The Novel mTOR Inhibitor RAD001 (Everolimus) Induces Antiproliferative Effects in Human Pancreatic Neuroendocrine Tumor Cells

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Abstract

Background/Aim: Tumors exhibiting constitutively activated PI(3)K/Akt/mTOR signaling are hypersensitive to mTOR inhibitors such as RAD001 (everolimus) which is presently being investigated in clinical phase II trials in various tumor entities, including neuroendocrine tumors (NETs). However, no preclinical data about the effects of RAD001 on NET cells have been published. In this study, we aimed to evaluate the effects of RAD001 on BON cells, a human pancreatic NET cell line that exhibits constitutively activated PI(3)K/Akt/mTOR signaling. ***Methods:*** BON cells were treated with different concentrations of RAD001 to analyze its effect on cell growth using proliferation assays. Apoptosis was examined by Western blot analysis of caspase-3/PARP cleavage and by FACS analysis of DNA fragmentation. ***Results:*** RAD001 potently inhibited BON cell growth in a dose-dependent manner which was dependent on the serum concentration in the medium. RAD001-induced growth inhibition involved G0/G1-phase arrest as well as induction of apoptosis. ***Conclusion:*** In summary, our data demonstrate antiproliferative and apoptotic effects of RAD001 in NET cells in vitro supporting its clinical use in current phase II trials in NET patients.

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Introduction

The phosphatidylinositol-3-kinase (PI(3)K)/Akt/mTOR pathway is crucial for the regulation of cell survival and proliferation. Growth factors of the insulin family, including insulin, insulin-like growth factor I (IGF-I) and IGF-II, initiate PI(3)K/Akt/mTOR signaling by activating insulin/IGF-I receptor tyrosine kinases (RTKs), which in turn leads to tyrosine phosphorylation of insulin receptor substrate (IRS) proteins and subsequent recruitment of PI(3)K. PI(3)K bound to IRS converts phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P$_2$) into the lipid second-messenger phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P$_3$), an event that is negatively regulated by phosphatase and tensin homologue deleted on chromosome 10 (PTEN). PtdIns(3,4,5)P$_3$ eventually recruits Akt to the plasma membrane thereby facilitating its activation by phospha-

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tidylinositol-3-dependent kinase1 (PDK1). Activated Akt promotes resistance to apoptosis and unrestricted cell growth through coordinated phosphorylation of multiple downstream targets. One major effector of Akt signaling is the serine/threonine protein kinase mammalian target of rapamycin (mTOR). Complexed with regulatory-associated protein of mTOR (raptor) and LST8 in mTORC1, mTOR acts as a key integrator of signals from growth factors and nutrients (e.g. amino acids and glucose) [1, 2]. Two well-characterized mTOR substrates are eukaryotic translation initiation factor 4E (eIF4E)-binding protein (4EBP1) and p70 ribosomal S6 kinase (p70S6K), both regulating transcription and translation initiation of critical growth genes. Activated p70S6K also mediates phosphorylation and subsequent proteasome-mediated degradation of IRS-1, the major substrate of the IGF-I/insulin receptors. Thus, p70S6K forms part of a powerful negative feedback mechanism of PI(3)K/Akt/mTOR signaling [3].

Activating mutations in one or another PI(3)K/Akt/mTOR pathway component, e.g. PTEN, occur in a wide range of tumors, contributing to resistance to apoptosis and unrestricted cell growth. Significantly, tumors harboring such mutations are hypersensitive to mTOR inhibitors [4, 5]. The rapamycin-derivative RAD001 (40-O-(2-hydroxyethyl)-rapamycin, Novartis Pharma) is a potent, orally bioavailable mTOR inhibitor. RAD001 induces growth inhibition in a variety of tumor cell lines in vitro and a range of animal models of cancer [6–9]. Moreover, RAD001 has been shown to sensitize tumor cells to conventional therapeutic antitumor agents and radiation [7, 10–12]. RAD001 is presently being investigated in clinical phase II trials in various tumor entities, including NETs.

NETs represent a rare and heterogeneous category of tumors. The majority of NETs show already local or distant metastasis at the time of diagnosis [13, 14]. Advanced stages are characterized mainly by hepatic metastases with a 5-year survival rate of less than 50% [14–16]. Currently available antiproliferative strategies against NETs of the GEP are only of moderate efficacy. While well-differentiated NETs of pancreatic origin demonstrate modest sensitivity to conventional chemotherapy regimens, no established chemotherapy protocol exists for NETs of midgut origin [15, 17–19]. Since lost expression of the PI(3)K antagonist PTEN occurs in 54% of poorly differentiated neuroendocrine carcinomas and 76% of all NETs display constitutive Akt phosphorylation [20, 21], a majority of NETs is likely to be accessible to targeted antiproliferative therapy with mTOR inhibitors. Here, we evaluate the in vitro effects of RAD001 on BON cells. This cell line, derived from a human pancreatic neuroendocrine tumor, exhibits constitutive Akt/mTOR phosphorylation due to an autocrine IGF-I loop [22–24]. We demonstrate that RAD001 induces potent antiproliferative effects due to G0/G1 cell cycle arrest and apoptosis. In summary, our data indicate the novel mTOR inhibitor RAD001 to be a promising agent for targeted antiproliferative NET therapy.

Materials and Methods

Reagents

DMEM/F12 (1:1) medium and penicillin/streptomycin were from Gibco/Invitrogen (Karlsruhe, Germany). Amphotericin B and fetal calf serum (FCS) were purchased from Biochrom (Berlin, Germany). RAD001 was kindly provided by Novartis Pharma (Basel, Switzerland). Antibodies against pp70S6 Kinase (#9234), p70S6 Kinase (#9202), pAkt (#9271), Akt (#9272), pGSK3 (#9331), GSK3 (#9315), cyclin D1 (#2926), p27Kip1 (#2552), caspase-3 (#9662) and PARP (#9542) were from Cell Signaling (Beverly, Mass., USA). Antibody against β-actin (#A5441) was from Sigma-Aldrich (St. Louis, Mo., USA). Horseradish peroxidase-conjugated secondary antibodies to mouse (#31432) or rabbit (#31452) IgG and chemiluminescent substrate SuperSignal West Dura Extended Duration Substrate were from Pierce (Rockford, Ill., USA).

Cell Culture

Human pancreatic neuroendocrine BON tumor cells were kindly provided by R. Göke (Marburg, Germany). BON cells were cultured in DMEM/F12 (1:1) medium supplemented with 10% FCS, 1% penicillin/streptomycin and 0.4% amphotericin B in a 5% CO_2 atmosphere.

Cell Proliferation Assay

For proliferation assays, BON cells were seeded into 96-well plates at a density of 2000 cells/well and grown for 24 h. The next day, medium was replaced by serum-rich medium (10% FCS) or serum-depleted medium (1% FCS) containing various concentrations of RAD001 (25, 30, 35, 40, 45, 50, 55 nM or 5, 10, 15, 20, 25, 30, 35 nM) for 72 h. The cell proliferation rate was measured with Cell Titer 96 kit (Promega, Madison, Wisc., USA) according to the manufacturer's instructions. Following 3 h of incubation with Cell Titer 96 solution, absorbance at 492 nm was determined using an ELISA plate reader.

Measurement of Apoptosis and Cell Cycle Analysis

Apoptosis and cell cycle distribution were analyzed using flow cytometry according to Nicoletti et al. [25]. Cells were scraped with a rubber policeman, washed with PBS and incubated in staining buffer containing 0.1% sodium citrate, 0.1% Triton X-100 (Sigma) and 50 µg/ml propidium iodide overnight. Sub-G1 events and cell cycle distribution were measured in a fluorescence-activated cell sorter, FACSCalibur (Becton Dickinson, Franklin Lakes, N.J., USA), using Cell Quest® software. Nuclei to the left
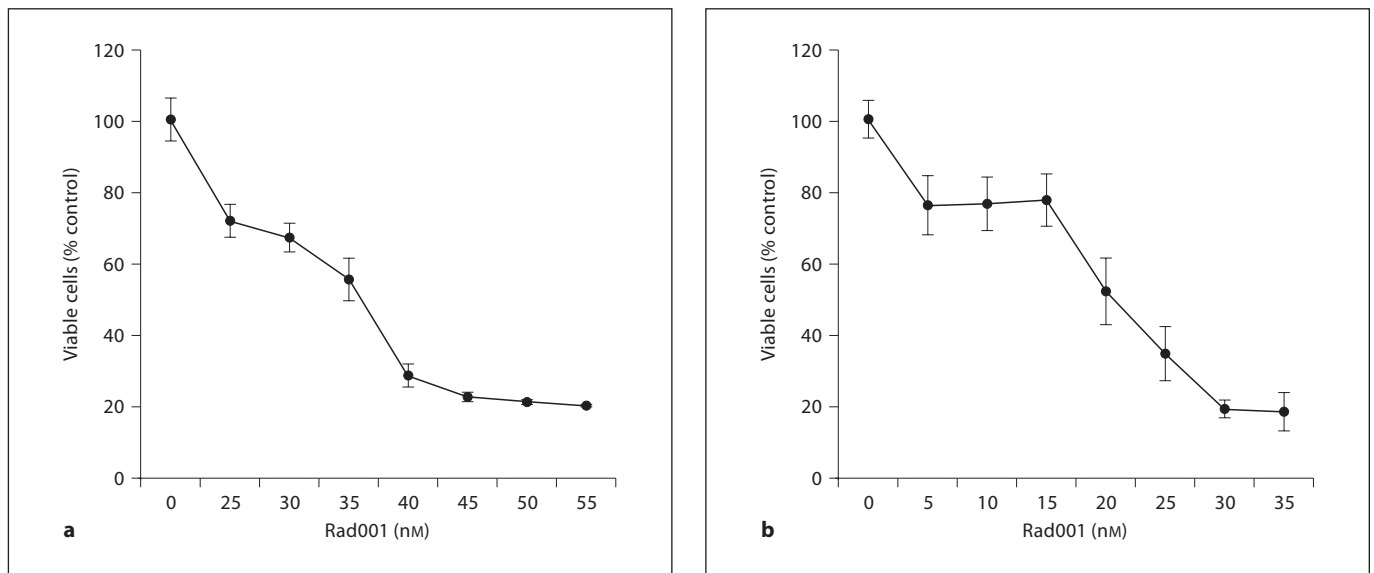
Fig. 1. RAD001 inhibits proliferation. BON cells were incubated with indicated concentrations of RAD001 in (**a**) serum-rich medium (10% FCS) or (**b**) serum-depleted medium (1% FCS) for 72 h. The cell proliferation rate was measured with Cell Titer 96 kit (Promega). Values are demonstrated as average and SD of 3 independently performed experiments. $p < 0.001$ vs. untreated control for all concentrations.

of the 'G1-peak' containing hypodiploid DNA were considered apoptotic.

Protein Extraction and Western Blotting

Protein extraction and Western blotting were performed as described recently [26, 27]. Briefly, cells were lysed in 500 μl lysis buffer. The lysates were centrifuged for 10 min at 4°C and 13,000 *g* and supernatants were diluted 1:1 with SDS sample buffer. Samples were boiled for 5 min and separated on a SDS polyacrylamide gel. Proteins were electrotransferred for 60 min onto PVDF membranes (Immobilone; Millipore, Eschborn, Germany) using a semi-dry Western-blot technique. After blocking in 2% non-fat dried milk, the membranes were incubated overnight in appropriate dilutions of antibodies against pp70S6K (1:2,000), pAkt (1:5,000), pGSK3 (1:5,000), cyclin D1 (1:2,000), p27Kip1 (1:1,000), caspase-3 (1:1,000) and PARP (1:20,000). After washing with PBS, the membranes were incubated with peroxidase-conjugated secondary antibody (1:25,000) for 2 h. The blots were washed and immersed in the chemiluminescent substrate for 30 min and exposed to XOMAT-AR film (Eastman Kodak, Rochester, N.Y., USA). Afterwards, the membranes were stripped and incubations with antibodies against p70S6K (1:1,000), Akt (1:5,000) and GSK3 (1:5,000) were performed as described above.

Statistical Analysis

National Institutes of Health Image 1.59 software was used for densitometric analysis of specific bands in Western blots. Statistical analysis was performed using a two-tailed Student's t test. $p < 0.05$ was considered statistically significant.

Results

RAD001 Inhibits Proliferation

To investigate the effects of RAD001 on the proliferation of neuroendocrine BON tumor cells, we performed proliferation assays. A 72-hour exposure to RAD001 inhibited BON cell proliferation in a dose-dependent manner, which was strongly dependent on serum concentration. When RAD001 was added to the cells in serum-rich medium (10% FCS), a concentration of 35 nM reduced cell proliferation by ~50% (fig. 1a). An equal reduction of cell proliferation was achieved with only 20 nM RAD001, when cells were cultured in serum-depleted medium (1% FCS; fig. 1b). For further experiments serum-depleted medium (1% FCS) was used.

RAD001 Inhibits Phosphorylation of p70S6K

BON cells have been shown to serum-independently exhibit constitutive phosphorylation of p70S6K due to an autocrine IGF-I loop [24]. In preliminary experiments, we also found p70S6K to be constitutively activated in BON cells, even after cultivation in serum-free medium for 5 days (data not shown). Treatment of BON cells with the mTOR inhibitor RAD001 (20 nM) potently inhibited p70S6K phosphorylation (fig. 2a).
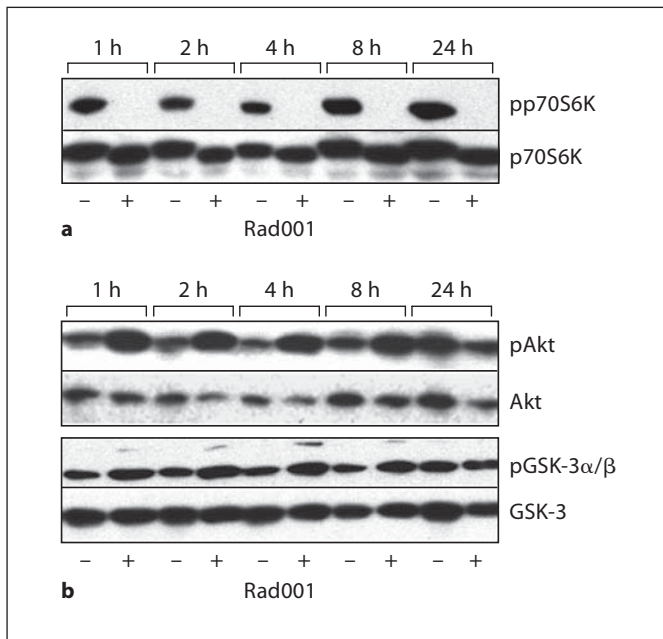
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Fig. 2. a RAD001 inhibits phosphorylation of p70S6K. BON cells were treated with 20 nM RAD001 for indicated times. Protein expression level of phosphorylated and total p70S6K was examined using Western blot analysis. One representative blot out of 3 performed experiments is shown. **b** RAD001 increases phosphorylation of Akt and its downstream target GSK-3α/β. BON cells were treated with 20 nM RAD001 for indicated times. Protein expression levels of phosphorylated and total Akt and GSK-3α/β were examined using Western blot analysis. For each protein, one representative blot out of 3 performed experiments is shown.

Fig. 3. a RAD001 induces G0/G1 cell cycle arrest. BON cells were incubated with indicated concentrations of RAD001 for 48 h. Subsequently, the proportion of cells in G0/G1-, S- and G2/M-phase was analyzed by flow cytometry. Values are demonstrated as average and SD of triplicate values from the same experiment and were confirmed by 3 independent separate experiments. * $p < 0.05$ for G0/G1-phase fraction vs. untreated control; +++ $p < 0.001$ for S-phase fraction vs. untreated control. **b** RAD001 decreases the protein level of cyclin D1 and induces p27Kip1 protein expression. BON cells were incubated with indicated concentrations of RAD001 for 24 h. Protein expression levels of cyclin D1 and p27Kip1 were examined using Western blot analysis. Equal loading was confirmed by detection of β-actin. For both proteins, one representative blot out of 3 performed experiments is shown.

RAD001 Increases Phosphorylation of Akt and Its Downstream Target GSK-3α/β

Rapamycin has recently been observed to upregulate the level of phosphorylated Akt in several human cancer cell lines [3, 28]. Consistently, mTOR inhibition by RAD001 significantly increased phosphorylation of Akt and its downstream target GSK-3α/β (fig. 2b).

RAD001 Induces G0/G1 Cell Cycle Arrest

The antiproliferative activity of mTOR inhibitors is assumed to be primarily due to G0/G1-phase arrest [6]. In BON cells, only high concentrations of RAD001 (≥ 25 nM) induced G0/G1-phase arrest, thereby significantly decreasing the fraction of BON cells in the S-phase (fig. 3a). Western blot analysis revealed RAD001 to dose-dependently decrease the protein level of cyclin D1, while only high concentrations of RAD001 (≥ 25 nM) strongly induced p27Kip1 protein expression (fig. 3b)
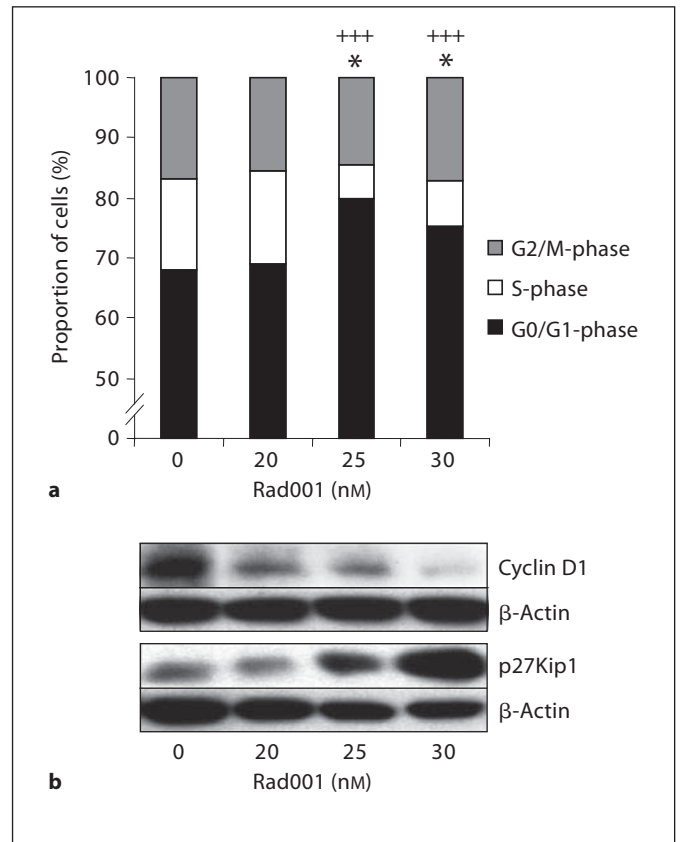
RAD001 Induces Apoptosis

In a relatively limited number of studies, rapamycin induced apoptosis, which seemed to be dose-independent [6]. To assess this issue, the extent of RAD001-induced apoptosis was studied by Western blot analysis of caspase-3/PARP cleavage and DNA fragmentation. A 24-hour exposure of BON cells to RAD001 dose-dependently decreased the amount of uncleaved caspase-3 and simultaneously increased the fraction of cleaved PARP
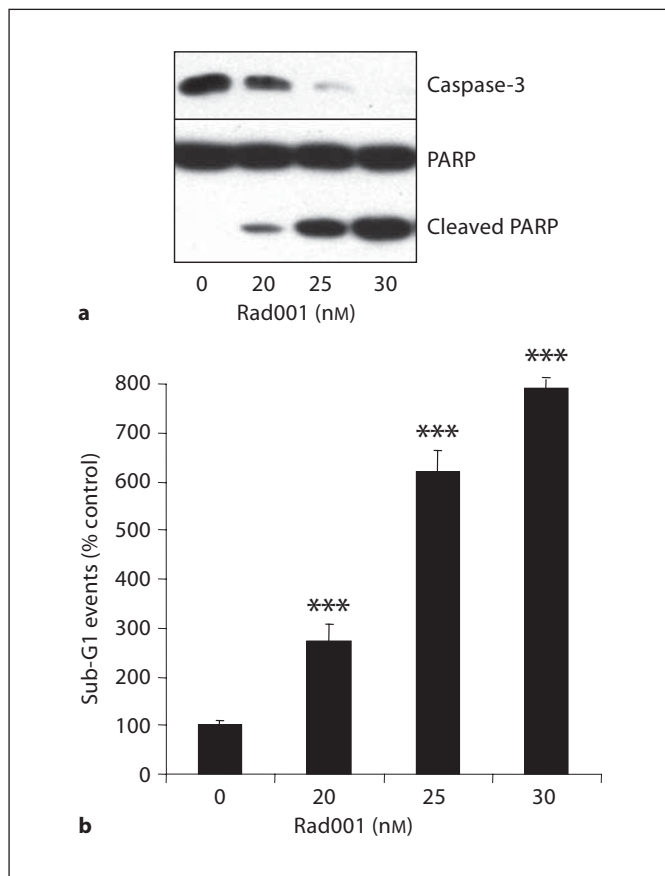
Fig. 4. RAD001 induces apoptosis. BON cells were incubated with indicated concentrations of RAD001. **a** After 24 h of incubation, the extent of caspase-3/PARP cleavage was examined using Western blot analysis. One representative blot out of three performed experiments is shown. **b** After 48 h of incubation, the proportion of cells with fragmented DNA was analyzed by flow cytometry. Values are demonstrated as average and SD of triplicate values from the same experiment and were confirmed by 3 independent separate experiments. *** $p < 0.001$ vs. untreated control.

(fig. 4a). In addition, treatment with RAD001 dose-dependently increased the number of sub-G1 events (fig. 4b).

Discussion

Constitutive activation of the PI(3)K/Akt/mTOR pathway seems to be a common mechanism, by which tumor cells promote cell growth and survival. Recent studies revealed activating mutations in the PI(3)K/Akt/mTOR pathway to occur in about one third to one half of all hu-

man tumors [1, 29, 30]. In such tumors, mTOR inhibition seems to be a promising strategy for overcoming resistance to apoptosis and unrestricted cell growth [4, 5]. Since 54% of poorly differentiated neuroendocrine carcinomas lack expression of the PI3K antagonist PTEN and 76% of all NETs display constitutive Akt phosphorylation, a majority of NETs is likely to be accessible to targeted antiproliferative therapy with mTOR inhibitors [20, 21]. BON cells exhibit constitutively activated PI(3)K/Akt/mTOR signaling due to an autocrine IGF-I loop and have been previously shown to be sensitive to rapamycin-mediated antiproliferative effects [24]. Here we demonstrate the novel rapamycin derivative RAD001 to potently induce growth inhibition in the human pancreatic neuroendocrine BON tumor cells in vitro. We found the growth inhibitory efficacy of RAD001 to be attenuated in serum-rich medium and suggest this to be due to (1) growth factors in the serum that stimulate alternative survival pathways thus partially rescuing cells from RAD001-mediated cell death or (2) binding and inactivation of RAD001 by serum components.

In our cell model, 50% growth inhibition was observed with 20 nM RAD001 in serum-depleted and with 35 nM RAD001 in serum-rich medium. Significantly, the peak blood concentrations (C_{max}) in humans after oral RAD001 doses of 0.5 mg, 1.0 mg, 2.0 mg and 4.0 mg were approximately 5 nM, 12 nM, 23 nM and 46 nM [31]. As in several clinical studies of mTOR-targeted cancer therapy RAD001 is well tolerated when administered in oral dosages of 5–10 mg/day [32, 33], RAD001 concentrations significantly inducing antiproliferative effects in neuroendocrine cancer will probably be achievable with a good safety profile.

It has been shown that rapamycin and its derivatives inhibit the binding of raptor to mTOR and thus block downstream phosphorylation of p70S6K and 4EBP1 [6]. Here, RAD001 treatment completely abrogated phosphorylation of p70S6K in BON cells. As p70S6K enhances the general translation capacity, its dephosphorylation might result in reduced cell size and motility. Several studies reported rapamycin and its derivatives to induce G0/G1 phase arrest due to increased turnover and impaired translation of cyclin D1 mRNA [34, 35]. Furthermore, rapamycin has been shown to increase the expression of the cyclin-dependent kinase (cdk) 2 inhibitor p27Kip1 at mRNA and protein level [36]. Consistently we found RAD001 to increase the number of BON cells in the G0/G1 phase and this in fact was associated with decreased cyclin D1 and increased p27Kip1 protein levels. Whereas G0/G1 phase arrest due to mTOR inhibition by rapamycin and RAD001 has been reported for many tumor cell lines,

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only a relatively limited number of studies showed rapamycin and its derivatives to induce apoptosis [6, 37]. In BON cells, RAD001 dose-dependently induced apoptosis as demonstrated by caspase-3 cleavage, PARP cleavage and DNA fragmentation. One link between mTOR inhibition and apoptosis might again be provided by p70S6K, as dephosphorylation of p70S6K has been suggested to facilitate activation of the pro-apoptotic protein BAD [6].

Recently, a study by O'Reilly et al. [3] demonstrated mTOR inhibition to result in an upregulation of PI(3)K/Akt/mTOR signaling by relieving the negative p70S6K-mediated feedback-inhibition on IRS-1. Consistently, we found that RAD001 increased Akt signaling in neuroendocrine BON tumor cells. As upregulation of Akt signaling has been hypothesized to attenuate the antiproliferative efficacy of mTOR inhibitors, combinatorial therapy with PI(3)K inhibitors can be suggested to be a promising approach to enhance the antiproliferative effects of RAD001 in NETs and other malignancies. In support of this hypothesis, combined administration of rapamycin and the PI(3)K inhibitor LY294002 has recently been demonstrated to exhibit additive antiproliferative effects on non-small cell lung cancer cells [28]. A phase II clinical trial, evaluating the effects of combinatorial therapy with RAD001 and the somatostatin analogue octreotide showed promising results in patients with advanced low grade neuroendocrine carcinoma with partial remission in 15% and stable disease in 19 from 32 patients [38]. As somatostatin also inhibits PI(3)K activity [39], these effects might be due to simultaneous inhibition of mTOR and an upstream inhibitory effect on PI(3) kinase. In summary, our present findings indicate the mTOR inhibitor RAD001 to be a promising agent for antiproliferative NET treatment. Given the upregulation of Akt phosphorylation by RAD001, the effectiveness of combined mTOR- and PI(3)K/Akt inhibition should be further investigated.

Acknowledgement

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