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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 treatment 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 (≥72 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 G_1 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 G_1 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 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 G_1- 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

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translational machinery. Activation of S6K1 itself is also tightly regulated by hierarchical phosphorylation events, which are dependent 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). Immunopurified 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* 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 antitumor 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 Iffa Credo (L'Abresque, 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 $NaHCO_3$, 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 mm^3. RAD001 was administered 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.v., 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 [$V = \pi/6\ (d_1 \times d_2 \times d_3)$, where d_1, d_2, and d_3 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 mm^3 or when skin overlaying the tumor exhibited evidence of necrosis. All protocols involving animals were approved by the Veterinäramt of Baselstadt, 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 significance 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 Extract 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 NaF, 1 mM EDTA, 6 mM EGTA, 15 mM PP_i, 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 non-tumor-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* treatments, treated with 2, 20, and 200 nM 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% CO_2. 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 antibodies: 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-eIF-4E (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 antimouse or antirabbit immunoglobulins in conjunction with the enhanced chemiluminescence 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 500 μl in ice-cold extraction buffer and adjusted to a final NP40 concentration of 0.1%. 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. Denatured 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 reprobing for eIF-4E protein (see above).

40*S* 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 immunoprecipitated 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 40*S* 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). [γ-^{32}P]phosphate incorporation into S6 was quantified using ImageQuant (Molecular Dynamics). 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. 1*A*; 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 treatment with RAD001 after vehicle controls were sacrificed due to tumor burden led to a prolonged low tumor growth rate with all treatment schedules, resulting in similar tumor burden after 17 days of treatment as compared with 5-FU (Fig. 1*A*). For all treatment schedules, RAD001 was well tolerated, with no significant body weight loss or mortalities observed (Fig. 1*B*; Table 1, Experiment 1). These results demonstrate that RAD001 is a well-tolerated antitumor agent in a rat model of pancreatic cancer and indicate a potential for intermittent administration schedules that may allow dissociation of antitumor from immunosuppressive effects.

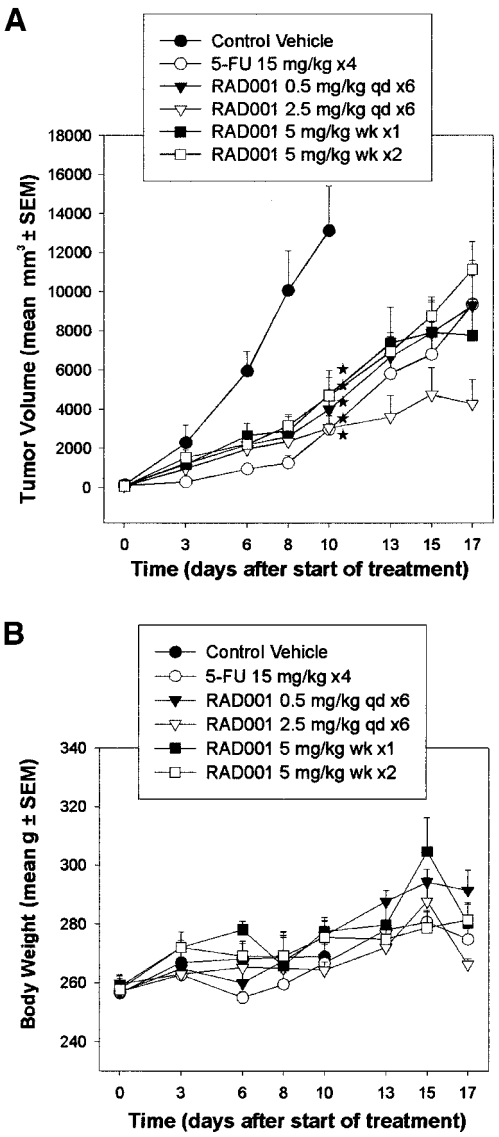

Fig. 1. Suppression of tumor growth by daily and intermittent dosing schedules of RAD001. Tumors were established in male Lewis rats by s.c. injection of CA20948 tumor suspension obtained from donor rats. Treatments started on day 4 after inoculation. Formulated RAD001 was diluted in a 5% glucose solution 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; 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.

RAD001 Modulates 4E-BP1 and S6K1 Activity in Tumor, Skin, and PBMCs Obtained from CA20948 Pancreatic Tumor-Bearing Rats. To investigate RAD001-specific effects on mTOR signaling *in vivo*, three CA20948 tumor-bearing rats were treated with vehicle or a single efficacious dose of RAD001 (5 mg/kg). Rats were sacrificed

[3] T. O'Reilly, H. A. Lane, and C. Heusser, unpublished data.

Table 1 *Effect of daily and intermittent RAD001 administration on CA20948 rat pancreatic tumor-bearing rats*

Compound	Schedule	Tumor response		Host response		
		% T/C[a]	Δ Tumor volume (mm^3)	Δ Body weight (g)	%Δ Body weight	Survival (alive/total)
Experiment 1						
Vehicle	2 ml/kg p.o. daily	100	12972 ± 2188	12 ± 8	5	8/8
5-FU	15 mg/kg i.v. 4× weekly	23	2863 ± 764[b]	18 ± 4	7	7/7
RAD001	0.5 mg/kg p.o. daily	30	3904 ± 856[b]	35 ± 7	14	7/7
RAD001	2.5 mg/kg p.o. daily	23	2959 ± 624[b]	7 ± 2	3	7/7
RAD001	5 mg/kg p.o. weekly	36	4652 ± 1220[b]	22 ± 5	8	7/7
RAD001	5 mg/kg p.o. twice weekly	36	4604 ± 928[b]	21 ± 3	8	7/7
Experiment 2						
Vehicle	2 ml/kg p.o. daily	100	12331 ± 1410	29 ± 2	14	8/8
RAD001	0.5 mg/kg p.o. daily	23	2894 ± 567[b]	30 ± 5	17	8/8
RAD001	0.5 mg/kg p.o. weekly	48	5951 ± 1739	36 ± 2	15	8/8
RAD001	5 mg/kg p.o. weekly	14	1708 ± 339[b]	32 ± 2	15	8/8
Experiment 3						
Vehicle	2 ml/kg p.o. weekly	100	19270 ± 3918	28.3 ± 2.1	10	8/8
RAD001	0.5 mg/kg p.o. weekly	48	9275 ± 1926	21 ± 2.4	8	8/8
RAD001	1 mg/kg p.o. weekly	45	8617 ± 1704	32.8 ± 2.7	12	8/8
RAD001	2 mg/kg p.o. weekly	32	6161 ± 1079[b]	24.9 ± 1.9	9	8/8
RAD001	3 mg/kg p.o. weekly	36	6869 ± 611[b]	24.3 ± 3.3	9	6/6
RAD001	5 mg/kg p.o. weekly	24	4680 ± 1593[b]	22.8 ± 1.7	8	8/8

[a] T/C, treated tumor *versus* control tumor size.
[b] $P < 0.05$ *versus* control, Dunnett test.

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.[4] In contrast, 4E-BP1 exhibited a decrease in Thr70 phosphorylation in tumor, skin, and PBMC extracts (Fig. 2*A*), 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,[4] 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 eIF-4E binding activity of 4E-BP1 was assessed using an *in vitro* 7-methyl-GTP-binding assay (Fig. 2*B*). Whereas similar levels of eIF-4E were recovered in the control- and RAD001-treated extracts, in two animals increased eIF-4E·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 eIF-4E in vehicle control-treated rats (Fig. 2*B*). After RAD001 treatment, only the lower migrating form was found bound to eIF-4E, with an associated loss of the upper band consistent with reduced 4E-BP1 phosphorylation levels (Fig. 2*A*). A similar 4E-BP1 doublet with eIF-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. 2*C*), *in vitro* kinase assay using 40*S* ribosomal subunits as a substrate revealed a statistically significant reduction in S6K1 activity in all extracts [Fig. 2*D*; 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, 40*S* ribosomal protein S6, in tumor extracts (Fig. 2*C*). A similar reduction was not observed in skin and PBMC extracts because these tissues exhibited no detectable S6 phosphorylation in control animals. Interestingly, a

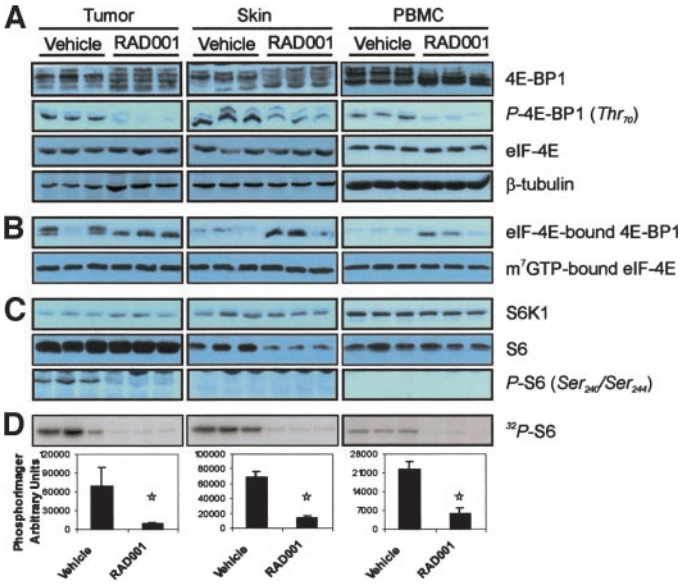

Fig. 2. RAD001 administration inhibits mammalian target of rapamycin signaling in CA20948 tumor-bearing rats. S.c. CA20948 tumor-bearing rats received a single administration of an efficacious dose of RAD001 (5 mg/kg) or vehicle and were sacrificed 24 h after administration (3 rats/group). Tumors, skin, and PBMCs were individually prepared and extracted as described in "Materials and Methods." Results from individual rats are presented. *A* and *C*, total protein was subjected to electrophoresis followed by immunoblot analysis. Membranes were probed for eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and phospho-threonine 70 4E-BP1 [*P-4E-BP1 (Thr$_{70}$)*] levels, with eukaryotic initiation factor 4E (eIF-4E) and β-tubulin levels acting as loading controls (*A*) or ribosomal protein S6 kinase 1 protein, S6 40*S* ribosomal protein, and phospho-serine 240/244 S6 [*P*-S6 (*Ser$_{240}$/Ser$_{244}$*)] levels (*C*). *B*, the level of 4E-BP1 bound to eIF-4E was measured by purification of 4E-BP1·eIF-4E complexes on 7-methyl-GTP-Sepharose, as described in "Materials and Methods," followed by immunoblot analysis. *D*, ribosomal protein S6 kinase 1 was immunoprecipitated from equal amounts of total protein extract, and activity was measured by *in vitro* kinase assay using 40*S* ribosomal subunits as a specific substrate, as described in "Materials and Methods." Phosphorimages (^{32}*P-S6*) and PhosphorImager quantifications of the kinase assay are presented. Data are means ± SD of $n = 3$ animals/group. *Stars* represent $P < 0.05$ *versus* vehicle-treated controls (Dunnett test).

[4] A. Boulay and H. A. Lane, unpublished data.

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. 2*D*), long-term effects on mTOR function were assessed using the 40*S* 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 48 h after administration, with some evidence of recovery after 72 h (80% and 62% inhibition at 48 and 72 h, respectively; Fig. 3*A*). In comparison, S6K1 derived from skin samples remained significantly inhibited for at least 72 h (72% inhibition at 72 h; Fig. 3*B*). 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. 3*C*). 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 5 and 0.5 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.

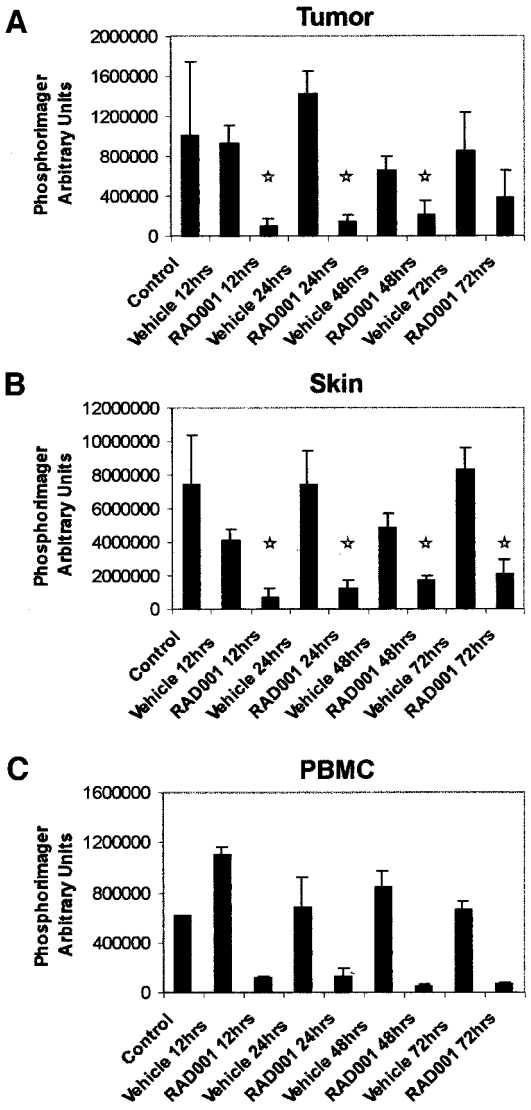

Fig. 3. RAD001 administration (5 mg/kg) causes prolonged inactivation of ribosomal protein S6 kinase 1 in tumors, skin, and PBMCs derived from CA20948 tumor-bearing rats. CA20948 tumor-bearing rats were treated once with 5 mg/kg RAD001 or vehicle (3 rats/group). After 12, 24, 48, and 72 h, tumor and skin samples were individually extracted. Blood obtained from rats within each treatment group was pooled, and peripheral blood mononuclear cells (PBMCs) were isolated and extracted. Assay of ribosomal protein S6 kinase 1 activity was performed using 40S ribosomal subunits as *in vitro* substrate. PhosphorImager quantifications of the S6 kinase assays are presented. *A* (*Tumor*) and *B* (*Skin*): data are means ± SD of $n = 3$ animals/group. *Stars* represent $P < 0.05$ *versus* untreated controls (Dunnett test). *C* (*PBMC*): data are means; *error bars* represent the range of duplicate assays.

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

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