RAD001 (everolimus) inhibits tumour growth in xenograft models of human hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and highly resistant to available chemotherapies. Mammalian target of rapamycin (mTOR) functions to regulate protein translation, angiogenesis and cell cycle progression in many cancers including HCC. In the present study, subcutaneous patient-derived HCC xenografts were used to study the effects of an mTOR inhibitor, RAD001 (everolimus), on tumour growth, apoptosis and angiogenesis. We report that oral administration of RAD001 to mice bearing patient-derived HCC xenografts resulted in a dose-dependent inhibition of tumour growth. RAD001-induced growth suppression was associated with inactivation of downstream targets of mTOR, reduction in VEGF expression and microvessel density, inhibition of cell proliferation, up-regulation of p27^{Kip1} and down-regulation of p21$^{Cip1/Waf1}$, Cdk-6, Cdk-2, Cdk-4, cdc-25C, cyclin B1 and c-Myc. Our data indicate that the mTOR pathway plays an important role in angiogenesis, cell cycle progression and proliferation of liver cancer cells. Our study provides a strong rationale for clinical investigation of mTOR inhibitor RAD001 in patients with HCC.

Keywords: liver cancer ● angiogenesis ● mTOR ● therapy

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and third highest cause of global cancer mortality [1]. More than 80% of HCC patients present at advanced disease stages where surgery is not an option, and in cases where surgical resections are performed, the 2-year recurrence rate is still as high as 50% [2, 3]. Although several chemotherapeutic agents have been used in HCC therapy, no adjuvant or palliative treatment modalities have been shown to be particularly effective (reviewed in ref. [4]). Large phase III studies have shown a 4–10.5% response rate in HCC treated with

doxorubicin [5, 6]. Recently, sorafenib (Nexavar®, Bayer and Onyx Pharmaceuticals, Leverkusen, Germany), which inhibits vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor, Raf, c-kit and flt-3, has been shown to inhibit HCC-induced proliferation and angiogenesis [7–9] and provides a significant improvement in overall survival in patients with HCC [10], suggesting that this class of agents may be effective in the treatment of HCC.

Cell growth and proliferation in multiple cancer types are regulated by mammalian target of rapamycin (mTOR) pathway though p70S6 kinase (p70S6K), ribosomal S6 protein (S6R), and eukaryotic initiation factor 4E-binding protein 1 (4EBP1) [11]. It has been reported that the *PIK3CA gene,* encoding p110α of the class IA PI3K, was mutated in approximately 35.6% of HCC cases [12]. The *PTEN* gene that negatively regulates phosphatidylinositol 3′-kinase (PI3K) was mutated, silenced or deleted in a number of tumour types including HCC (reviewed in ref. [13]). Tumours displaying aberrantly high PI3K-dependent signalling often displayed enhanced sensitivity to mTOR inhibitors (reviewed in ref. [14]).

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mTOR activation has been shown to be important for the secretion of VEGF [15]. Rapamycin could inhibit tumour growth [16], progression [17] and metastasis [18] through anti-angiogenic activity related to impaired production of VEGF or inhibition of cell proliferation [19]. In HCC, total p70S6K expression was positively correlated with tumour nuclear grade, and inversely correlated with tumour size [20]. Complete remission of lung metastases [21] and tumour-free survival [22] were observed in patients on rapamycin after a liver transplant. These observations make mTOR and its downstream targets exciting novel targets for HCC therapy. mTOR inhibitors are currently in clinical trials as novel anti-cancer agents [23–25].

RAD001 (Everolimus) is a novel mTOR inhibitor that exhibits improved aqueous solubility for oral administration [26]. RAD001 is efficacious in both preclinical and clinical transplant trials [27]. RAD001 therapy was shown to inhibit the growth of cell lines [28] and tumour growth [29], improve the efficacy of replicating adenoviruses that target colon cancer [30], block the cell cycle [31] and sensitize tumour cells to cisplatin-induced apoptosis [32].

In the present study, we report that RAD001 potently inhibited tumour growth through inhibition of downstream targets of mTOR, angiogenesis and positive cell cycle regulators.

Material and methods

Reagents

RAD001 (everolimus) was supplied by Novartis, Institutes for Biomedical Research, Oncology, Basel, Switzerland. Antibodies against p70S6K, Akt, cleaved caspase-3, mTOR, S6R, 4EBP1 and phosphorylation-specific antibodies against ERK1/2 Thr^{202}/Tyr^{204}, mTOR Ser^{2448}, p70S6K Thr^{421}/Ser^{424} and Thr^{389}, S6R Ser$^{235/236}$ and Ser$^{240/244}$, 4EBP1 Thr^{70} and Ser$^{37/46}$, Akt Ser^{308} and Ser^{473} were obtained from Cell Signaling Technology (Beverly, MA, USA). The antibodies against cyclin D1, cyclin B1, cyclin A, Cdk-2, Cdk-4, Cdk-6, cdc-2, p21$^{Cip1/Waf1}$, p16^{INK4a} and p27^{Kip1} and α-tubulin were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). CD31, VEGF and Ki-67 antibodies were from Lab Vision (Fremont, CA, USA). The chemiluminescent detection system was supplied by Amersham, Pharmacia Biotech (Arlington Heights, IL, USA).

Effects of RAD001 on the growth of subcutaneous patient-derived HCC xenografts

The study received ethics board approval at the National Cancer Centre of Singapore and Singapore General Hospital. All mice were maintained according to the 'Guide for the Care and Use of Laboratory Animals' published by the National Institutes of Health, USA.

Patient-derived HCC xenografts were subcutaneously implanted into male *SCID* mice of 9–10 weeks age (Animal Resources Centre, Canning Vale, West Australia). Four xenograft lines (2-1318, 5-1318, 26-1004 and 30-1004) were minced under sterile conditions as described [35]. Except

for 26-1004, the 2-1318, 5-1318 and 30-1004 lines were derived from HBV positive HCC. The 26-1004 has wild-type p53. Two lines (2-1318 and 5-1318) exhibit a mutation in codon 249 of the *p53* gene. The 30-1004 has a frame shift mutation in codon 270 of the *p53* gene. A mutational analysis reveals that one of the 26-1004 has a 16 base pair deletion in exon 8 of the *PTEN* gene.

RAD001 as microemulsion (2% RAD001) was suspended in water at an appropriate concentration. For the dose-response experiments, mice bearing tumours were orally administered 1, 1.5, 2, 2.5 and 5 mg/kg RAD001 daily or 200 μl vehicle for 18 days. Each treatment group was comprised of fourteen animals. Treatment started on day 7 after tumour implantation. By this time, the HCC xenografts reached the size of approximately 100 mm^3. Tumour growth was monitored at least twice weekly by Vernier calliper measurement of the Length and Width of tumour. Tumour volume was calculated as follows: Tumour volume = [(Length) × (Width^2) × (π/6)]. Animals were sacrificed on day 18 during treatment. Body weight and tumour weight were recorded.

Efficacy of RAD001 was determined by % T/C, where T and C are the median weight (mg) of RAD001-treated and vehicle-treated tumours at day 18 during the treatment, respectively. Ratios of less than or equal to 42% are considered an active response (Drug Evaluation Branch of the Division of Cancer Treatment, NCI criteria).

Western blot analysis

To determine changes in indicated proteins, 3–4 independent tumours from vehicle- and RAD001-treated mice (day 18 during treatment) were homogenized separately in lysate buffer as described [33]. To study expression of the mTOR signalling pathway in HCC xenografts, tumours from 2-1318 (passage 8), 26-1004 (passage 9), 5-1318 (passage 7) and 30-1004 (passage 8) lines were used. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). One hundred μg of proteins from a single tumour were subjected to Western blot analysis as previously described [33]. All primary antibodies were used at a final concentration of 1 μg/ml. The blots were then visualized with a chemiluminescent detection system (Amersham).

Immunohistochemical analysis

Vehicle- and RAD001-treated tumours were collected on day 18 during treatment. They were fixed and paraffin-embedded. Five μM sections were cut, dewaxed, rehydrated and subjected to antigen retrieval. After blocking endogenous peroxidase activity and reducing non-specific background staining, the sections were incubated with the primary antibodies against CD31, VEGF, cleaved caspase-3 and Ki-67 (overnight at 4°C). Immunohistochemistry was performed as described [33]. For Ki-67, only nuclear immuno-reactivity was considered positive. Ki-67 index and caspase-3 positive cells were the number of labelled cells among at least 500 cells per region and then expressing them as percentage values. For the quantification of mean vessel density in sections stained for CD31, 10 random 0.159 mm^2 fields at ×100 magnification were captured for each tumour and microvessels were quantified. For VEGF expression, at least 20 high-power fields were chosen randomly, and 2000 cells were counted. VEGF-positive cells were expressed as percentage of total cells counted.

Determination of serum VEGF

Animals were sacrificed on day 18 during treatment and approximately 500–800 µl of blood was collected per mouse. The concentration of human VEGF in the sera of vehicle- and RAD001-treated mice was measured using Endogen Human VEGF ELISA kits (Pierce Biotechnology, Rockford, IL, USA) according to the protocol of the manufacturer. The sensitivity for VEGF kits is <8.0 pg/ml. The absorbance at 450 nm was measured using an ELISA plate reader (Benchmark Plus microplate spectrophotometer, Bio-Rad).

Statistical analysis

Body weight, tumour weight at sacrifice, serum VEGF levels, mean vessel density, Ki-67 index, VEGF expression and percentage of cleaved caspase-3-positive cells were analysed by ANOVA without repeated measures.

Results

We first investigated mTOR signalling in four patient-derived HCC xenografts. Figure 1 shows that there was no observable difference among the four xenografts in either total or phosphorylated mTOR at Ser^{2448}, a site that is phosphorylated by Akt [34]. However, activation of p70S6K at Thr^{389}, S6R at $Ser^{240/244}$ and 4EBP1 at $Ser^{37/46}$ was observed in all xenografts. Among four HCC xenografts studied, 5-1318 expressed highest levels of phospho-Akt at Ser^{308}, phospho-p70S6K at Thr^{389} and phospho-4EBP1 at Thr^{70} (Fig. 1). High levels of phospho-S6R at $Ser^{240/244}$ were observed in 2-1318 and 30-1004 lines. As expected, phosphorylation of Akt at Ser^{473} was high in xenograft line 26-1004, which has a 16 bp deletion in exon 8 of the *PTEN* gene. Despite high levels of phospho-Akt at Ser^{473}, phosphorylation of p70S6K at Thr^{389} and Thr^{70} in 26-1004 was not elevated (Fig. 1). Taken collectively, our results suggest that downstream targets of the mTOR pathway were activated in patient-derived HCC xenografts.

We next examined anti-tumour activity of RAD001 in these xenografts. As shown in Figure 2A, RAD001 inhibited the growth of HCC xenografts in a dose-dependent manner ($P < 0.01$). The xenograft line 26-1004, which has a 16 base pair deletion in exon 8 of the *PTEN* gene, had the greatest sensitivity to RAD001 as measured by percentage of growth inhibition (Fig. 2A). No overt toxicity of RAD001 was observed during the course of treatment as defined by weight loss, unkempt appearance or mortality, and behaviour. Since the dose of 2.5 mg/kg daily gave maximal growth inhibition, we selected this dose for our subsequent studies. Reduction of tumour growth rates rather than producing regressions was noticed in all xenografts studied (Fig. 2B–D). The growth suppression was seen approximately 1 week after treatment. RAD001, when given at a dose of 2.5 mg/kg daily, significantly inhibited the growth of all four xenograft lines ($P < 001$, Fig. 3). The T/C ratio, where T and C are the median weight (mg) of RAD001-treated and vehicle-treated tumours at day 18 during the
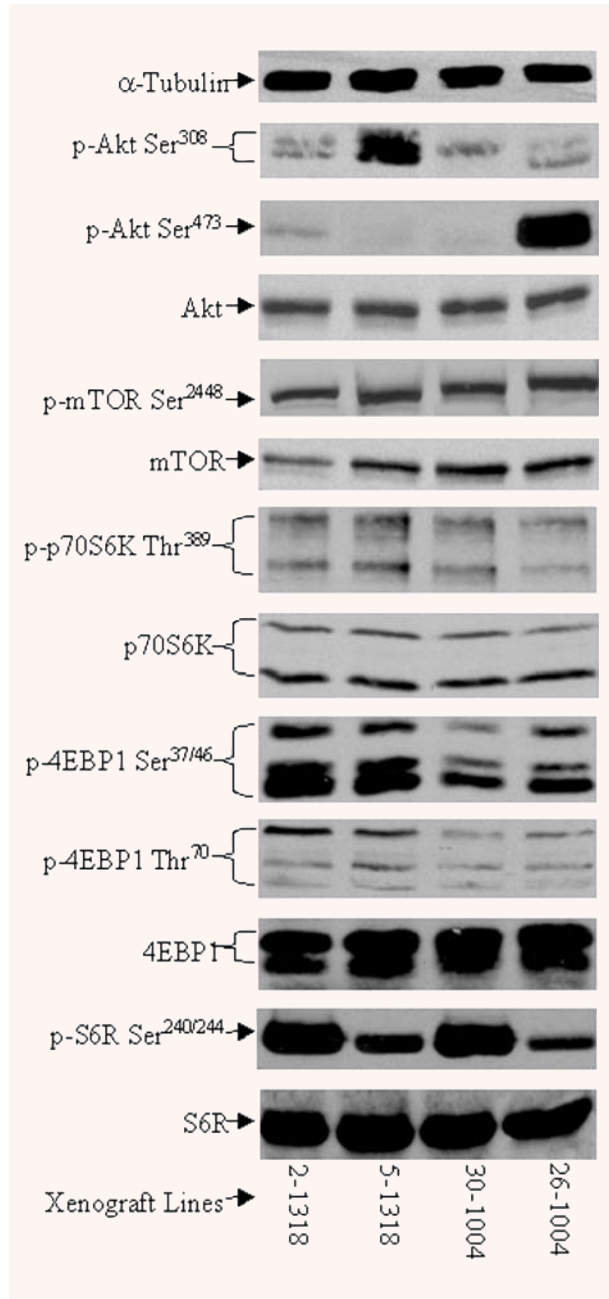
Fig. 1 Expression of the mTOR pathway in patient-derived HCC xenografts. Tumours from 2–1318 (passage 8), 26-1004 (passage 9), 5-1318 (passage 7) and 30-1004 (passage 8) lines were homogenized in lysate buffer as described in Materials and Methods. One hundred micrograms of proteins were analysed by Western blotting using both total and phosphorylation-specific antibodies against components of the Akt/mTOR pathway as described [33].
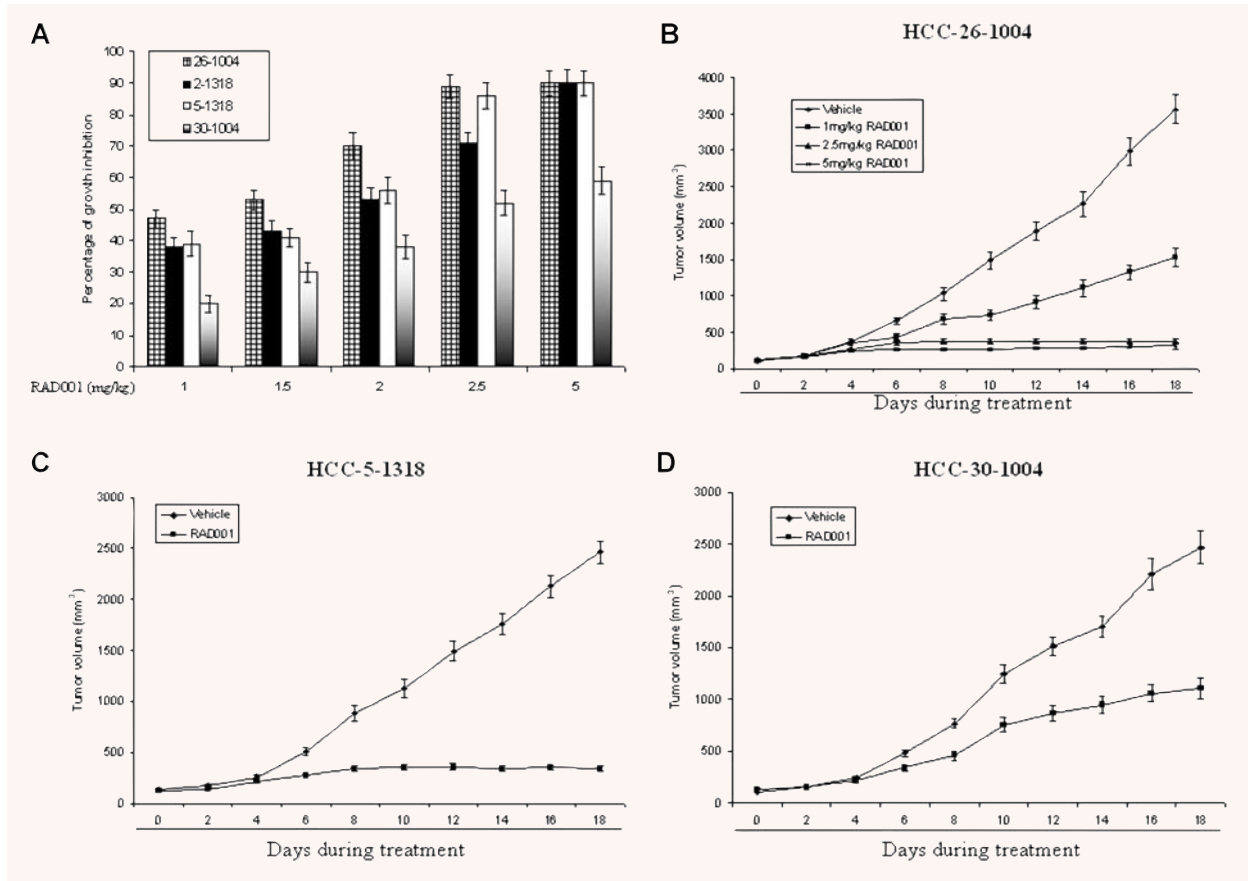
Fig. 2 Effects of RAD001 on growth inhibition of patient-derived HCC xenografts. Indicated xenograft lines were s.c. implanted in *SCID* mice as described in Materials and Methods. For the dose-response experiment, mice bearing xenografts were treated with vehicle or five doses (1, 1.5, 2, 2.5 and 5 mg) of RAD001 per kg body weight daily for 18 days as described in Materials and Methods. To study the effects of RAD001 on growth rate, mice bearing 26-1004 tumours were treated with vehicle or three doses of RAD001 (1, 2.5 and 5) mg/kg for 18 days. For 5-1318 and 30-1004 xenografts, only 2.5 mg dose was used. Each treatment arm involved 14 independent tumour-bearing mice representing the same xenograft line. Tumour growth was measured and calculated as described in Materials and Methods. Mean volume ± S.E. at a given time for vehicle- or RAD001-treated tumours is shown. The differences seen were statistically significant ($P < 0.01$). Experiments were repeated three times with similar results.

treatment, respectively, for 26-1004, 2-1318, 5-1318 and 30-1004 xenografts was 0.11, 0.19, 0.14 and 0.48, respectively. Since the ratios of less than 0.42 were observed in three of four lines studied, it was considered an active response (Drug Evaluation Branch of the Division of Cancer Treatment, NCI criteria).

To understand the potential mechanisms of RAD001 action, we investigated the phosphorylation status of downstream targets of mTOR. Figure 4 shows that phosphorylation of mTOR at Ser^{2448} was not significantly altered by RAD001 treatment. However, the levels of phospho-p70S6K at Thr^{389} and Thr$^{421/Ser424}$, and phospho-4EBP1 at Ser^{70} in RAD001-treated tumours were significantly reduced ($P < 0.01$). Complete inhibition of S6R phosphorylation was also observed in RAD001-treated tumours, confirming that reductions in S6R and 4EBP1 phosphorylation paralleled reduc-

tions in p70S6K activity in these tumours. There was no impact of RAD001 on ERK1/2, Akt and eIF4E phosphorylation (Fig. 4).

Since alterations in cell cycle proteins have been associated with cellular proliferation and clinical outcome [35], we investigated the status of the cell cycle regulators in vehicle- and RAD001-treated tumours after 18 days of treatment. Figure 4 shows that while no alterations in the levels of cyclin D1 and Wee1 were detected in any of the treatment arms, significant increase in p27^{Kip1} and reductions in the levels of p21^{WAF1}, Cdk-2, Cdk-4, Cdk-6, cdc-25C, c-Myc, cdc-2 and cyclin B1 in RAD001-treated tumours were observed ($P < 0.01$). These suggest that RAD001 treatment may block the cell cycle in late G1/S phase *in vivo*.

We next examined the anti-proliferative and apoptotic effects of RAD001 *in vivo*. As shown in Table 1, the number of Ki-67-positive
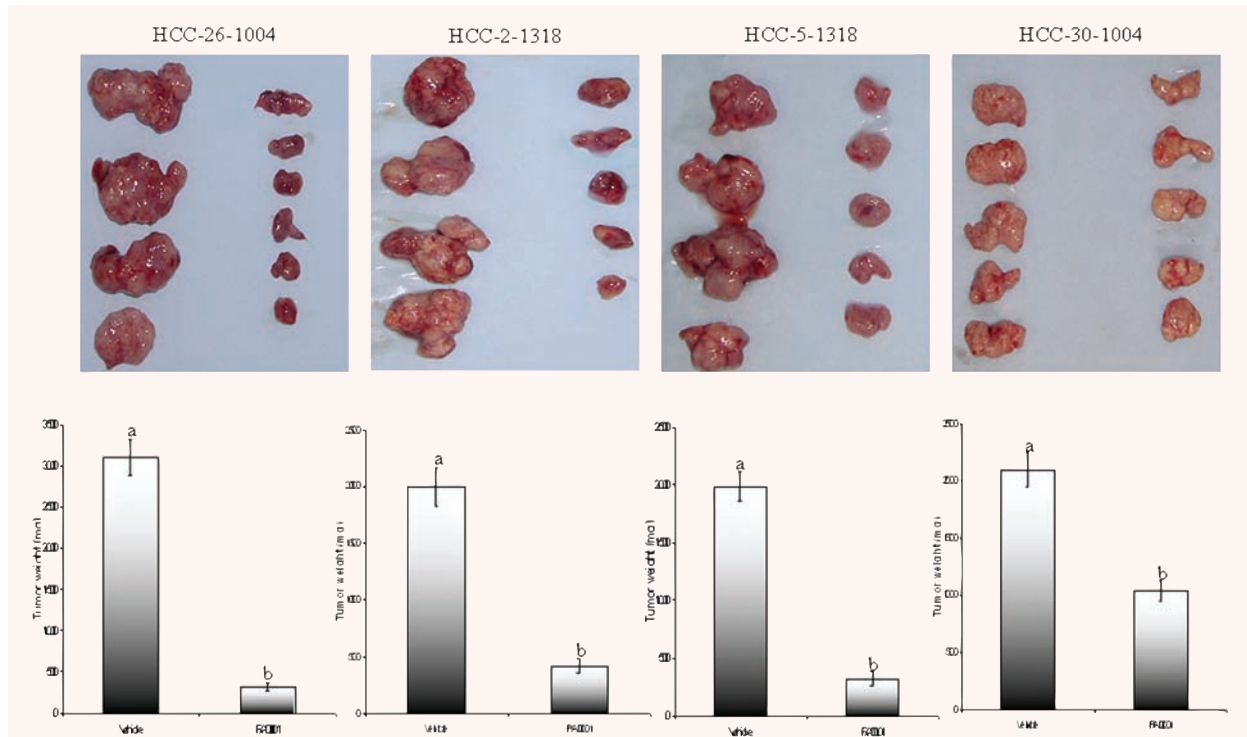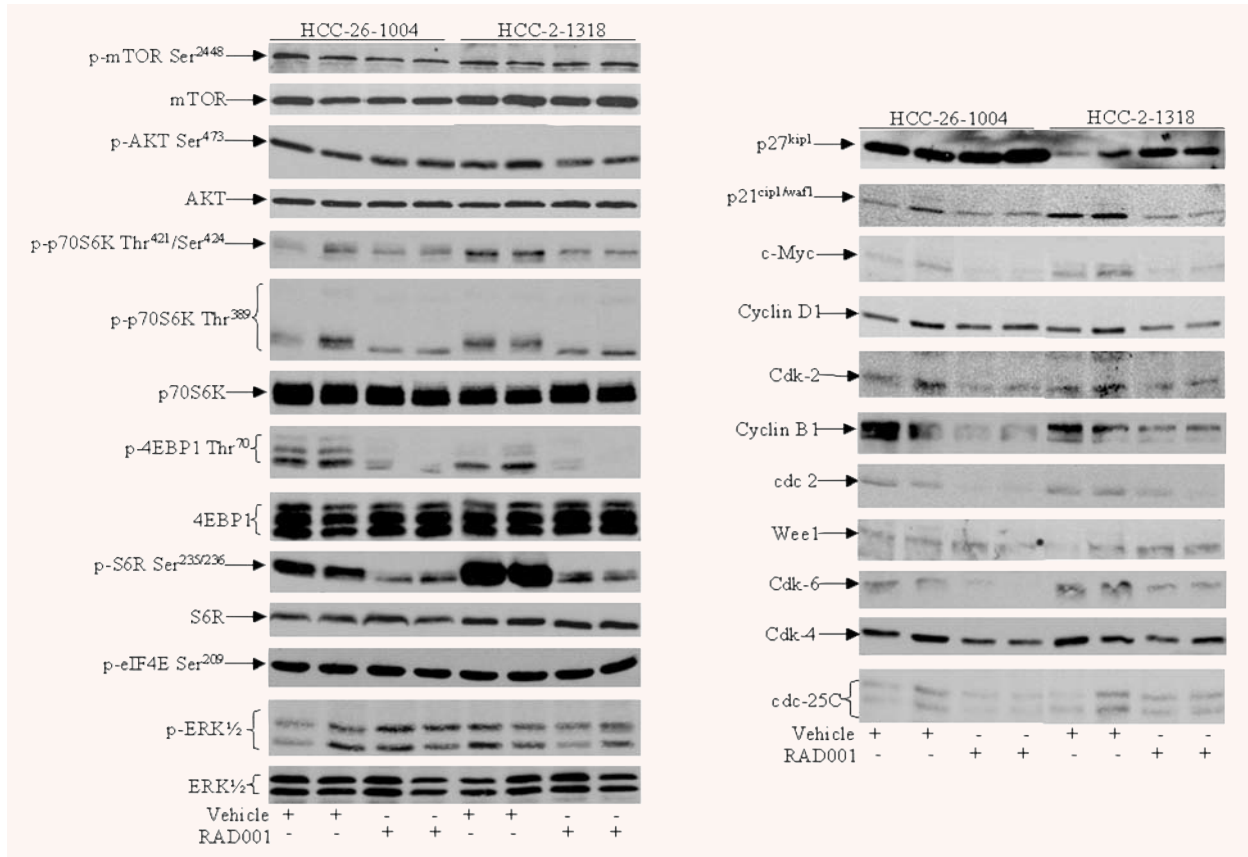
Fig. 3 Effects of RAD001 on growth of 26-1004, 5-1318, 2-1318 and 26-1004 xenografts. The indicated xenografts were s.c. implanted in *SCID* mice as described in Materials and Methods. Mice bearing HCC xenografts were treated vehicle and 2.5 mg RAD001 daily for 18 days as described in Table 1. Representative vehicle- and RAD001-treated tumours and the corresponding tumour weight for indicated lines of HCC xenografts are shown. Bars with different letters indicate $P < 0.01$ as determined by ANOVA.

cells in RAD001-treated tumours was significantly reduced ($P < 0.01$). The percentage of cells stained for cleaved caspase-3 was not significantly different between vehicle- and RAD001-treated tumours, suggesting that RAD001 does not cause apoptosis in those tumours (Table 1).

Because mTOR activation is important for the production of VEGF [36, 37] and tumour angiogenesis [15], we studied the VEGF expression and blood vessel formation in vehicle- and RAD001-treated tumours. Immunohistochemistry revealed that approximately 62% $\pm$ 9%, 69% $\pm$ 6%, 70% $\pm$ 8% and 43% $\pm$ 6% cells in 2-1318, 5-1318, 26-1004 and 30-1004 xenografts were positive for VEGF, respectively. Treatment with RAD001 significantly decreased VEGF-positive cells ($P < 0.01$, Table 1). The median number of CD31-positive microvessels in vehicle- and RAD001-treated tumours was significantly reduced ($P < 0.01$) (Table 1). We next determined the circulating levels of VEGF in vehicle- and RAD001-treated mice by ELISA. As shown in Figure 5, high levels of VEGF were detected in sera collected from vehicle-treated mice and VEGF levels were significant lower in the sera derived from RAD001-treated animals ($P < 0.01$). Low circulating VEGF was observed in mice bearing 30-1004 xenograft. This correlates well with the number of VEGF-positive cells in this tumour.

Discussion

It is well known that HCC is highly resistant to available chemotherapy agents, administered either alone or in combination [38]. Since downstream targets of mTOR are activated in HCC [20] and given that the mTOR pathway can be activated by a number of growth factors, receptor tyrosine kinases [39, 40] and HCV replication [41], inhibition of this pathway could have profound effects on the development and progression of HCC. We previously report the characterization of HCC xenografts established directly from human HCC tumours [33]. The advantages of patient-derived xenografts as opposed to xenograft derived from cell lines include the fact that they retain both tumour cells as well as stromal cells and have intact tissue architecture [42]. Thus patient-derived xenografts are expected to exhibit cellular and tissue characteristics that are very similar to the original tumours and to demonstrate better pre-clinical and clinical concordance. However, patient-derived xenografts also have certain limitations as HCC models – due to the use of immunocompromised mice, we are unable to investigate the role of the immune system in HCC [43]. Also, subcutaneous or orthotopic implantation of HCC

Fig. 4 Effects of RAD001 on cell cycle regulators and the phosphorylation status of mTOR and its downstream targets in patient-derived HCC xenografts. 26-1004, 2-1318, 5-1318, and 30-1004 HCC xenografts were s.c. implanted in *SCID* mice as described in Materials and Methods. Mice bearing HCC xenografts were treated vehicle and 2.5 mg RAD001 daily for 18 days as described in Table 1. Lysates from vehicle- and RAD001-treated 26-1004 and 2-1318 tumours were subjected to Western blot analysis described [33]. Blots were incubated with the indicated antibodies. Representative blots are shown. Differences in the levels of the indicated proteins between vehicle- and RAD001-treated groups were statistically significant ($P < 0.01$), as analysed by ANOVA. Similar results were obtained for 5-1318 and 30-1004 xenografts. Experiments were repeated at least three times with similar results.

tissues are performed in animals with non-cirrhotic livers, we are currently unable to effectively recapitulate the role of cirrhosis.

In the present study, we show that downstream targets of mTOR are pivotal in the growth and angiogenesis of HCC. RAD001 effectively inhibits the growth of four patient-derived xenograft lines. The effect of RAD001 is that of reduction of tumour growth rates without causing regressions, and resulting in T/C values in the range of approximately 8.5–48%. These effects occur within the dose range of 2.5–5 mg/kg/day. Treatment is well tolerated, in most cases permitting significant increases in body weight. A comparison of RAD001 activity against established lesions (treatment beginning 7 days after tumour implantation when primary tumours are detectable) and freshly injected tumour implantation (treatment beginning 1 day after injection) showed compatible activity irrespective of a delay in treatment initiation (data not shown).

In the present study, we show that RAD001-induced growth inhibition associates with inactivation of p70S6K and S6R, activation of transcriptional repressor 4EBP1, up-regulation of p27[Kip1] and inhibition of Cdk-2, Cdk-4, Cdk-6, c-Myc, cyclin B1, cdc-25C and p21[Cip1/Waf1]. RAD001 does not cause apoptosis as demonstrated by an increase in both cleaved PARP and cleaved caspase-3. RAD001 also exerts an anti-angiogenic effect as part of its anti-tumour activity by inhibiting VEGF expression. It remains to be determined whether tumour-associated endothelial cells are the targets for inhibition by RAD001 because they express VEGF receptor and are dependent on VEGF for proliferation and survival [44, 45]. As shown in Figure 2, RAD001-treated tumours grow at a very low rate, and the size attained at the end of the experiment is approximately 8–45% of the control tumours. Based on these data, we hypothesize that RAD001-mediated inhibition of mTOR

Table 1 Effects of RAD001 on body weight at sacrifice, cell proliferation, microvessel density, VEGF expression and apoptosis of four patient-derived HCC xenografts

Lines of xenografts	Treatments	Body weight (g)	Microvessel density*[1]	Ki-67 index (%)	Cleaved caspase-3 (%)	VEGF-expression (%)
2-1318	Vehicle	21.6 ± 0.9	27 ± 8	29 ± 7	11.6 ± 4	62 ± 9
	RAD001	22.7 ± 0.8	11 ± 5*	12 ± 4*	10.1 ± 3	34 ± 6*
5-1318	Vehicle	22.9 ± 0.7	25 ± 7	18.6 ± 7	8.6 ± 4	69 ± 6
	RAD001	23.5 ± 0.9	9 ± 4*	8.9 ± 4*	5.7 ± 3	34 ± 7*
26-1004	Vehicle	22.1 ± 1.0	29 ± 9	23.9 ± 8	12.3 ± 4	70 ± 8
	RAD001	23.5 ± 0.8	8.4 ± 5*	8.6 ± 5*	10.2 ± 4	24 ± 7*
30-1004	Vehicle	23.4 ± 0.7	18.6 ± 7	15.4 ± 6	7.2 ± 2.9	43 ± 6
	RAD001	24.6 ± 0.8	9 ± 5*	8.9 ± 5*	6.4 ± 1.5	29 ± 5*

[1]Mean microvessel density of 10 random 0.159 mm^2 fields at 100 magnification; * indicates $P < 0.01$.
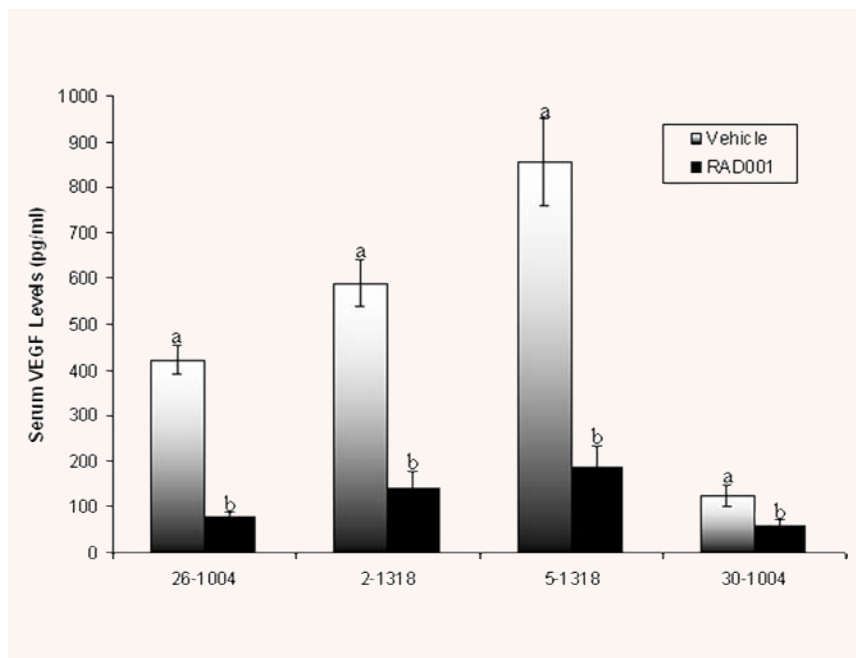
Fig. 5 Effects of RAD001 on serum VEGF levels. 26-1004, 2-1318, 5-1318 and 30-1004 HCC xenografts were s.c. implanted in *SCID* mice as described in Materials and Methods. Mice bearing HCC xenografts were treated vehicle and 2.5 mg RAD001 daily for 18 days as described in Table 1. Serum VEGF levels in vehicle- and RAD001-treated mice were determined using Endogen Human VEGF (Pierce Biotechnology, Rockford, IL, USA) ELISA kit as described by the manufacturer. The sensitivity for VEGF was <8.0 pg/ml. Bars with different letters indicate $P < 0.01$ as analysed by ANOVA. Experiments were repeated at least three times with similar results.

and its target protein-p70S6 kinase, S6R and 4EBP1, results in enhancement of p27^{kip1} translation and suppression of VEGF, p21$^{Cip1/Waf1}$, cdc-2, Cdk-4, cyclin B1, Cdk-2 and c-Myc expression. These potential kinetic changes would block angiogenesis and cycle progression, the ultimate anti-angiogenic and anti-proliferative effects of RAD001 on HCC. This hypothesis is supported by previous study [17] and our present data showing that RAD001-treated tumours have lesser VEGF-positive cells and fewer CD31-positive blood vessels compared to vehicle-treated tumours. In addition, mTOR inhibitor-induced G0/G1 cell cycle arrest is associated with an up-regulation of p27^{Kip1} [46].

The exact molecular determinants predict responsive of tumour cells to RAD001 is still unclear. Currently, the activation status of the PI3K/Akt/mTOR/p70S6K pathway may be indicative of responsiveness to rapamycin (reviewed in ref. [14]). In the present study, RAD001 inhibits tumour growth without suppressing

the phosphorylation of mTOR at ser^{2448}. It remains to be determined whether phosphorylation of this site is important for determining the likely *in vivo* effects of mTOR inhibitors. In this study, the 26-1004 xenograft has a non-functional form of a tumour suppressor PTEN and expresses high levels of phospho-Akt at Ser^{473} (Fig. 1). As loss of PTEN or constitutive/hyper-activation of Akt has been suggested to sensitize tumours to the mTOR inhibitors [47–49], we expect that 26-1004 xenograft would be more sensitive to RAD001. Indeed, the growth and proliferation of 26-1004 xenografts displays slightly enhanced sensitivity to RAD001. A similar correlation is not observed when performing the same analysis with phosphorylated mTOR at Ser^{2448} or phospho-p70S6K Thr^{389} and Thr$^{421/Ser424}$ or phospho-4EBP1 at Thr^{70}. Our preliminary data suggest that a correlation between the anti-proliferative activity of RAD001 and the levels of S6R phosphorylation on serines 240 and 244 may exist (data not shown). Hence patients with non-functional PTEN/activated PI3K/Akt pathway and/or high levels of phospho-S6R in their tumours may respond to mTOR inhibitors in a potent manner.

The potential use of rapamycin and its analogues in treatment of HCC has been reported previously [21, 22]. Complete remission of lung metastases is observed in a patient on rapamycin after liver transplant for metastatic HCC [21]. Stippel *et al.* [22] report a tumour-free survival in another HCC patient given rapamycin after a liver transplant. This patient underwent a bilateral salphingo-oophorectomy for HCC metastases and immunosuppression was switched to rapamycin monotherapy. Fourteen months after this procedure, the patient was asymptomatic with stable liver function. This observation indicates that beside the surgery, the changing of the immunosuppression regimen may contribute to the patient outcome. It has been reported that the activities of PI3K and Akt, as well as the activity of their downstream target, mTOR, were increased in HCV-replicating cells [41]. Furthermore, the activation of the PI3K/Akt pathway was at least partially responsible for the suppression of HBV replication [50]. It remains to be determined whether inhibition of the mTOR pathway will result in suppressing HCV replication in patients with HCV-related HCC but enhancing HBV replication in those with HBV-related tumours.

Here we show that RAD001 inhibits tumour growth when given alone, suggesting that at least some patients may be amenable to single agent therapy. As for all potential anti-cancer drugs, inhibitors of the mTOR pathway may not be very effective as individual therapeutic agents because HCC tumours possess more than one genetic defect [51]. Recent study indicates that mTOR inhibition by rapamycin triggers rapid and sustained activation of PI3K/Akt survival pathway in human lung and other types of cancer cells [52]. Furthermore, clinical results indicate that in tumour tissue from patients receiving RAD001 there is hyperphosphorylation of Akt [53]. This appears to be a consequence of increased stability of IRS-1 and up-regulation of the IGF-1 signalling pathway [53]. In the present study, up-regulation of phospho-Akt is not observed in RAD001-treated tumours (Fig. 4). The exact mechanisms responsible for this remain to be illustrated. It is possible that the effects of RAD001 on activation of PI3K/Akt vary depending upon cell and tumour types, duration of exposure to RAD001 and timing of the assessment of phospho-Akt. Experiments are underway to determine these possibilities. For maximal therapeutic benefit, it may be necessary to combine RAD001 with other signal transduction inhibitors or conventional chemotherapeutic drugs. It has been demonstrated that a combination of rapamycin plus the ABL tyrosine kinase inhibitor, Imatinib, synergistically inhibits the proliferation of BCR/ABL-transformed myeloid and lymphoid cell lines [54]. It is likely that treating patients with a combination of RAD001 plus other molecular-targeted agents such as IGF receptor inhibitors or a multi-targeted kinase inhibitor such as sorafenib may be the good approach for treating HCC. In the meantime, a multitude of other targeted agents have become available that should also be tested in combination with RAD001. The optimal combinations can be elucidated over time.

In summary, we have demonstrated that oral delivery of RAD001 causes growth inhibition of patient-derived HCC xenografts. Our data implicate the important roles of the mTOR pathway and its downstream targets in liver cancer cell proliferation and angiogenesis. Our data, coupled with previous studies [21, 22], have indicated that RAD001 may be a useful drug for the treatment of HCC.

Acknowledgements

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