Mammalian target of rapamycin inhibitors: rapamycin and RAD001 (everolimus) induce anti-proliferative effects in GH-secreting pituitary tumor cells in vitro

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Abstract

The effect of mammalian target of rapamycin (mTOR) inhibitors on pituitary tumors is unknown. Akt overexpression was demonstrated in pituitary adenomas, which may render them sensitive to the anti-proliferative effects of these drugs. The objective of the study was to evaluate the anti-proliferative efficacy of the mTOR inhibitor, rapamycin, and its orally bioavailable analog RAD001 on the GH-secreting pituitary tumor GH3 and MtT/S cells and in human GH-secreting pituitary adenomas (GH-omas) in primary cell cultures. Treatment with rapamycin or RAD001 significantly decreased the number of viable cells and cell proliferation in a dose- and time-dependent manner. This was reflected by decreased phosphorylation levels of the downstream mTOR target p70S6K. Rapamycin treatment of GH3 cells induced G0/G1 cell cycle arrest. In other tumor cell types, this was attributed to a decrease in cyclin D1 levels. However, rapamycin did not affect cyclin D1 protein levels in GH3 cells. By contrast, it decreased cyclin D3 and p21/CIP, which stabilizes cyclin D/cyclin-dependent kinase 4 (cdk4) complexes. Rapamycin inhibited FCS-induced retinoblastoma phosphorylation and subsequent E2F-transcriptional activity. In response to decreased E2F activity, the expression of the E2F-regulated genes cyclin E and cdk2 was reduced. Our results showed that mTOR inhibitors potently inhibit pituitary cell proliferation, suggesting that mTOR inhibition may be a promising anti-proliferative therapy for pituitary adenomas. This therapeutic manipulation may have beneficial effects particularly for patients harboring invasive pituitary tumors resistant to current treatments.
including solid tumors, lymphomas, melanomas, and CNS tumors (Cantley & Neel 1999, Simpson & Parsons 2001). Tumors bearing these mutations are particularly susceptible to mTOR inhibitors. Rapamycin analogs, such as RAD001 (Everolimus, Novartis Pharma), were shown to effectively inhibit growth and proliferation of various tumor cells in vitro and in animal models (Beuvink et al. 2005, Faiivre et al. 2006). RAD001 is currently being evaluated in various cancer entities alone or in combination with chemotherapy, biological, and radiation therapy (Wanner et al. 2006).

Rapamycin and its analogs were shown to block G1/S cell cycle transition by decreasing cyclin D1 expression and increasing p27 expression (Hashemolhosseini et al. 1998). G1/S cell cycle transition is triggered by the activation of cyclin-dependent kinase (cdk) 4 and 6, by the D-type cyclins (cyclin D1–3) (Sherr & Roberts 1995). Activated cdk4 and 6 phosphorylate retinoblastoma (Rb), which becomes inactivated and releases E2F factors (Weinberg 1995). E2F-dependent transcriptions result in the expression of cyclin E, cyclin A, and cdk2, which complex to further hyperphosphorylate pRb and irreversibly enter the cells into the S-phase (Sherr 2000).

Pituitary adenomas constitute 10% of all intracranial neoplasms and have an 1:1000 prevalence (Daly et al. 2007). As for many tumor types, it has recently been shown that the Akt pathway is overexpressed and activated in human pituitary adenomas (Musat et al. 2005), and in spontaneously developed TSH-secreting pituitary tumors of a knockin mutant mouse harboring a mutation in the thyroid hormone receptor-β gene (Lu et al. 2008). Thus, pituitary tumors may be sensitive to the anti-proliferative effects of mTOR inhibitors. The aim of the present study was to investigate the direct in vitro effects of rapamycin and RAD001 on the rat pituitary tumor cell lines GH3 and MtT/S and on dispersed human GH-secreting pituitary adenomas.

Materials and methods

Reagents

Antibodies against: total p70/S6K, pp70/S6K (Thr389); total Akt, pAkt (Ser473), total mTOR, pmTOR (Ser2448), cyclin D1, cyclin D3, and pRb (Ser780) were all from Cell Signaling Technology (Beverly, MA, USA) and were all diluted 1:1000 in 5% nonfat dry milk in Tris-buffered saline (TBS)/Tween 20. Antibodies against: cyclin E, p21/Cip1, and cdk2 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and were all diluted 1:1000 in 5% nonfat dry milk in TBS/Tween 20. HRP-conjugated secondary antibodies were purchased from DAKO (Glostrup, Denmark) and Cell Signaling Technology. Rapamycin was from Calbiochem (La Jolla, CA, USA), and RAD001 was kindly provided by Novartis Pharma AG. Both were dissolved in DMSO.

Cell culture and pituitary adenomas

GH3 and MtT/S cells (Ooi et al. 2004) were cultured in high glucose DMEM supplemented with 10% FCS, 2.2 g/l NaHCO3, 10 mM HEPES, 2 nM glutamine, 2.5 mg/l amphotericin B, 105 U/l penicillin–streptomycin at 37 °C, and 5% CO2.

Samples of nine GH-secreting pituitary macroadenomas were obtained with informed consent during curative transsphenoidal surgical resection, in accordance with methods approved by the local Institutional Review Board. They were diagnosed by clinical, biochemical, radiological, and histological findings. Pituitary GH-secreting adenomas were mechanically dispersed and enzymatically dissociated using 0.35% collagenase and 0.1% hyaluronidase as previously described (Rubinfeld et al. 2006), and were cultured in low glucose DMEM supplemented with 10% FCS.

Cell viability

For cell viability assays, GH3 and MtT/S cells (~1×10⁴/well) and GH-secreting adenoma cells (~5×10⁴/well) were seeded in 96-well tissue culture plates. The following day, GH3 and MtT/S cells were treated with 0.5, 10, and 20 nM of either rapamycin (GH3 only) or RAD001 for 24 and 48 h, while GH-secreting adenoma cells were incubated with RAD001 (0.5, 10, 20, and 100 nM) for 48 h. DMSO served as control and was added to the cultures at concentrations equivalent to the highest concentration of rapamycin or RAD001 tested in each experiment. Cell viability of GH3, MtT/S, and cultured GH-secreting adenomas was measured using a cell proliferation assay with XTT reagent (Biological Industries, Beit Haemek, Israel), according to kit instructions. This assay is based on a colorimetric method in which tetrazolium salt XTT is reduced to colored formazan by mitochondrial enzymes. All assays were performed in six replicates.

Cell viability of cultured GH-secreting adenomas was also examined in MEM medium with d-valine substituted for l-valine (Biological Industries) containing 2% FCS to inhibit fibroblasts’ proliferation.
Drug toxicity was tested by counting trypan blue-stained cells. Treatment of GH3 cells with 20 nM rapamycin or RAD001 for 24 h had no effect on the percentage of trypan blue-stained cells (cells treated with vehicle – 20%, rapamycin – 23%, and RAD001 – 17%).

Cell counting
GH3 cells were seeded in triplicate in tissue culture dishes. The next day, the cells were treated with the indicated concentrations of either rapamycin or RAD001 and incubated for 24 or 48 h. DMSO served as control. Cells were harvested using trypsin, and then counted using a hemocytometer and a phase microscope.

BrdU
GH3 cells (~2×10^4/well) were seeded in 96-well tissue culture plates. The following day, cells were treated with RAD001 (1 and 20 nM) for 24 and 48 h. BrdU assay was conducted according to the manufacturer’s instructions (BrdU Cell Proliferation Assay, Calbiochem). Briefly, the BrdU label was added to cultures and allowed to incubate for 6 h at 37 °C, after which the medium was removed and the cells were fixed for 30 min at room temperature. Anti-BrdU antibody was then added for 60 min and cells were washed with washing buffer. Peroxidase Goat Anti-Mouse IgG HRP conjugate was added for 30 min, and the cells were washed with washing buffer and distilled H2O. Substrate solution was added for 15 min and stop solution was added to complete the reaction. Absorbance of the cells was measured at 450 nm.

Cell cycle analysis
Cell cycle was analyzed by flow cytometry after propidium iodide staining. GH3 cells were plated in 6 cm plates and subjected to the indicated treatments for 24 h. Cells were scraped with a rubber policeman, washed with PBS, and fixed in 70% ethanol for 30 min or overnight at 20°C. Cells were then washed with PBS and resuspended in staining buffer containing propidium iodide (50 μg/ml), RNase A (100 μg/ml), and 0.1% Triton X-100. Analysis was done in a fluorescence-activated cell sorter, FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA), using CellQuest and ModFit Software.

Transfection and reporter assay
The E2F-Luc construct (Mercury pathway profiling system, Clontech Laboratories, Inc.) has the E2F-responsive element upstream to the TATA box of the herpes simplex virus thymidine kinase promoter and the reporter gene luciferase.

GH3 cell transfection was performed with SuperFect (Qiagen GmbH). Cells (3×10^5) were transfected for 3 h with 1 μg of E2F-Luc plasmid, left in cell growth medium overnight, and treated with rapamycin for 6 h. Luciferase activity was measured by a Berthold luminometer. The pEGFP-C2 vector (Clontech) encoding an optimized variant of the green fluorescent protein (GFP) was used as control of the transfection efficiency. Data are expressed as the ratio of E2F-relative luciferase activity to GFP absorbance. Each experiment was performed in triplicate.

Protein extraction and western blotting
GH3 cells and GH-secreting pituitary adenoma cells were serum starved (0.1% FCS) for 16–24 h. Different concentrations of either rapamycin or RAD001 (5, 10, and 20 nM) were added to cell medium for 10 min and 30 min respectively. GH3 and MtT/s cells were also treated for 24 h. Cell lysates were prepared using RIPA buffer supplemented with protease inhibitor cocktails and phosphatase inhibitor cocktails, all from Sigma, and equal protein aliquots were loaded on 10% SDS-PAGE, transferred into nitrocellulose membrane, blocked, and incubated overnight at 4 °C with antibody against the desired protein. After three washes in TBS/Tween 20, the membranes were incubated with the secondary antibody for 60 min. Immunodetection was performed by using the ECL detection system (Santa Cruz Biotechnology) followed by autoradiography of the immunoblots. In the case of GH3 and MtT/s, each western blot was repeated in lysates obtained from three independent experiments. The optical density of the appropriately sized bands was measured employing the VersaDoc Imaging System (Bio-Rad Laboratories, Inc).

Statistical analysis
Results were expressed as mean±S.E.M. Data were analyzed by one-way ANOVA, and P values <0.05 were considered significant.

Results
Rapamycin and RAD001 decrease the number of viable cells
GH3 and MtT/S cells were cultured and incubated with either rapamycin or RAD001 (0.5, 10, and 20 nM) for 24 and 48 h and an XTT assay was then performed. A significant inhibitory effect of both rapamycin and RAD001 on the number of viable GH3 cells was observed already after 24 h exposure (Fig. 1A).
The magnitude of the reduction in viable GH3 cells was similar for both inhibitors (40–50%) after 24 h incubation, although RAD001 exhibited a dose-dependent inhibition, whereas the maximal effect of rapamycin was achieved already at 0.5 nM. Treatment of MtT/S cells with RAD001 resulted in a significant decrease in the number of viable cells at concentrations of 10 and 20 nM after 24 and 48 h (Fig. 1B), although to a lower extent (15–25%) than that observed in GH3 cells.

**Figure 1** Rapamycin and RAD001 decrease the number of viable cells. GH3 cells (A), MtT/S cells (B), and GH-secreting adenomas, GH 1–7 (C), were incubated with the indicated concentrations of rapamycin (GH3 cells) and RAD001 for the indicated times (GH3 and MtT/S cells) or for 48 h (GH 1–7). Cell viability was examined using a cell proliferation assay with XTT reagent. Values are demonstrated as the average and S.E.M. of three independently performed experiments in GH3 and MtT/S cells and as average and S.D. of six wells compared with control wells (100%). (A) *P < 0.0001 versus cells treated with vehicle for all doses and times used with rapamycin or RAD001. #P < 0.05 for GH3 cells treated with 10 and 20 nM RAD001 versus cells treated with 0.5 nM. (B) *P < 0.05 for MtT/S cells treated with RAD001 versus cells treated with vehicle. (C) *P < 0.05, **P < 0.001 for GH-secreting adenoma cells treated with RAD001 versus cells treated with vehicle. (D) Two GH-secreting adenomas, GH 8–9, were incubated with the indicated concentrations of RAD001 for 48 h in D-val MEM supplemented with 2% FCS or in DMEM containing 10% FCS. Values are demonstrated as the average and S.D. of six wells compared with control wells (100%). *P < 0.05, **P < 0.0001 versus cells treated with vehicle.
Incubation of cells from three GH-secreting adenomas (adenomas 1–3) with RAD001 (0.5 and 20 nM) for 48 h in DMEM containing 10% FCS, significantly reduced the number of viable cells by 25–30% (Fig. 1C). Incubation of another four GH-secreting adenomas (adenomas 4–7) with 10 nM and 100 nM RAD001 decreased the number of viable cells after 48 h by 30–70% in three cases (Fig. 1C). One case (GH5) did not respond to any of these doses.

Since adenoma cell cultures are contaminated with fibroblasts, it was essential to confirm that reduction in viable cell numbers was not due to the inhibition of fibroblasts growth. Therefore, cell viability of another two GH-secreting adenomas (adenomas 8–9) was examined either in cell cultures grown in D-val MEM (inhibits fibroblast growth) supplemented with 2% FCS or in DMEM containing 10% FCS, both treated with 0.5 and 20 nM RAD001 for 48 h. D-val MEM was supplemented with only 2% FCS (when compared with DMEM) as FCS contains l-val, which may facilitate fibroblasts growth. In both adenomas, the reduction in viable cells was comparable with either D-val MEM or DMEM except for the 0.5 nM treatment in GH8 (Fig. 1D).

**Rapamycin and RAD001 inhibit proliferation of GH3 cells**

Cell-counting and BrdU experiments were performed in order to verify the effects of rapamycin and RAD001 on proliferation of GH3 cells. Treatment with either rapamycin (1, 10, and 20 nM) or RAD001 (1 and 20 nM) caused a significant dose-dependent decrease in cell number, when compared with control cells at 24 h (20–30% with rapamycin and 20–35% with RAD001, Fig. 2A) and 48 h (25–50% with rapamycin and RAD001). Incubation of GH3 cells with RAD001 (1 and 20 nM) resulted in significant reduction in BrdU incorporation at 24 h (15–30%) and 48 h (25–35%, Fig. 2B).

**Rapamycin and RAD001 inhibit the phosphorylation of p70S6K in GH3 cells and GH-adenomas**

We assessed the effect of rapamycin and RAD001 (5, 10, and 20 nM) on the phosphorylation of the mTOR effector, p70S6K, at Thr389 in GH3 cells and two cultured human GH-secreting pituitary adenomas. Treatment of 10 and 30 min with each of the inhibitors potently abrogated p70S6K phosphorylation in GH3 cells (Fig. 3A). Similar effect on the phosphorylation of p70S6K was observed after the treatment of cultured GH-secreting adenomas with RAD001 (Fig. 3B). These data show that GH-secreting pituitary tumor cells are sensitive to rapamycin analogs.
Rapamycin induces G0/G1 cell cycle arrest

We next examined whether the anti-proliferative effects of rapamycin in GH3 cells reflect G0/G1 cell cycle arrest, which was previously reported in various tumor cells (Faivre et al. 2006). Cells were incubated with the indicated concentrations of rapamycin for 24 h and were analyzed by flow cytometry. G0/G1 arrest was significantly induced already at 5 nM (20%, Fig. 4A).

Rapamycin reduces cyclin D3 protein levels

An early event in the G1 phase is the synthesis of D-type cyclins and their association with cdk4 and 6. Rapamycin was reported to inhibit the G1/S cell cycle progression by decreasing cyclin D1 transcription. Western-blot analysis revealed no changes in cyclin D1 protein levels following 24 h treatment with rapamycin in GH3 cells (Fig. 4B) and MtT/S cells (data not shown), but it decreased cyclin D3 expression in GH3 cells (Fig. 4B). These data suggest that in GH3 cells, cyclin D3, but not D1, is involved in rapamycin-induced cell cycle arrest. Rapamycin treatment did not affect cdk4 or 6 protein levels (data not shown).

Cdk4 complexes with D-type cyclins were shown to stabilize by binding p21/Cip1 (LaBaer et al. 1997). Rapamycin treatment decreased p21/Cip1 expression in GH3 cells (Fig. 4B).

Rapamycin decreases Rb phosphorylation levels, E2F-transcriptional activity and cyclin E and Cdk2 expression

The limiting factor in G1/S cell cycle progression is Rb phosphorylation. Cdk4 associated with D-type cyclins phosphorylates Rb at Ser780 early in the G1 phase. In GH3 cells, rapamycin treatment decreased phosphorylated Rb-Ser780 levels (Fig. 4B).

Hyperphosphorylated Rb releases E2F-transcriptional factors to drive the transcription of genes, such as cyclin E, which will lead the cells to the late G1-phase and S-phase entry. The inability of rapamycin-treated cells to proceed to the S-phase was reflected by the significant decrease in E2F-transcriptional activity.
Accordingly, in GH3 cells, rapamycin decreased the E2F-regulated genes cyclin E and cdk2 (Fig. 4C). This contrasts with observations done in T cells (Kawamata et al. 1998). Cyclin E/cdk2 activity is inhibited by the cdk-inhibitory protein p27/Kip1. Previous studies have highlighted p27/Kip1 as an important mediator of rapamycin’s induced cell cycle arrest (Luo et al. 1996). However, GH3 cells do not express p27/Kip1 (Qian et al. 1996), and still respond to rapamycin treatment, confirming previous observation in mice lacking p27/Kip1 (Nakayama et al. 1996). These data suggest that in GH3 cells, rapamycin inhibits the cyclin E–cdk2 complex by decreasing the availability of its components rather than interfering with its activity.

**The effects of rapamycin and RAD001 on the phosphorylation of Akt and mTOR**

An important setback in mTOR inhibitor treatment observed in other cancers is the elimination of the p70S6K negative feedback loop, which leads to increased pAkt–Ser473 levels in treated cells and subsequent treatment resistance. We evaluated the effect of rapamycin and RAD001 (5, 10, and 20 nM) on pAkt–Ser473. No significant change was observed at
any time point with either of the inhibitors in GH3 cells (Fig. 5A) or with RAD001 in MtT/S cells (Fig. 5B).

Interestingly, 24 h rapamycin treatment significantly decreased pmTOR–Ser2448 levels in GH3 cells (Fig. 5C).

**Discussion**

The present study demonstrates for the first time that rapamycin and RAD001 are potent cell proliferation suppressors of rat pituitary cells and human pituitary cells derived from GH-secreting adenomas. Our results indicate a reduction in viable cells as a result of mTOR inhibition, observed by the XTT assay. Cell counts and BrdU incorporation further point to a decrease in cell numbers and cell proliferation respectively, caused by G0/G1 cell cycle arrest, which was analyzed by FACS and further confirmed by the reduction in cyclin D3 and E expression, E2F-transcriptional activity, and inhibition of FCS-induced Rb phosphorylation. Treatment with mTOR inhibitors significantly inhibited the phosphorylation of the mTOR effector, p70S6K, and of mTOR itself, suggesting that mTOR inhibition can affect the PI3K/Akt/mTOR pathway in pituitary cells, resulting in significant anti-proliferative effects. Furthermore, this sensitivity of pituitary adenoma cells to mTOR inhibitors points to a constitutively activated mTOR pathway in these cells and indicates that mTOR is a growth-promoting factor, which may be involved in pituitary tumorigenesis. Thus, our results may be compatible with the observation that Akt is overexpressed and activated in human pituitary tumors (Musat et al. 2005), and with the observation that in a model of rats that carry a germline

Figure 5 (A) The effects of rapamycin and RAD001 on Akt phosphorylation. GH3 (A) and MtT/S (B) cells were incubated with the indicated concentrations of rapamycin (GH3 cells) or RAD001 for 24 h. Protein expression levels of phosphorylated and total Akt were examined by western-blot analysis. One representative blot out of three performed is shown. The barographs represent mean and S.E.M. densitometric values of three experiments. (C) Rapamycin inhibits the phosphorylation of mTOR. GH3 cells were incubated with the indicated concentrations of rapamycin for 24 h. Protein expression levels of phosphorylated and total mTOR were examined by western-blot analysis. One representative blot out of three performed is shown. The barographs represent mean and S.E.M. densitometric values of three experiments. *P < 0.0001 versus cells treated with vehicle for all doses used.
**Tsc2**-inactivating mutation, rapamycin treatment induced regression of established pituitary tumors (Kenerson et al. 2005). In normal rat pituitary cells, rapamycin was shown to inhibit basal proliferation and insulin-, cAMP-, and estradiol-induced proliferation of cells (Kawashima et al. 2000), as well as prolactin release (Wera et al. 1995a,b). Human prolactin gene was expressed in GH3 cells and its transcription was inhibited by rapamycin (Wera et al. 1995a,b).

The majority of the previous *in vitro* studies have reported that induction of G0/G1 arrest is the primary mechanism by which mTOR inhibitors exert their antiproliferative effects (Fairev et al. 2006). Consistent with these findings was the increase in the GH3 cell subpopulation at the G0/G1 phase induced by rapamycin. Interestingly, cyclin D3, but not cyclin D1, protein expression was reduced by rapamycin and RAD001 (studied in GH3 cells), which was also shown in human breast cancer (Garcia-Morales et al. 2006), lymphoma (Hipp et al. 2005, Wanner et al. 2006), and myeloma (Stromberg et al. 2004) cell lines. Additional mechanism, which may contribute to reduced activity of cdk4/6-cyclin D complexes by rapamycin, is the down-regulation of p21, which apart from its function as cdk’s inhibitor appears to stabilize these complexes (LaBaer et al. 1997, Gaben et al. 2004). Furthermore, as the cell cycle inhibitor p27Kip1 is not expressed in the GH3 cell line (Qian et al. 1996) and its expression levels in human pituitary adenomas are low (Bamberger et al. 1999, Lidhar et al. 1999), p27-independent mechanisms, such as the inhibition of p21 expression, may be involved in rapamycin-induced cell cycle arrest of these cells. In addition to these impacts of rapamycin on mid-G1 phase events and on Rb phosphorylation early in the G1 phase, the inhibition of G1 phase progression by rapamycin is also reflected in the late-G1 phase, thus cyclin E and cdk2 expression levels were reduced, as well as E2F-transcriptional activity.

Treatment of GH3 cells with rapamycin and RAD001 abrogated the phosphorylation of mTORC1 substrate p70S6K as expected, and interestingly reduced the phosphorylation of mTOR at Ser2448. This may be explained by the finding that phosphorylation of Ser2448 occurs in a feedback fashion, catalyzed by the downstream target of mTOR, p70S6K (Chiang et al. 2005), and not directly by Akt. Therefore, the phosphorylation of mTOR may be a consequence of p70S6K activity and may indicate mTOR activity. Thus, the decreased phosphorylation of mTOR by rapamycin or RAD001 may serve as another indication for the inhibition of mTOR and p70S6K activities.

Continuous mTOR inhibitor treatment was suggested to actually overactivate the PI3K pathway by blocking the negative feedback loop downstream to p70S6K, resulting in treatment resistance (O’Reilly et al. 2006). In this study, we did not observe any changes in pAkt–Ser473 levels after 24 h in GH3 cells treated with rapamycin or RAD001, or in MtT/S cells treated with RAD001, suggesting high sensitivity, although development of resistance at later time points cannot be ruled out.

As mTOR controls cell growth, inhibiting mTOR could decrease cell volume, thus leading to tumor shrinkage. Drugs commonly used for the treatment of GH-secreting pituitary adenomas, such as the somatostatin analogs octreotide and lanreotide, have been reported to cause tumor shrinkage in ~40% of patients (Melmed et al. 2005). The reason for this somatostatin analog-induced tumor shrinkage was found to be a decrease in cell volume rather than apoptosis. Therefore, it can be speculated that treatment with mTOR inhibitors could decrease tumor volume in acromegalic patients who do not respond to the standard somatostatin analog treatment.

In summary, the findings of our *in vitro* study suggest that mTOR inhibitors may be a novel promising anti-proliferative treatment modality for patients with GH-secreting pituitary adenomas who do not respond to somatostatin receptor ligands.

### Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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