measure of mitochondrial activity, or SRB assay, a measure of protein content. The percentage of growth inhibition was calculated and standardized to that of untreated controls. The MTT assay showed significant growth inhibition of BON cells after rapamycin treatment (Fig. 1A). This growth inhibition caused by rapamycin treatment was also apparent in BON cells through SRB assay (Fig. 1B). The effect of rapamycin on BON cell proliferation was further evaluated through
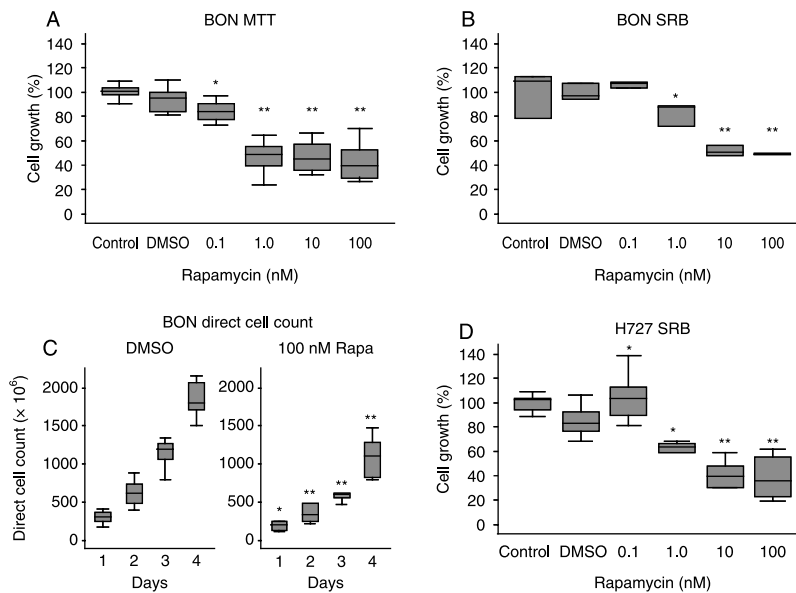
Figure 1 Rapamycin significantly inhibits cell proliferation in BON and H727 cells. (A) BON cells were plate treated with rapamycin at varying concentrations for 4 days. Cell growth was determined afterwards by MTT assay in three independent experiments. (B) BON cells were treated with increasing concentrations of rapamycin for 4 days. Experiment done in triplicate wells and growth inhibition was determined by an SRB assay. (C) BON cells were plated into six-well plates at 2×10^5 cells/well. After overnight incubation, triplicate wells were treated with either DMSO or 100 nM rapamycin. Control and rapamycin-treated cells were harvested every 24 h, and viable cell count was determined in two independent experiments. (D) H727 cells were treated with rapamycin for 4 days, and dosage-dependent cell growth inhibition was determined by an MTT assay in two independent experiments. In each case, the cell growth was normalized to untreated controls and compared with vehicle controls. The mid-line of each box is at the median for the data represented by that box. The upper and lower edges of each box mark the 75th and 25th percentiles of the data respectively. The upper and lower bars mark the 95th and 5th percentiles of the data respectively. $*P < 0.05$ and $**P < 0.0001$.

a direct cell count. BON cells were treated with either DMSO or 100 nM rapamycin and harvested every 24 h up to 4 days and the number of viable cells was determined through Trypan blue exclusion. Treatment with rapamycin began to significantly inhibit cell proliferation by 24 h of treatment (Fig. 1C). Rapamycin treatment of H727 cells, a human bronchial carcinoid cell line, also led to significant antiproliferative effects in a dosage-dependent manner (Fig. 1D). These results suggest that both cell lines are rapamycin sensitive and that treatment with rapamycin produces antiproliferative effects in a dosage-dependent manner.

### Rapamycin inhibits mTOR activity but induces Akt activity

Next, in order to determine the effect of rapamycin on cell signaling, the phosphorylation of Akt and mTOR's downstream targets after treatment with rapamycin for 2 and 24 h was evaluated in BON and H727 cells using western blot analysis. As expected, after rapamycin treatment, BON and H727 cells showed a dramatic decline in phospho-S6K1 levels as well as markedly decreased levels of phospho-4E-BP1 (Fig. 2A and B).

Upon immunoblotting with total 4E-BP1 antibodies, an increase in faster migrating species of 4E-BP1 was also associated with rapamycin treatment, also demonstrating that rapamycin effectively inhibited the phosphorylation of 4E-BP1 in both cell lines.

Although rapamycin inhibited the phosphorylation of mTORC1's downstream targets, it was also observed to induce activation of Akt kinase as evident by an increase in the level of Akt phosphorylation. The increase in pAkt was observed at 2 h and was maintained after 24 h of treatment. These results are consistent with other studies done by Sun *et al*. (2005) and O'Reilly *et al*. (2006) who also observed that rapamycin-induced pAkt in several human cancer cell lines. Induction of Akt phosphorylation has been proposed to be an undesirable consequence of mTOR inhibition since it may potentially limit rapamycin's antitumor activity.

Octreotide does not produce significant antiproliferative effects as a single agent or in combination with rapamycin

As rapamycin analogs are currently in clinical trials in combination with octreotide for neuroendocrine
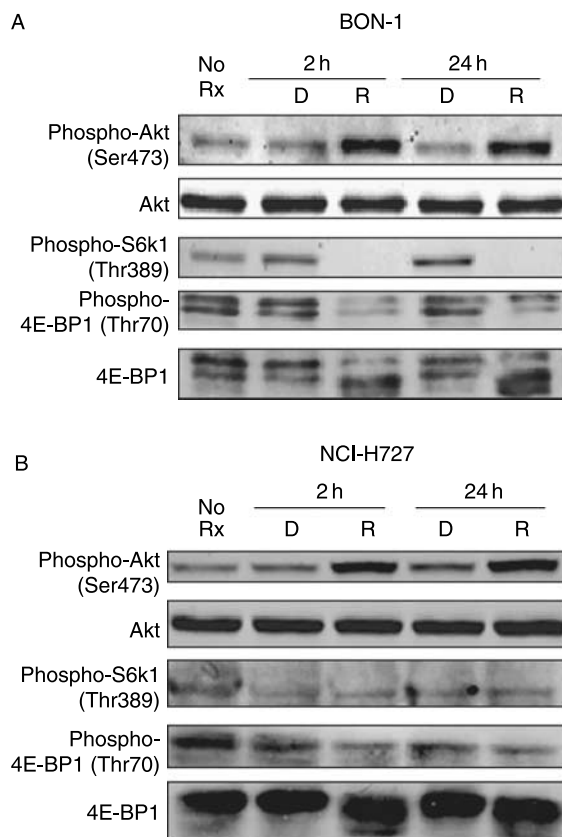
Figure 2 Rapamycin inhibits S6K1 phosphorylation, dephosphorylates 4E-BP1, but induces Akt phosphorylation in both BON and H727 cell lines. (A) BON and (B) H727 cells were grown in medium supplemented with 10% FBS and treated DMSO vehicle (D) or 100 nM rapamycin (R) for 2 and 24 h before harvesting. Total cell lysates were resolved by SDS-PAGE. Western blot analysis was performed using antibodies against phospho-Akt (Ser473), total Akt, phospho-S6K1 (Thr389), phospho-4E-BP1 (Thr70), and total 4E-BP1. Total Akt showed adequate protein quantitation in the samples.

tumors, we sought to determine the growth inhibitory effect of octreotide as a single agent, and in combination with rapamycin. BON and H727 cells were treated with varying concentrations of octreotide with or without 100 nM rapamycin for 4 days. Cell proliferation was then measured by SRB assay and percent growth inhibition was determined by comparison with vehicle-treated controls. It was observed that even at high concentrations, octreotide did not significantly inhibit cell growth in BON cells (Fig. 3A). In H727 cells, only very high concentrations (100 µM) of octreotide led to a small ($<20\%$) but statistically significant growth inhibition ($P = 0.0031$, Fig. 3C).

When BON and H727 cells were treated with a 100 nM rapamycin in combination with increasing concentrations

of octreotide, octreotide was not found to enhance the antiproliferative effects of rapamycin in either cell line, even at high concentrations (Fig. 3B and D).
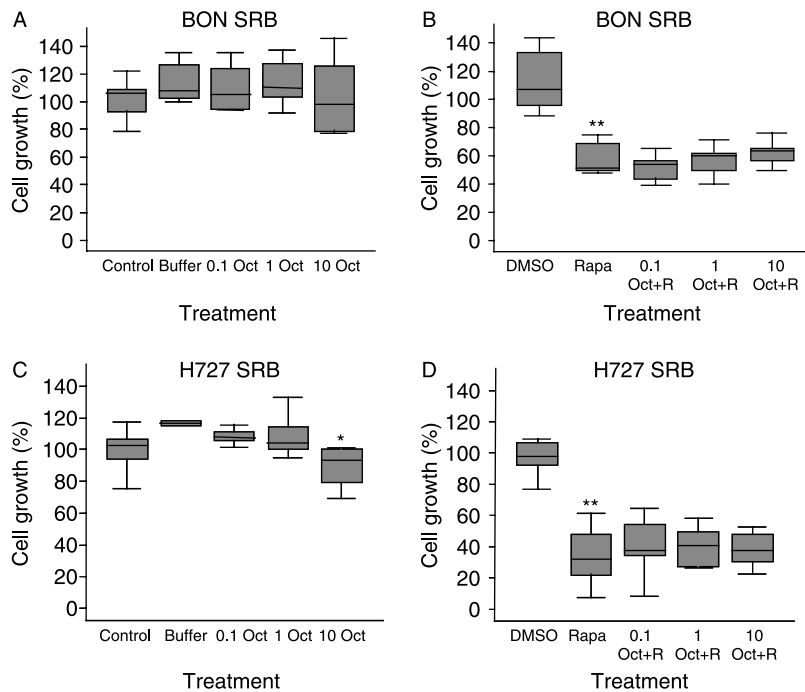
Octreotide does not inhibit rapamycin-induced Akt activation

To evaluate the effects of octreotide combined with rapamycin on the Akt/mTOR signaling pathway, we determined the expression and phosphorylation of Akt and mTORC1 targets by western blot analysis (Fig. 4A and B). As expected, rapamycin alone inhibited S6K1 phosphorylation to nearly undetectable levels while increasing pAkt levels. Octreotide as a single agent did not alter the phosphorylation of Akt or S6K1 in comparison with the controls. When in combination, octreotide and rapamycin down-regulated mTORC1 signaling, similar to rapamycin alone, but octreotide did not decrease rapamycin-induced Akt phosphorylation.

Rapamycin and octreotide antitumor activity *in vivo*

As octreotide is also known to have indirect effects, such as central effects on GH release and proposed effects on angiogenesis (Susini & Buscail 2006) that may modulate *in vivo* growth, we next evaluated the effect of octreotide and rapamycin, alone and in combination, in mice bearing BON xenografts. BON cells were injected into the upper right flanks of male BALB/c mice, and 8 days after inoculation, mice were divided into six groups and treated with long-acting octreotide (Sandostatin LAR) alone (30 mg/kg), octreotide vehicle, rapamycin alone (15 mg/kg), rapamycin vehicle DMSO, rapamycin and octreotide, and combination vehicle. Treatment for octreotide and vehicle were given s.c. once on first day of treatment, while rapamycin and its vehicle DMSO were given i.p. once a week for 30 days.

The *in vivo* model demonstrated a significant decrease ($P < 0.001$) in tumor volume on day 30 in mice treated with 15 mg/kg rapamycin (average volume, 200 mm^3) compared with that in control mice (597 mm^3; Fig. 5). Treatment with rapamycin in combination with octreotide showed reduction in tumor volume (255 mm^3) compared with that seen in control (476 mm^3) but this difference did not reach statistical significance ($P = 0.0363$; Bonferroni correction significance level of 0.008 due to multiple tests). Mice treated with octreotide alone (471 mm^3) did not demonstrate a significant decrease in tumor volume in comparison with its control (567 mm^3). Neither did mice treated with octreotide alone demonstrate a significant decrease in tumor volume

Figure 3 Octreotide does not enhance rapamycin-mediated cell growth inhibition. (A) BON cells were treated with octreotide buffer (OB) or 0, 0.1, 1.0, and 10 μM octreotide for 4 days. Cell growth was measured afterwards by an SRB assay. (B) BON cells were treated with 100 nM rapamycin and 0, 0.1, 1.0, and 10 μM octreotide for 4 days. Growth inhibition was determined by an SRB assay. Rapamycin significantly inhibited growth compared with DMSO control. The addition of octreotide did not significantly inhibit growth compared with rapamycin alone. (C) H727 cells were treated with OB or increasing concentrations of octreotide for 4 days and cell growth inhibition was analyzed by an SRB assay. (D) H727 cells were treated with 100 nM rapamycin and various concentrations of octreotide for 4 days. Experiment done in triplicate wells and growth inhibition was determined by an SRB assay in three independent experiments. The mid-line of each box is the median. The upper and lower edges of each box represent the 75th and 25th percentiles of the data respectively. The upper and lower bars mark the 95th and 5th percentiles of the data respectively. Statistical significance $*P < 0.01$ and $**P < 0.0001$. 'Oct', octreotide and 'R', rapamycin.

compared with the remaining treatment groups (rapamycin, rapamycin and octreotide). In fact, mice treated with octreotide alone had statistically significantly larger tumor volume ($471\ mm^3$) than mice treated with either rapamycin alone ($200\ mm^3$, $P < 0.001$) or mice treated with rapamycin in combination with octreotide ($255\ mm^3$, $P = 0.0023$). Taken together, these results demonstrate that octreotide does not enhance rapamycin's efficacy in tumor growth suppression.

Since treatment of BON tumors with octreotide did not produce significant tumor suppression, the plasma octreotide levels were assessed on day 30. Six plasma samples, each pooled separately from three octreotide-treated mice, were assessed. Octreotide delivery was indeed achieved with an average plasma level of 3238 pg/ml and median plasma level of 767 pg/ml (range 149–13 901 pg/ml).

Discussion

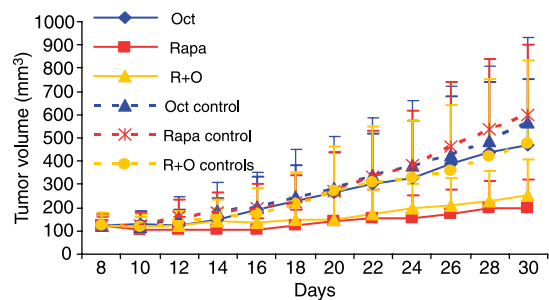As many tumors demonstrate activation of the PI3K/Akt/mTOR signaling pathway, this pathway has

emerged as a promising therapeutic target for many cancer types. The objective of our study was to determine the antitumor effects of rapamycin and octreotide as single agents and to assess the nature of their interactions in combination. We found that *in vitro*, rapamycin is not synergistic or additive with octreotide. Rapamycin as a single agent was observed to significantly inhibit cell proliferation and inhibit mTOR activity while inducing Akt phosphorylation in both carcinoid tumor cell lines. Octreotide showed limited antiproliferative effects alone, and it did not overcome the rapamycin-induced Akt phosphorylation in the combination treatment. Furthermore, we showed that rapamycin alone leads to significant tumor reduction in tumor growth *in vivo*, whereas octreotide treatment alone did not inhibit tumor growth. We also found that rapamycin in combination with octreotide *in vivo* did not show significant tumor suppression compared with controls and rapamycin treatment.

The mTOR signaling pathway can be activated through multiple mechanisms, including increased
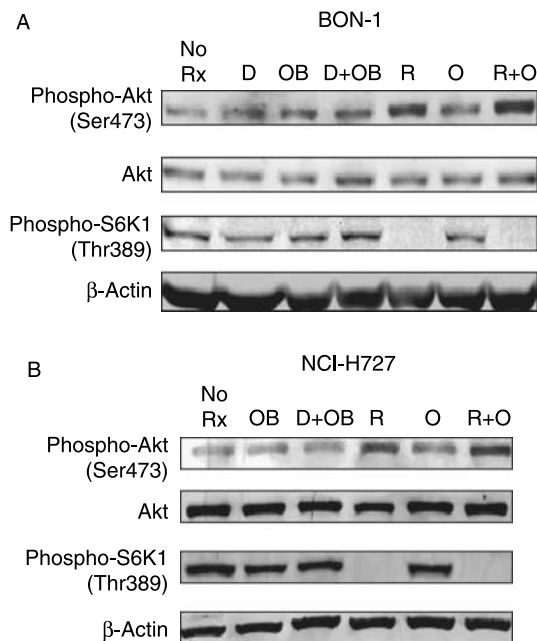
Figure 4 Octreotide does not inhibit rapamycin-induced Akt phosphorylation, and S6K1 phosphorylation is unchanged in the presence of octreotide in both cell lines. (A) BON and (B) H727 cells in 10% FBS medium were treated with octreotide buffer (OB), DMSO and octreotide buffer (D+OB), rapamycin (R), octreotide (O), or the combination (R+O), for 2 h. The control group was left untreated. Cell lysates of all samples were analyzed by western blot using anti-pAkt (Ser473), anti-Akt, and anti-pS6K1 (Thr389). The proteins were normalized by anti-β-actin. antibody.

Figure 5 Rapamycin significantly suppresses tumor progression while octreotide demonstrates little antitumor activity as a single agent and in combination. BON xenografts were established in 4-week-old male mice. At the average tumor volume of about 120 mm^3, they were treated with: long-acting octreotide at 30 mg/kg, rapamycin at 15 mg/kg, octreotide and rapamycin, octreotide vehicle, DMSO, or DMSO and octreotide vehicle (R+O control). Treatment groups contained 12 mice each and control groups had 11 mice per group. Tumor size was recorded every 2 days until the last day of treatment. Average tumor volume per group is shown; bars indicate S.D.

signaling through growth factor receptors, and loss of tumor suppressor PTEN. Most neuroendocrine tumors, including carcinoids, express IGF-I and IGF-I receptors (IGF-IRs; von Wichert *et al.* 2000). Although most carcinoids express PTEN, 54% of poorly differentiated neuroendocrine tumors demonstrate loss of PTEN expression (Wang *et al.* 2002). Frequent loss of 10q, the site of the *PTEN* gene has been reported in sporadic islet cell carcinoma. In addition, altered subcellular localization of PTEN has also been reported in islet cell carcinoma (Perren *et al.* 2000). Evidence from patients with genetic mutations in *TSC2*, *NF1*, and *VHL* genes have also implicated a role for aberrations in mTOR signaling in the neuroendocrine tumors (Yao 2007). In preclinical work, cancer cells with activation of PI3K/Akt signaling, regardless of mechanism of activation, have shown sensitivity to rapamycin (Noh *et al.* 2004). Shah *et al.* (2006) reported that 76% of the neuroendocrine tumors demonstrate activation of Akt. Taken together, these data suggest that mTOR is a promising target for neuroendocrine tumors. Indeed, we demonstrated that rapamycin treatment was associated with significant

inhibition of cell proliferation and growth in neuroendocrine BON tumor cells as well as bronchial carcinoid cell line, H727 *in vitro*, and in BON cells *in vivo*. Our results are consistent with a recent report by Zitzmann *et al.* (2007) which demonstrated that everolimus inhibited *in vitro* BON cell growth through G0/G1 arrest as well as apoptosis.

Although rapamycin treatment led to significant inhibition of cell proliferation, it did induce Akt phosphorylation in both carcinoid tumor cell lines. This finding is consistent with previous reports that rapamycin increases Akt activation in some cancer cell lines, a finding that has been attributed to the loss of mTOR/S6K-dependent feedback inhibition of signaling, with loss of phosphorylation and degradation of insulin response substrate-1 (Shi *et al.* 2005, Sun *et al.* 2005, O'Reilly *et al.* 2006). Similarly, Zitzmann *et al.* (2007) observed increased pAkt levels after treatment of BON tumor cells with everolimus. Furthermore, an increase in pAkt levels was also noted in tumors from patients with different tumor types treated with everolimus on a phase I trial (O'Reilly *et al.* 2006). As Akt is a known pro-survival factor, it has been proposed that rapalog-induced Akt activation may limit the efficacy of these mTOR inhibitors. Whether this is indeed the case at this time is unclear, especially as many cell lines sensitive to rapamycin's growth inhibitory effects demonstrate activation of Akt in response to treatment (Akcakanat *et al.* 2007), as also seen with BON and H727 cells in this study. Interestingly, in a phase II trial of temsirolimus in neuroendocrine tumors, when tumor biopsies were performed at baseline and were compared

after 2 weeks of treatment, an increase in pAkt was associated with an increased time to progression. Thus, an increase in pAkt may be simply a marker of biological inhibition of mTOR signaling, rather than a marker of tumor resistance. However, preclinical studies demonstrate inhibitors of upstream signaling, such as PI3K inhibitor LY294002, or inhibitors of IGF-IR, NVP-AEW541 (small molecule kinase inhibitor, Novartis) and A12 (monoclonal antibody to IGR-IR, ImClone Systems, New York, NY, USA) can inhibit this feedback loop activation of Akt, and in combination therapy may have improved antitumor activity (Shi *et al.* 2005, Sun *et al.* 2005, O'Reilly *et al.* 2006).

As octreotide has been reported to inhibit Akt phosphorylation in different cell types (Charland *et al.* 2001, Theodoropoulou *et al.* 2006), we hypothesized that combined treatment of rapamycin with octreotide would minimize the upstream activation and thus enhance the antitumor activity of both agents. However, we did not observe inhibition of pAkt with octreotide treatment and also an inhibition of rapamycin-induced Akt phosphorylation in the combination treatment. Further, our results suggests that octreotide has no synergistic or additive growth inhibitory relationship with rapamycin in either neuroendocrine tumor cell line tested. In contrast, octreotide has been reported to inhibit cell growth *in vitro* in some studies (Charland *et al.* 2001). Using BON cells, Evers reported that the long-acting somatostatin analog was effective in inhibiting tumor growth when administered at the time of tumor placement (Evers *et al.* 1989); however, when treatment began after established tumor growth, no significant inhibitory effects were noted by treatment with octreotide as a single agent (Evers *et al.* 1991). This suggests that small variations in study design such as tumor burden at initiation of therapy may affect the *in vivo* efficacy of octreotide. Another possible explanation for our results is that there may be other indirect mechanisms in which both agents interact which we could not analyze. Effects on hormone secretion, chromogranin A levels, or on angiogenesis might also be further investigated in order to gain insight on octreotide's antitumor effect in combination with rapamycin.

The low antitumor activity of octreotide observed in our study may also be due to some limitations in our study. Although octreotide has been suggested to mediate most of its antiproliferative effects through somatostatin receptors, its potential antitumor effects may be attenuated in our *in vitro* experiments where octreotide's role in hormone secretion regulation, antiangiogenesis, and other indirect mechanisms cannot be studied. There are also some limitations of

the xenograft model used in our study. First, we selected a nonorthotopic xenograft model to allow for efficient quantitation of tumor volumes and assessment of the interaction between rapamycin and octreotide. Although in this model we did not observe significant growth inhibition with octreotide alone and any octreotide-mediated enhancement of rapamycin's *in vivo* growth inhibitory effect, we cannot exclude the possibility that differences in cell signaling due to the microenvironment may lead to different results in other models such as in orthotopic liver metastasis models. Secondly, in our study, we used delivery of long-acting octreotide, an approach previously reported to achieve high plasma levels of octreotide in mice, as well as *in vivo* growth inhibition through an indirect mode (Weckbecker *et al.* 1997, Celinski *et al.* 2003). In humans, monthly dosing of octreotide LAR is monitored with octreotide levels, and steady-state levels are achieved after 8–12 weeks of treatment (Rubin *et al.* 1999). Monthly dosing of octreotide LAR achieves steady-state levels of 1154 ± 748 pg/ml and $10\,925 \pm 5330$ at 10 and 60 mg/month dosing respectively (Woltering *et al.* 2005). We observed a median plasma level of 767 pg/ml in our study; thus we did achieve clinically relevant plasma octreotide concentrations, within the range that have been reported after one dose of octreotide LAR given at 10 or 20 mg (Rubin *et al.* 1999). The octreotide concentrations in five of six pooled serum samples tested were within the range of steady-state serum levels achieved with 10 mg/month or greater dosing in humans. However, our study was limited by variability in serum octreotide levels; this may in part be due to technical challenges in delivering small volumes of drug suspended in emulsion form. Therefore, we cannot exclude the possibility that achieving more consistent, and higher levels of octreotide may have a more prominent antitumor effect.

Results from a current phase II trial of everolimus and depot octreotide given in combination to patients with advanced low-grade neuroendocrine carcinoma suggests that the treatment is well tolerated and this combination may have antitumor activity (Yao 2007, Yao *et al.* 2007). Relative contributions of octreotide and everolimus in this regimen are not yet determined. Our results in neuroendocrine cells suggest that rapamycin has a strong growth inhibitory effect *in vitro* and *in vivo*, while octreotide has limited antiproliferative effects as a single agent and exhibits no synergistic relationship with rapamycin *in vitro* nor *in vivo*. Thus, our preclinical data would suggest that the more effective component in the everolimus and octreotide combination regimen may be the mTOR

inhibitor. Notably, results from a multicenter phase II study of weekly temsirolimus in advanced progressive neuroendocrine tumors has recently been published (Duran *et al*. 2006). Single agent temsirolimus demonstrated some clinical activity in this population: two patients (5.4%) achieved a confirmed partial response and a third patient had an unconfirmed partial response at the end of cycle 8 but discontinued therapy (not due to toxicity). Ongoing and planned RADIANT (RAD001 in advanced neuroendocrine tumors) trials will evaluate the efficacy of everolimus alone in a phase II trial, and will determine the effect of octreotide alone versus octreotide and everolimus in advanced carcinoid tumors in a phase III trial (Duran *et al*. 2007, Yao 2007).

In summary, we have shown that rapamycin effectively inhibits cell proliferation and cell growth *in vitro* and *in vivo* as a single agent. Octreotide as a single agent had limited antiproliferative effects *in vitro* and no apparent effect on tumor suppression *in vivo*. As combined agents, octreotide did not enhance rapamycin's antitumor activity; however, due to possible limitations in our models and to the success of current combinational therapy of mTOR inhibitors with octreotide in clinical trials, further studies are needed to dissect any potential interaction between mTOR and somatostatin inhibitors. More insight into the mechanism of action of mTOR inhibitors is needed to best select patients for therapy with these agents alone and in combination with other therapies.

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