Antitumor Efficacy of Intermittent Treatment Schedules with the Rapamycin Derivative RAD001 Correlates with Prolonged Inactivation of Ribosomal Protein S6 Kinase 1 in Peripheral Blood Mononuclear Cells

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ABSTRACT

The orally bioavailable rapamycin derivative RAD001 (everolimus) targets the mammalian target of rapamycin pathway and possesses potent immunosuppressive and anticancer activities. Here, the antitumor activity of RAD001 was evaluated in the CA20948 syngeneic rat pancreatic tumor model. RAD001 demonstrated dose-dependent antitumor activity with daily and weekly administration schedules; statistically significant antitumor effects were observed with 2.5 and 0.5 mg/kg RAD001 administered daily (treated tumor versus control tumor size (T/C), 23% and 23–30%, respectively), with 3–5 mg/kg RAD001 administered once weekly (T/C, 14–36%), or with 5 mg/kg RAD001 administered twice weekly (T/C, 36%). These schedules were well tolerated and exhibited antitumor potency similar to that of the cytotoxic agent 5-fluorouracil (T/C, 23%). Moreover, the efficacy of intermittent treatment schedules suggests a therapeutic window allowing differentiation of antitumor activity from the immunosuppressive properties of this agent. Detailed biochemical profiling of mammalian target of rapamycin signaling in tumors, skin, and peripheral blood mononuclear cells (PBMCs), after a single administration of 5 mg/kg RAD001, indicated that RAD001 blocked phosphorylation of the translational repressor eukaryotic initiation factor 4E-binding protein 1 and inactivated the translational activator ribosomal protein S6 kinase 1 (S6K1). The efficacy of intermittent treatment schedules was associated with prolonged inactivation of S6K1 in tumors and surrogate tissues (>2 h). Furthermore, detailed analysis of the dose dependency of weekly treatment schedules demonstrated a correlation between antitumor efficacy and prolonged effects (>7 days) on PBMC-derived S6K1 activity. Analysis of human PBMCs revealed that S6K1 also underwent a concentration-dependent inactivation after RAD001 treatment ex vivo (>95% inactivation with 20 nm RAD001). In contrast, human PBMC-derived eukaryotic initiation factor 4E-binding protein 1 was present predominantly in the hypophosphorylated form and was unaffected by RAD001 treatment. Taken together, these results demonstrate a correlation between the antitumor efficacy of intermittent RAD001 treatment schedules and prolonged S6K1 inactivation in PBMCs and suggest that long-term monitoring of PBMC-derived S6K1 activity levels could be used for assessing RAD001 treatment schedules in cancer patients.

INTRODUCTION

RAD001 (everolimus), an orally bioavailable derivative of rapamycin, is a macrolide antifungal antibiotic that demonstrates potent antiproliferative effects against a variety of mammalian cell types. Specifically, RAD001 inhibits cytokine-driven lymphocyte proliferation (1), as well as the proliferation of human tumor-derived cells grown either in culture or as tumors in animal models (2, 3). As a result of these properties, RAD001 is being clinically developed both as an immunosuppressant for prevention of allograft rejection (Certican; Ref. 1) and as a novel therapeutic in the fight against human cancer (2–4).

RAD001, like rapamycin, binds with high affinity to a ubiquitous intracellular receptor, the immunophilin FKBP12. This complex specifically interacts with the mammalian target of rapamycin (mTOR) protein kinase; inhibiting downstream signaling events (5). The mTOR kinase is a member of the phosphoinositide kinase-related kinase family, which consists of high molecular weight serine/threonine kinases involved in cell cycle checkpoint control (6). Several lines of evidence suggest that mTOR acts as a sensor for stress (7) and the availability of amino acids (8–10) or intracellular ATP (11). In the presence of mitogens and sufficient nutrients, mTOR relays a signal to translational regulators, specifically enhancing the translation of mRNAs encoding proteins essential for cell growth (12) and progression through the G1 to S transition (13, 14). Consistent with targeting the mTOR pathway, treatment of mammalian cells with rapamycin has been shown to inhibit these signaling events, mimicking a starvation phenotype (15) and leading to growth retardation and accumulation of cells in G1 phase (16). The mechanism of growth stimulus and nutrient level integration by mTOR is, as yet, not fully understood. However, an increasing body of evidence suggests the involvement of the phosphatidylinositol 3’-kinase/Akt/TSC/Rheb pathway (12, 17–23). Indeed, it has been suggested that, in tumor cells, the activation status of the Akt pathway may be indicative of responsiveness to rapamycin or its derivatives (24–27).

mTOR is a part of a multisubunit complex that contains the regulatory proteins raptor (28, 29) and GβL (30). The mTOR complex signals to at least two downstream effectors, the translational repressor protein eukaryotic initiation factor 4E (eIF-4E)-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase 1 (S6K1). These share an evolutionary conserved amino acid motif, the TOS motif, that functions as a docking site for raptor (31–33). Binding of 4E-BP1 to the translational activator eIF-4E is modulated by mTOR-dependent phosphorylation of specific serine and threonine residues (5). Ser37 and Ser46 are constitutively phosphorylated, acting as priming sites for the mitogen-induced, rapamycin-sensitive phosphorylation of Thr70 and Ser65 (34). After a final phosphorylation event at Ser65, 4E-BP1 dissociates from eIF–4E (35), thereby allowing the reconstitution of a translationally competent initiation factor complex (eIF–4F; Ref. 5). eIF–4F activation results in the translation of a subset of capped mRNA containing highly structured 5′-untranslated regions and encoding proteins involved in G1- to S-phase progression (13, 14). Mitogen-induced activation of the S6K1 is also dependent on mTOR function and has been implicated in the translational regulation of mRNAs possessing a 5′-terminal oligopyrimidine tract (36–38). 5′-Terminal oligopyrimidine tract mRNAs are characterized by a stretch of 4–14 pyrimidines located at their extreme 5′ terminus and typically encode ribosomal proteins as well as components of the
translational machinery. Activation of S6K1 itself is also tightly regulated by hierarchical phosphorylation events, which are depend- ent on the activation of various signal transduction pathways and culminate in the phosphorylation of the rapamycin-sensitive site Thr389, an event closely paralleling kinase activation (12, 39). Immuno- purifed mTOR has been shown to autophosphorylate on Ser2481 (40) and to phosphorylate Ser37, Ser46, and Ser65 on 4E- BP1 in vitro (11, 34, 41, 42). However, some of these events have been demonstrated to be resistant to antiproliferative concentrations of rapamycin (40–42). It is therefore unclear what role mTOR kinase activity plays per se in rapamycin-sensitive signaling events.

Because mTOR couples nutrient/growth factor availability to cell growth and proliferation in a variety of cell types, there is a potential for developing rapamycin derivatives such as RAD001 as novel inhibitors of the deregulated cell growth characteristic of human cancers. Consistent with this, RAD001 inhibits the proliferation of a wide variety of human solid tumor cell lines both in vitro in cell culture and in vivo in animal xenograft models (2, 3, 27, 43, 44).

Furthermore, antiproliferative effects of RAD001 in posttransplant lymphoproliferative disorder-like B cell lines have been observed in vitro (11, 34, 41, 42). However, some of these events have been demonstrated to be resistant to antiproliferative concentrations of rapamycin (40–42). It is therefore unclear what role mTOR kinase activity plays per se in rapamycin-sensitive signaling events.

Because mTOR couples nutrient/growth factor availability to cell growth and proliferation in a variety of cell types, there is a potential for developing rapamycin derivatives such as RAD001 as novel inhibitors of the deregulated cell growth characteristic of human cancers. Consistent with this, RAD001 inhibits the proliferation of a wide variety of human solid tumor cell lines both in vitro in cell culture and in vivo in animal xenograft models (2, 3, 27, 43, 44).

Furthermore, antiproliferative effects of RAD001 in posttransplant lymphoproliferative disorder-like B cell lines have been observed in vitro and in vivo (45, 46). In the present study, we have demonstrated that RAD001 displays significant antitumor activity in the syngeneic CA20948 rat pancreatic tumor model. Equivalent activity was observed with daily and intermittent treatment schedules, suggesting the possibility of a therapeutic window allowing differentiation of anti-tumor activity from the immunosuppressive properties of this agent. Detailed biochemical analysis of the mTOR effectors 4E-BP1 and S6K1 in tumor, skin, and peripheral blood mononuclear cell (PBMC) extracts obtained from RAD001-treated rats suggests that modulation of 4E-BP1 activity and significant inactivation of S6K1 are associated with antitumor activity. Furthermore, the efficacy observed using intermittent treatment schedules is paralleled by long-term down-regulation of S6K1 activity in all three tissues. We also provide evidence that the duration of S6K1 inactivation in PBMCs correlates with the dose-dependent suppression of tumor growth observed with weekly regimens. Moreover, unlike 4E-BP1 phosphorylation, S6K1 activity can be reproducibly measured in human PBMCs and represents a potentially valuable pharmacodynamic biomarker by which to monitor RAD001 treatment schedules in cancer patients.

MATERIALS AND METHODS

Drug Preparation. RAD001 (everolimus) is a derivative of rapamycin [40-O-[2-hydroxyethyl]-rapamycin; Ref. 47]. For animal studies, RAD001 was formulated at 2% (w/v) in a microemulsion vehicle, which was diluted to the appropriate concentration in 5% (w/v) glucose solution just before administration by gavage. For in vitro and ex vivo analyses, RAD001 was prepared in DMSO before addition to cell culture or human volunteer blood samples.

Antitumor Efficacy Studies and Statistical Analyses. Male Lewis rats were purchased from Ifla Creo (L’Abresle, France) and allowed food and water ad libitum. A suspension of CA20948 tumor cells (obtained from donor rats because this line is nonculturable in vitro) in Ham’s F-12 medium supplemented with 10% FCS, 0.1 g/100 ml NaHCO3, 1% penicillin, and 1% fungizone was injected s.c. into the left flank of rats. Treatment of randomized rats started when the tumors reached about 100 mm3. RAD001 was administra- ted p.o. daily at 0.5 or 2.5 mg/kg (∼6/week), twice weekly at 5 mg/kg, or weekly at 0.5, 1, 2, 3, or 5 mg/kg. A volume of vehicle equivalent to the highest dose of RAD001 administered in the experiment was used as a negative control. As a positive control, the cytotoxic agent 5-fluorouracil (5-FU; ICN Pharmaceuticals Inc., Costa Mesa, CA) was administered at a near maximum tolerated dose (15 mg/kg, i.e., ∼4×/week, 2 days treatment/2 days rest), which gives maximal antitumor effect. Tumors were measured every day or every other day with a caliper, and the volumes were calculated by using the formula of an ellipsoid [V = π/6 × d1 × d2 × d3], where d1, d2, and d3 represent the three largest diameters. Animals were also weighed the same day tumors were measured. The animals were sacrificed when either their tumor burden exceeded 25,000 mm3 or when skin overlaying the tumor exhibited evidence of necrosis. All protocols involving animals were approved by the Veteri- nary Service, Basel, Switzerland.

Results are presented as mean ± 1 SEM or as percentage of T/C (mean increase of tumor volumes of treated animals divided by the mean increase of tumor volumes of control animals multiplied by 100). The statistical signifi- cance of differences between treatment and control groups were determined by ANOVA followed by the Dunnett test. Statistical analyses on body weight were performed by ANOVA followed by Tukey’s test, and for comparison between weight at start and end of the experiment for individual animals, the paired t test was used. The level of significance was set at P < 0.05. Statistical calculations were performed using SigmaStat 2.03 (Jandel Scientific).

Rat-Derived and Human Volunteer-Derived Tissue/PBMC Protein Ex- tract Preparation. CA20948 tumor-bearing rats were given 0.5, 1, 2, or 5 mg/kg RAD001 or an equivalent volume of vehicle. At the indicated times after administration, rats were sacrificed, and tumor and shaved skin samples (for 0.5 and 5 mg/kg RAD001 doses) were dissected and weighed. Samples were rinsed in ice-cold PBS and immediately extracted in ice-cold extraction buffer [50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 20 mM Nα, 1 mM EDTA, 6 mM EGTA, 15 mM PPi, 30 mM p-nitrophenyl phosphate, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, and 0.1% NP40] with a constant ratio of 45 mg tumor/ml extraction buffer and 90 mg skin/ml extraction buffer, using a PT3000 Polytron (probe PT-DA 3012/2S; Kinematica AG) or a hand-held PT2100 Polytron (probe PT-DA 2112/2EC), respectively. Lysates were cleared by centrifugation for 30 min at 12,000 × g at 4°C. Supernatants were subsequently aliquoted, snap frozen on dry ice, and stored at −80°C. In the case of skin samples, before further analysis, samples were centrifuged for 20 min at 436,000 × g at 4°C to remove the fat fraction.

Blood (for 0.5, 1, 2, and 5 mg/kg RAD001 doses) from tumor-bearing and normal-bearing rats was withdrawn into syringes containing EDTA [0.5% (w/v)] final] and then placed into an ice-cold tube and mixed. Unless otherwise stated, the blood from individual animals within the same treatment group was analyzed separately. The blood was immediately centrifuged for 20 min at 430 × g at 4°C. The PBMCs, deposited at the interface between the RBCs and the plasma, were collected and pelleted by centrifugation for 5 min at 3000 × g at 4°C. PBMCs were washed with 10 ml of ice-cold PBS and then repelleted by centrifugation for 5 min at 3000 × g at 4°C. Cell pellets were resuspended in ice-cold extraction buffer containing 1% NP40 at the fixed ratio of 500 μl extraction buffer/10 ml initial blood volume. The cells were sheared by vigorous pipetting and then centrifuged for 30 min at 12,000 × g at 4°C. Supernatants were aliquoted, snap frozen on dry ice, and stored at −80°C. Human blood from healthy volunteers was collected under medical supervision into tubes containing either sodium citrate (BD Vacutainer 9NC; BD Vacutainer Systems, Plymouth, United Kingdom) or EDTA (BD Vacutainer K3E) as an anticoagulant. The blood was either immediately processed or, for ex vivo tumors, treated with 2, 20, and 200 μl RAD001 or DMSO vehicle for 30 min at room temperature. Human PBMCs were isolated and extracted as described for rat PBMCs.

A549 Cell Culture and Protein Extract Preparation. A549 human lung carcinoma cells (CCL185) were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium (Amimed, Allschwil, Switzerland) supplemented with 10% FCS, 2 mM l-glutamine, and 100 μg/ml penicillin/streptomycin at 37°C and 5% CO2. Cell lysates were prepared as described previously (48).

Immunoblot Analysis. Cell lysates (30–40 μg) were electrophoretically resolved on denaturing SDS polyacrylamide gels (SDS-PAGE), transferred to polyvinylidene difluoride (Millipore Corp., Bedford, MA), and probed with the following primary antiserum and anti-S6 (provided by J. Mestan; Oncology Research, Novartis Pharma AG, Basel, Switzerland); anti-4E-BP1 (kindly provided by N. Sonenberg; McGill University, Montreal, Quebec, Canada); anti-eIF4E [kindly provided by S. J. Morley; University of Sussex, Brighton, United Kingdom]; anti-phospho-4E-BP1 Thr70, anti-S6K1, and anti-phospho-S6 Ser240/Ser244 (all from Cell Signaling Technology Inc., Beverly, MA); and anti-β-tubulin (Tub2.1; Sigma, St. Louis, MO). “Decorated” proteins were revealed using horseradish peroxidase-conjugated antiniouse or antirabbit immunoglobulins in conjunction with the enhanced chemilumines- cence procedure (Amersham Pharmacia Biotech Inc., Buckinghamshire, United Kingdom).
Affinity Purification of 4E-BP1-eIF-4E Complexes with 7-Methyl-GTP-Sepharose. Rat tumor (1 mg), skin (0.7 mg), or PBMC (0.25 mg) extracts were diluted to a final volume of 1 ml (tumor and skin) or 500 µl (PBMC) with ice-cold extraction buffer and adjusted to a final NP40 concentration of 1%. The 4E-BP1-eIF-4E complexes were affinity purified with 20 µl of 7-methyl-GTP-Sepharose beads (Amersham Pharmacia Biotech Inc., Piscataway, NJ) by gentle rotation for 2.5 h at 4°C. Proteins retained on the beads were washed twice with extraction buffer in the absence of NP40 and resuspended in 15 µl of Laemmli buffer. Dena- tured samples were subjected to 15% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were first immunoblotted for 4E-BP1 protein, followed by stripping as described previously (49) and re-probing for eIF-4E protein (see above).

405 Ribosomal S6 Kinase Assay. Rat tumor (1 mg), skin (0.7 mg), or PBMC (0.25 mg) extracts were diluted to a final volume of 1 ml (tumor and skin) or 500 µl (PBMC) with ice-cold extraction buffer and adjusted to a final NP40 concentration of 1%. Human-derived PBMC extracts (0.8–1 mg) were diluted to a final volume of 750 µl with ice-cold extraction buffer (final NP40 concentration, 1%). In some experiments, human-derived PBMC extracts were first precleared with 20 µl of 50% protein A-Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) by rotating for 20 min at 4°C. S6K1 was immuno-nprecipitated from all extracts by addition of 2.5 µl of the M5 S6K1-specific polyclonal antibody and incubation on ice for 1 h, followed by retrieval of immunocomplexes with 20 µl of 50% protein A-Sepharose. S6K1 activity was measured using rat liver 405 ribosomal subunits as a specific substrate, as described previously (50), except that p-nitrophenyl phosphate was omitted in the reaction mixture. Phosphorylated S6 was resolved by 12.5% SDS-PAGE and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Phosphorylation of S6 was resolved by 12.5% SDS-PAGE and analyzed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Where appropriate, the statistical significance of differences between treatment groups and untreated control groups was determined using ANOVA or ANOVA on ranks followed by the Dunnett test. The level of significance was set at P < 0.05. Statistical calculations were performed using SigmaStat 2.03 (Jandel Scientific). Coefficient of variation is defined as SD divided by the mean and multiplied by 100.

RESULTS

Intermittent RAD001 Treatment Schedules Display Antitumor Efficacy. Short-term exposure to rapamycin in vitro has long-term antiproliferative effects on tumor cell lines (51), suggesting that intermittent treatment schedules may retain antitumor activity. Furthermore, daily oral administration of RAD001 is effective in rat models of autoimmune disease and allotransplantation (47, 52), whereas we have found that weekly (5 mg/kg) RAD001 dosing schedules have reduced immunosuppressive properties in rats as compared with daily treatment (2.5 mg/kg): 66 ± 18% and 98 ± 1% inhibition of IgG antibody response after dinitrophenol-coupled keyhole limpet hemocyanogen immunization, respectively.3 With these observations in mind, we evaluated whether RAD001 treatment schedules, with potentially reduced immunosuppressive properties, could elicit antitumor responses. Daily versus intermittent RAD001 administration schedules were compared using the s.c. CA20948 rat pancreatic tumor model. Vehicle was used as a negative control, and the cytotoxic agent 5-FU was used as a positive control (Fig. 1; Table 1, Experiment 1). RAD001 treatment at 0.5 or 2.5 mg/kg/day, six times a week, resulted in antitumor activity characterized by statistically significant inhibition of tumor growth as compared with vehicle controls [treated tumor versus control tumor size (T/C), 30% and 23%, respectively; P < 0.05 after 10 days of treatment; Fig. 1A; Table 1, Experiment 1]. Statistically significant tumor growth suppression was also observed after intermittent administration of 5 mg/kg RAD001 twice a week (T/C, 36%) or once a week (T/C, 36%). Moreover, all RAD001 treatment schedules suppressed tumor growth to a similar extent as the cytotoxic 5-FU (T/C, 23%). Continued

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3 T. O’Reilly, H. A. Lane, and C. Heusser, unpublished data.

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Fig. 1. Suppression of tumor growth by daily and intermittent dosing schedules of RAD001. Tumors were established in male Lewis rats by s.c. injection of CA20948 tumor suspension obtained from donor rats. Treatments started on day 4 after inoculation. Formulated RAD001 was diluted in a 5% glucose solution and administered p.o. daily at a dose of 0.5 or 2.5 mg/kg (qd × 6, 6 times/week) or once (wk × 1) or twice (wk × 2) weekly at 5 mg/kg RAD001. Vehicle and 5-fluorouracil (5-FU; 4 times/week) were administered as negative and positive controls, respectively. Tumor volumes were measured (A), and rats were weighed (B) as described in "Materials and Methods." Vehicle control-treated rats were sacrificed on day 10 due to tumor burden. Data are means ± SEM (n = 7–8 animals/group). Stars represent P < 0.05 versus vehicle controls.
24 h later, and protein extracts were prepared from tumors, skin, and PBMCs. By immunoblot analysis, mTOR could be detected in tumor and PBMC extracts; however, neither mTOR expression nor phosphorylation on Ser2448 was modified on RAD001 treatment. In contrast, 4E-BP1 exhibited a decrease in Thr70 phosphorylation in skin, and PBMC extracts (Fig. 2A), a phenomenon associated with changes in 4E-BP1 electrophoretic mobility, particularly striking in PBMCs. This observation is consistent with previous work demonstrating dephosphorylation of 4E-BP1 on Thr70 in tumors derived from mouse xenograft models after five daily treatments with an ester of rapamycin CCI-779 (1 h after last administration; Ref. 53). Interestingly, the phosphorylation of another rapamycin-sensitive residue (Ser65; Refs. 5, 34, and 35) was unaffected by RAD001 treatment, indicating that RAD001-insensitive phosphorylation of this site can occur as reported previously (54).

To determine whether the decreased phosphorylation state of 4E-BP1 resulted in a change in functionality, the elf4e-4E binding activity of 4E-BP1 was assessed using an in vitro 7-methyl-GTP-binding assay (Fig. 2B). Whereas similar levels of elf4e-4E were recovered in the control- and RAD001-treated extracts, in two animals increased elf4e-4E-BP1 complex formation was clearly observed in skin and PBMC samples after RAD001 treatment. In tumor samples, two electrophoretically distinct forms of 4E-BP1 protein were bound to elf4e-4E in vehicle control-treated rats (Fig. 2B). After RAD001 treatment, only the lower migrating form was found bound to elf4e-4E, with an associated loss of the upper band consistent with reduced 4E-BP1 phosphorylation levels (Fig. 2A). A similar 4E-BP1 doublet with elf4e-4E binding activity has been observed previously in proliferating cells/tissue (29, 54) and presumably reflects differential 4E-BP1 phosphorylation states within the proliferating tumor.

To further assess the effect of RAD001 administration on the mTOR pathway, S6K1 protein and activity levels were also analyzed (Fig. 2, C and D). Whereas S6K1 protein levels were unaffected by RAD001 treatment (Fig. 2C), in vitro kinase assay using 40S ribosomal subunits as a substrate revealed a statistically significant reduction in S6K1 activity in all extracts [Fig. 2D; 83% (tumors), 80% (skin), and 75% (PBMC); all P < 0.05 versus vehicle-treated controls]. This reduction in S6K1 activity was associated with the dramatic dephosphorylation of its physiological substrate, 40S ribosomal protein S6, in tumor extracts (Fig. 2C). A similar reduction was not observed in skin and PBMC extracts because these tissues exhibited no detectable S6 phosphorylation in control animals. Interestingly, a
reduction in S6 protein expression was observed in RAD001-treated skin, but not in tumor or PBMC extracts. A similar phenomenon has been reported previously in tumors after treatment of mice bearing human prostate cancer xenografts with CCI-779 (24). Moreover, the translation of S6 (as a 5′-terminal oligopyrimidine tract mRNA) has been shown to be specifically inhibited by rapamycin in 3T3 cells (36). It is not known why, in this model, RAD001 treatment only has effects on S6 expression in skin; however, differential downstream effects of mTOR pathway inhibition, depending on the tissue source, are a plausible possibility (54). Taken together, these data demonstrate that both 4E-BP1 and S6K1 pathways are affected in tumors, skin, and PBMC samples obtained from CA20948 tumor-bearing rats after a single administration of an efficacious dose of RAD001.

Prolonged Inactivation of S6K1 in Tumors, Skin, and PBMCs Correlates with the Efficacy of Intermittent RAD001 Treatment Schedules. To investigate whether the antitumor efficacy of intermittent RAD001 treatment schedules is associated with prolonged effects on the mTOR pathway, CA20948 tumor-bearing rats were treated with a single dose of RAD001 (5 mg/kg) or vehicle, and tumor, skin, and PBMC extracts were prepared 12, 24, 48, or 72 h after administration. Because S6K1 was significantly inactivated 24 h after a single RAD001 administration in all tissues analyzed (Fig. 2D), long-term effects on mTOR function were assessed using the 40S kinase assay (Fig. 3). Tumor and skin extracts were obtained from each of 3 rats/treatment group, whereas PBMC extracts were obtained from pooled blood from each treatment group. A dramatic reduction in S6K1 activity was already observed in tumors, skin, and PBMCs 12 h after RAD001 administration (91%, 91%, and 82% inhibition, respectively; all \(P < 0.05\) versus untreated controls; Fig. 3). In contrast, treatment with vehicle did not significantly modulate S6K1 activity as compared with untreated controls (Fig. 3). Moreover, RAD001 treatment resulted in the sustained inactivation of S6K1 in all tissues. In tumors, statistically significant inhibition of S6K1 was maintained up to 72 h after administration, with some evidence of recovery after 72 h (80% and 62% inhibition at 48 and 72 h, respectively; Fig. 3A). In comparison, S6K1 derived from skin samples remained significantly inhibited for at least 72 h (72% inhibition at 72 h; Fig. 3B). Although a statistical analysis could not be performed on the pooled PBMC samples, S6K1 activity was also dramatically inhibited for up to 72 h in these samples (82% inhibition at 72 h; Fig. 3C). Thus, consistent with the antitumor efficacy of intermittent 5 mg/kg RAD001 treatment schedules in CA20948 tumor-bearing rats, a single administration of 5 mg/kg RAD001 resulted in long-term inactivation of S6K1 in tumors, skin, and PBMCs.

The Antitumor Efficacy of Intermittent RAD001 Treatment Schedules Is Dose Dependent: Correlation Between Efficacy and Prolonged Effects on mTOR Effectors in Rat PBMCs. Following the observation that intermittent RAD001 (5 mg/kg) treatment schedules significantly inhibited tumor growth, we explored the effect of RAD001 dose on the efficacy of weekly treatment schedules (Table 1, Experiments 2 and 3). As expected, 5 mg/kg/week RAD001 significantly suppressed CA20948 tumor growth as compared with vehicle controls (T/C, 14% and 24% at 7 and 8 days, respectively; \(P < 0.05\)). In contrast, although 0.5 mg/kg RAD001 caused a significant inhibition of tumor growth when administered daily (T/C, 23%), weekly administration of the same dose did not significantly affect tumor growth (T/C, 48%; \(P > 0.05\)). This apparent dose dependency of weekly RAD001 schedules was confirmed by a more stringent analysis comprising doses between 0.5 and 5 mg/kg (Table 1, Experiment 3). Statistically significant antitumor responses were observed with 3 and 2 mg/kg RAD001 (T/C, 36% and 32%, respectively), but not with 1 mg/kg (T/C, 45%). Interestingly, 3 mg/week elicited a similar antitumor response (T/C, 36%) as 0.5 mg/kg/day (×6/week; T/C, 30% and 23%). Because both these schedules involve administration of 3 mg/kg RAD001 per week, these data indicate that, with the same total RAD001 exposure, intermittent dosing schedules can elicit equivalent antitumor responses as daily schedules.

To further investigate the dose dependency of weekly schedules in terms of effects on mTOR signaling in a surrogate tissue, the duration of S6K1 inactivation in response to a single administration of 0.5 versus 5 mg/kg RAD001 was determined in PBMCs derived from three non-tumor-bearing rats (Fig. 4, A and B). Whereas in vehicle controls, no effect on S6K1 activity could be observed (24 h after administration), a single administration of 5 mg/kg RAD001 resulted in statistically significant, prolonged inactivation of the S6K1 for up to 7 days (99% and 86% inhibition after 24 h and 7 days, respectively; \(P < 0.05\)). In comparison, 0.5 mg/kg RAD001 caused a significant inhibition of PBMC-derived S6K1 activity 24 h after administration.
Dose-dependent effects of RAD001 on ribosomal protein S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) in peripheral blood mononuclear cells (PBMCs) obtained from non-tumor-bearing rats. Rats were treated with a single optimal (A and C) versus suboptimal (B and D) RAD001 dose (5 and 0.5 mg/kg, respectively) or vehicle (3 rats/group). At the times indicated, PBMC samples were collected and individually extracted. A and B. S6K1 was immunoprecipitated from equal amounts of protein extract, and S6K1 activity was assayed using 40S ribosomal subunits as a substrate. PhosphorImager quantification of the kinase assays are presented (means ± SD of n = 3 animals/group). Stars represent P < 0.05 versus untreated controls (Dunnett test). C and D. equal amounts of PBMC extracts were resolved by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, and probed for 4E-BP1 protein, phospho-threonine 70 4E-BP1 (P-4E-BP1 Thr70), or β-tubulin as a loading control. Arrows denote hypophosphorylated (bottom arrow) and hyperphosphorylated (top arrow) forms of 4E-BP1 protein.

Extracts: RAD001 Induces Concentration-Dependent S6K1 Inactivation Ex Vivo. To evaluate the potential of using mTOR effectors as biomarkers to evaluate RAD001 treatment schedules, we assessed whether basal S6K1 activity could also be measured in human PBMC extracts obtained from healthy volunteers. Human blood was collected into tubes containing either sodium citrate or EDTA as an anticoagulant, and PBMC extracts were prepared. Subsequent assay of S6K1 activity demonstrated that activity could indeed be detected in non-challenged human PBMCs derived from unrelated donors (Fig. 6A). Interestingly, S6K1 activity was reproducibly higher when the blood was initially collected in EDTA as compared with sodium citrate, a phenomenon potentially related to the different chelating properties of these anticoagulants. Using EDTA, a coefficient of variation of 10% was obtained among six assays on PBMC extracts prepared separately from the same blood donor, indicating good reproducibility of preparation (Fig. 6B). Accordingly, equivalent S6K1 protein levels were detected in the same extracts by immunoblot analysis (Fig. 6B). These results demonstrate that, in analogy with the rat PBMC data, basal S6K1 activity can be detected in human PBMCs. However, unlike control rat PBMC extracts (see Fig. 2A), there was no evidence of Thr70 phosphorylation in any of the human PBMC extracts analyzed, an observation correlating with the fact that most of the 4E-BP1 protein was present in the hypophosphorylated/fast migrating state (when compared with 4E-BP1 derived from proliferating human tumor cells; Fig. 7A, DMSO). Ex vivo treatment of whole blood with 20 nM RAD001 for 30 min did not further increase protein mobility (Fig. 7A, RAD001), suggesting that 4E-BP1 is largely active as a translational repressor in human PBMCs. Hence, unlike the situation...
protein extracts were analyzed by immunoblot for S6K1 and duplicate kinase assays are presented. As internal controls, equal amounts of PBMC/H11350 inhibition with concentrations led to almost complete inactivation of S6K1 (donor 1 and 2, respectively). Furthermore, increasing RAD001 concentrations of RAD001 for 30 min, followed by isolation, extraction, and assay of PBMC-derived S6K1 activity (Fig. 7A). Treatment with 2 nM RAD001 diminished PBMC-derived S6K1 activity as compared with DMSO vehicle controls (44% and 63% inhibition in donor 1 and 2, respectively). Furthermore, increasing RAD001 concentrations led to almost complete inactivation of S6K1 (>95% inhibition with >20 nM RAD001 in donor 1 and 2). These results demonstrate that RAD001 treatment of human blood ex vivo results in a concentration-dependent inactivation of PBMC-derived S6K1, supporting the notion that changes in PBMC-derived S6K1 activity could serve as a biomarker when assessing treatment schedules with rapamycin derivatives such as RAD001 in clinical trials for cancer.

**DISCUSSION**

The mTOR pathway plays a major role in cell proliferation by coupling cell growth with G1-S progression. Compounds targeting the mTOR pathway have potential, therefore, for application in cancer treatment modalities (2, 3, 4). In this context, RAD001 potently inhibits the proliferation of numerous tumor cell lines in vitro and inhibits the growth of a range of human xenografts in nude mice (2, 27, 43–46). Rapamycin and the rapamycin ester CCI-779 also present antitumor activity in a number of animal models of cancer (2, 24–26, 53, 55–59). However, although human pancreatic tumors have been reported in abstract form to be sensitive to CCI-779 (reviewed in Ref. 2), and mTOR/S6K1 signaling appears to be required for pancreatic cancer cell proliferation (60, 61), the work presented here is the first full publication demonstrating significant antitumor efficacy of a rapamycin derivative in an animal model of pancreatic cancer. Orally administered RAD001 was found to be well tolerated and to elicit antitumor potency equivalent to that of the cytotoxic agent 5-FU. Moreover, similar responses were achieved with daily or weekly RAD001 administrations, indicating that frequent drug administration is unnecessary to maintain an antitumor response. Although weekly rapamycin dosing schedules have been used previously (55, 56), a comparative analysis addressing the efficacy of daily versus weekly administration had not been performed. The fact that weekly RAD001 administration produces statistically significant antitumor responses in the CA20948 model is supported by a number of experimental observations. First, in vitro pulse treatment with either RAD001 (43) or rapamycin (51) causes prolonged down-regulation of the mTOR pathway in tumor cell lines. Indeed, Hosoi et al. (51) postulated that this phenomenon was due to the slow dissociation rate of the rapamycin-FKBP12 complex. Second, prolonged effects of CCI-779 on xenograft tumor growth were evident after cessation of daily treatment schedules (24, 53, 57), and antitumor responses have been

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* A. Boulay, T. O’Reilly, and H. A. Lane, unpublished data.
reported in Phase I clinical trials with weekly CCI-779 administration (2, 4).

One advantage of administering RAD001 intermittently in oncology is the avoidance of prolonged immunosuppression (1). In this context, the minimal effective dose of RAD001 in stringent rat kidney and heart allotransplantation models is ≥5 mg/kg administered daily (47, 52). Moreover, the immunosuppressive capacity of RAD001 (everolimus in combination with cyclosporin) in transplant patients has been related to maintenance of blood drug trough levels (1, 62), suggesting that constant drug exposure is required to provide clinically relevant immunosuppression. The demonstration that weekly administration of RAD001 (at doses of 2–5 mg/kg) is sufficient to elicit a significant antitumor response indicates that the above premise does not apply to oncology. Indeed, in support of this notion, as compared with daily RAD001 administration (2.5 mg/kg), a 5 mg/kg weekly RAD001 regimen allows a 20-fold higher T-cell-dependent antibody response, as measured by serum IgG antibody titers after immunization of rats with dinitrophenol-coupled keyhole limpet hemocyanogen.3 Hence, intermittent dosing allows for differentiation between immunosuppressive and antitumor effects, a possibility also suggested from preliminary clinical data (2, 4). The basis of this is presumably related to the biology of T cells as compared with tumor cells. In this respect, rapamycin potently prevents resting T cells from entering the cell cycle in response to interleukin 2 but has little effect on proliferating T cells (63, 64). This may explain why constant drug exposure is required in the immunosuppression setting, as opposed to the antitumor setting where the proliferation of cycling tumor cells is potently inhibited (2, 4) for long periods (51). This possibility is worthy of further investigation.

A limited analysis of the effects of rapamycin derivatives on mTOR effectors in tumor material derived from xenograft models was reported previously (24, 53). Until now, however, a comprehensive analysis had not been performed. Similarly, the possibility that the efficacy of intermittent treatment schedules correlates with long-term effects on the mTOR pathway in tumors and surrogate tissues had not been addressed. This prompted us to profile RAD001-mediated effects on mTOR signaling in CA20948 tumors and normal rat tissues. Mitogen-induced, multisite phosphorylation of the translational suppressor protein eIF-4E-BP1 is known to cause its release from the initiation factor eIF-4E, thereby facilitating formation of the eIF-4F complex and derepression of cap-dependent mRNA translation (2, 5). Indeed, the 4E-BP1 protein has been proposed to be a direct substrate for the mTOR kinase (34, 41, 42). Moreover, rapamycin treatment of cell lines decreases 4E-BP1 phosphorylation, resulting in increased affinity for eIF-4E in vitro (2, 5). Consistent with these observations, a single administration of 5 mg/kg RAD001 to three tumor-bearing rats reproducibly inhibited 4E-BP1 phosphorylation in tumors, skin, and PBMCs at 24 h, in accordance with changes in 4E-BP1 electrophoretic mobility and increased 4E-BP1/eIF-4E association. In the same animals, S6K1 signaling was virtually abolished in all tissues. The physiological downstream target of the S6K1 is the S6 40S ribosomal protein (12, 65). Hence, reductions in S6 phosphorylation are expected to parallel S6K1 inactivation, as observed in CA20948 tumor extracts. However, because S6 phosphorylation could not be detected in either skin or PBMC control extracts, no such correlation could be made in these tissues. This failure to detect S6 phosphorylation could reflect a reduced proliferation index as compared with the aggressively growing CA20948 tumors. Strikingly, and in agreement with previous in vitro analyses (43, 51), tumors, skin, and PBMC extracts derived from rats treated with a single 5 mg/kg RAD001 dose demonstrated prolonged inactivation of the S6K1 for ≥72 h. Taken together, these data suggest that RAD001-specific effects on 4E-BP1 and S6K1 activity can be reproducibly observed in tumors and surrogate tissues. Moreover, long-term effects of RAD001 on S6K1 activity occur with a dose of RAD001 known to elicit significant antitumor responses with intermittent treatment schedules.

The observation that the mTOR pathway is affected for long periods of time in tumors and PBMCs is consistent with preliminary pharmacokinetic studies performed in CA20948 tumor-bearing rats. Pharmacokinetic measurements after a single RAD001 administration (5 mg/kg, over a 72 h period) demonstrated good bioavailability/efficient tumor penetration (maximal concentrations in blood and tumor, ~200 and ~700 nm, respectively) and prolonged residency [RAD001 half-life, ~20–22 h].7 Unfortunately, a precise correlation of pharmacokinetic parameters with antiproliferative effects in tumors is difficult in this model because of the inability to determine in vitro IC50 values with the nonculturable CA20948 line. However, the efficient tumor accumulation and relatively long half-life of RAD001 provide further rationale for the long-term effects observed in this model.

Sequential tumor sampling is difficult in the clinical setting, necessitating some reliance on surrogate tissue to assess pharmacodynamic effects of antitumor agents. For this reason, the possibility of using PBMCs as a source for biomarker analysis when assessing RAD001 treatment schedules was evaluated. Detailed efficacy experiments demonstrated that antitumor response to weekly administration of RAD001 was dose dependent. Moreover, significant antitumor responses were associated with long-term effects on the mTOR pathway in PBMCs. Interestingly, PBMC-derives 4E-BP1 was unaffected by a suboptimal RAD001 dose (0.5 mg/kg), despite transient effects on S6K1 activity. This suggests that S6K1 is a more sensitive marker of RAD001 exposure in PBMCs than 4E-BP1. Indeed, all doses of RAD001 evaluated elicited a dramatic inhibition of PBMC-derived S6K1 after 24 h. However, the rate at which S6K1 activity subsequently recovered differed, with RAD001 doses that were efficacious with weekly schedules causing more profound long-term effects on S6K1 activity (≥7 days). The demonstration that the mTOR pathway is affected in PBMCs for a week after administration of 5 mg/kg RAD001 may be interpreted as being contrary to our observations that weekly treatment with this dose is suboptimal in terms of suppression of T-cell-dependent antigen responses. To reconcile these observations, one has to consider that T- and B-cell proliferative responses to foreign antigen presentation occur mainly in the secondary lymphoid organs (64). Here we assayed S6K1 derived from PBMCs, a source that does not reflect the situation in these organs. We therefore speculate that, using weekly schedules, there is a possibility to recover T-cell responses, a phenomenon that may also reflect the pharmacokinetic characteristics of RAD001.

To most efficiently exploit the pharmacological profile of targeted agents such as RAD001, it is important to carefully monitor the dose given to a cancer patient, especially considering the observation that rapamycin can be less effective as an antitumor agent in animal models if overdosed (59). The ease of human PBMC preparation suggests that this could be a valuable surrogate tissue when establishing treatment regimens for RAD001 in clinical trials for oncology. Based on this premise, S6K1 activity could be reproducibly assayed in PBMC extracts prepared from healthy volunteers, and RAD001 treatment of whole blood ex vivo resulted in concentration-dependent inactivation of the kinase. In contrast, despite promising results in tumor extracts derived from xenograft models (53) and suggestions that 4E-BP1 phosphorylation could be used as a confirmatory measure of mTOR inhibition in PBMCs (66), we have shown that 4E-BP1 phosphorylation cannot be detected in human PBMCs. During the

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7 T. O’Reilly and L. McMahon, unpublished data.
revision of this manuscript, others (66, 67) also reported on the potential for PBMC-derived S6K1 activity measurements to aid pharmacodynamic evaluation of rapamycin derivatives. Analysis of cancer patient-derived PBMCs after i.v. administration of 25, 75, and 250 mg CCI-779 demonstrated inactivation of PBMC-derived S6K1 for up to 8 days, with no evidence of dose dependency at the doses used (66–68). Although a limited feasibility study in nine patients indicated an association between time to disease progression and the degree of inhibition of S6K1 24 h after CCI-779 administration, no conclusions were drawn regarding the predictive nature of this biomarker or associated implications of the long-term S6K1 inactivation observed in patients (67). Our data provide a strong experimental rationale for analyzing long-term effects on PBMC-derived S6K1 activity when establishing weekly administration schedules. Indeed, recent Phase I trials with weekly administration of RAD001 in patients with advanced cancer have demonstrated a clear association between RAD001 dose and the recovery of PBMC-derived S6K1 activity over a 2-7-day period (69). The value of these observations in terms of prediction of patient response is now being pursued in clinical trials of RAD001 in oncology.

ACKNOWLEDGMENTS

We thank Dr. N. Sonenberg (McGill University, Montreal, Canada) for kindly supplying the 4E-BP1 antibody, Dr. S. J. Morley (University of Sussex, Brighton, United Kingdom) for kindly supplying the eIF-4E antibody, and Dr. J. Mestan (Oncology Research, Novartis Pharma AG, Basel, Switzerland) for supplying the S6 antibody. We thank Drs. W. Schuler and R. Sedrani (Transplantation Research, Novartis Pharma AG) for providing RAD001 and much appreciated advice. We thank Beatrice Engriser (Johnson Control, Basel, Switzerland) for her contribution to this study, and Drs. S. M. Maira and P. Fuerst (Oncology Research, Novartis Pharma AG) for comments on the manuscript. We extend special thanks to the volunteers who provided blood samples for this study.

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RAPAMYCIN DERIVATIVE RAD001: EFFICACY AND BIOMARKERS


Antitumor Efficacy of Intermittent Treatment Schedules with the Rapamycin Derivative RAD001 Correlates with Prolonged Inactivation of Ribosomal Protein S6 Kinase 1 in Peripheral Blood Mononuclear Cells

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