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RAD001 (Everolimus) Inhibits Growth of Prostate Cancer in the Bone and the Inhibitory Effects Are Increased by Combination With Docetaxel and Zoledronic Acid

Todd M. Morgan¹, Tiffany E.M. Pitts¹, Ted S. Gross², Sandra L. Poliachik², Robert L. Vessella¹, and Eva Corey^{1,*}

¹Department of Urology, University of Washington School of Medicine, Seattle, Washington

²Department of Orthopaedics and Sports Medicine, University of Washington School of Medicine, Seattle, Washington

Abstract

INTRODUCTION—mTOR activity is increased in advanced prostate cancer (CaP) as a result of a high rate of PTEN mutations. RAD001 (Everolimus) is a new orally available mTOR inhibitor. The objective of our study was to evaluate the effects of RAD001 on the growth of CaP in the bone, both alone and in combination with docetaxel and zoledronic acid.

METHODS—C4-2 CaP cells were injected into tibiae of mice and the animals were treated with RAD001, docetaxel, and zoledronic acid alone or in combination. Histomorphometrical analysis, serum PSA measurements, bone mineral density (BMD), and μ CT were used to determine the effects of treatment on tumor and bone.

RESULTS—All three agents alone decreased tumor volume, and RAD001 and docetaxel also decreased levels of serum PSA by 68% and 65%, respectively (both $P < 0.01$). Combinations of the agents were more effective in decreasing tumor volume than single agents. Three-drug treatment showed the greatest effect: 64% inhibition versus control ($P < 0.01$). Treatment with RAD001 interfered with the weight loss associated with growth of this tumor in the bone (non-RAD001 groups: 4.0% decrease in body weight, $P = 0.0014$; RAD001 groups: increase of 3.6% in body weight, $P = 0.0037$).

CONCLUSIONS—RAD001 inhibited growth of C4-2 cells in bone, an effect augmented by addition of docetaxel and zoledronic acid. Moreover RAD001 had a significant impact on maintenance of body weight. RAD001 may hold promise for its effects on both metastatic CaP and the important syndrome of tumor cachexia.

Keywords

mTOR; RAD001; prostate cancer; bone metastasis; combination chemotherapy

INTRODUCTION

Patients with advanced prostate cancer (CaP) suffer from the severe consequences of advanced disease, which commonly include bone metastases and cachexia. At present, there is no curative or effective long-term treatment for this stage of the disease. Therapeutic

modalities that would eliminate or slow the growth of advanced metastatic CaP and improve quality of life are therefore of great interest.

Mutations or chromosomal abnormalities in cancer cells often result in altered cell-cycle regulation and growth-signal transduction. Pathways playing roles in these processes may be strategic targets for cancer therapy. One such pathway of possible clinical relevance to CaP is the *PTEN* tumor-suppressor pathway. Although deletion and mutation of the *PTEN* gene have been reported only infrequently in primary CaP [1], a high rate of mutations and deletions is seen in metastatic lesions of CaP as well as CaP cell lines and xenografts [2]. Absence of PTEN expression is correlated with a Gleason score 7 or higher in patients with advanced CaP [3]. Additionally, a number of other recent reports support a tumor-suppressor role of PTEN in CaP [4–8].

Deregulated signaling through the mTOR pathway is a prominent consequence of *PTEN* inactivation. Consistent with this, PTEN-negative tumors are particularly good targets for mTOR inhibitors [9,10]. mTOR, originally identified as the mammalian target of rapamycin, is a serine/threonine kinase that plays a central role in control of translation and also regulates anti-apoptotic signals (reviewed in Castedo et al. [11]). The potential clinical utility of inhibition of the mTOR pathway in CaP is supported by published reports of inhibition of CaP growth by rapamycin and its derivatives [12–17]. RAD001 (Everolimus), an orally bioavailable mTOR inhibitor, induces apoptosis of epithelial cells and completely reverses the neoplastic phenotype of mice expressing human AKT1 in the prostate [18]. It has also been shown to decrease tumor growth in a pancreatic cancer model [19].

Because of significant heterogeneity of CaP tumors and their capacity to mutate and adapt, treatment with a single agent is unlikely to result in prolonged clinical responses. Therefore, it is important to identify agents that may be useful in combination [20–22]. There are two prevalent ways of selecting agents for combination therapy: identification of compounds that act by clearly distinct mechanisms and selection of compounds affecting different points in a specific signaling pathway critical to cancer progression. In this study we have chosen the former strategy, evaluating the simultaneous inhibition of mTOR (RAD001) and tubulin polymerization (docetaxel, TAX), while also targeting the bone microenvironment (zoledronic acid, ZOL). Our choice of these compounds for combined testing with RAD001 was based on the following reasoning. TAX, an inhibitor of tubulin depolymerization, is the only agent that has been shown to prolong survival in patients with advanced CaP and is considered a standard therapy for these patients. However, this survival advantage was modest (1.9–2.4 months), and the drug is also associated with significant adverse side effects [23,24]. ZOL is a bisphosphonate which exerts beneficial effects in CaP patients with bone metastases, reducing both pain and skeletal related events [25–30]. Our previously published results suggested that the most likely mode of action of ZOL in retarding the growth of bone metastases is indirect, involving diminished availability of growth factors otherwise released through osteolysis, although we cannot rule out the possibility that ZOL directly inhibits the growth of CaP cells as well [26]. Based on these facts we hypothesize that the combination of RAD001, TAX, and ZOL—all acting on different pathways—will result in more pronounced inhibition of CaP tumor growth in the bone environment than any single treatment alone.

METHODS

Cell Line

The C4-2 cell line, a subline of LNCaP cells [31,32], is an androgen-independent line that expresses the androgen receptor and mutated *PTEN*. Cells were maintained under standard tissue culture conditions as previously described.

Intra-Tibial Tumors

All animal procedures were performed in compliance with the University of Washington Institutional Animal Care and Use Committee, and NIH guidelines. Four- to 6-week-old male SCID mice (Fox Chase SCID mice, Charles River, Wilmington, MA) were injected with approximately 2×10^5 cells into the proximal end of the right tibia as we have described previously [33–35]. Tumor growth was monitored by serum levels of prostate specific antigen (PSA). Mice were randomized into groups when the tumors were well established in the bone (mean of serum PSA >5 ng/ml in each group; 4–5 weeks after tumor-cell injections).

RAD001 and Docetaxel Dose Determinations

To gauge the efficacy of RAD001 and TAX in treating intra-tibial C4-2 tumors, we conducted initial experiments with two different doses of each drug. RAD001 for these studies was provided by Novartis Institute for BioMedical Research, Oncology, Basel, Switzerland. In the RAD001 portion of the study, 14 animals with tumors were randomized to three groups: (1) a control group receiving placebo gavage; (2) a group receiving 5 mg/kg RAD001 gavage five times per week; and (3) a group receiving 10 mg/kg RAD001 gavage five times per week. In the TAX portion of the study, 14 animals were randomized to three groups: (1) a control group receiving weekly placebo intraperitoneal (IP) injections; (2) a TAX group which received 10 mg/kg TAX via weekly IP injections; and (3) a TAX group which received 20 mg/kg TAX via weekly IP injections. Two mice were excluded from analysis due to tumor invasion of muscle, indicated by PSA > 100 ng/ml (one each in control group and TAX 20 mg/kg group). In both studies, blood samples were collected weekly for determination of serum PSA levels (IMx Total PSA assay, Abbott Laboratories, Abbott Park, IL). Body weight was measured weekly and BMD was measured (PIXImus Lunar densitometer, GE Healthcare, Waukesha, WI) prior to sacrifice. Animals were sacrificed 5 weeks after enrollment in the RAD001 study and 4 weeks after enrollment in the TAX study, since significant differences in PSA were observed after a shorter duration of TAX treatment.

Effects of RAD001, Docetaxel, and Zoledronic Acid on Growth of Tumor in the Bone

Animals with established tumors were randomized into eight groups treated with single agents or combinations of agents. After exclusion of animals with tumor in the muscle at the end of the study, each group had 7–9 animals (total 66 animals). Dosing regimens were determined based on the results of the preliminary individual studies with RAD001 and TAX and our previously published results with ZOL [25,26]. We sought to use low doses to elicit better-defined differences between groups and also to minimize adverse side effects of the treatments in a manner consistent with clinical practice. The selected dosing regimens were RAD001: 2.5 mg/kg gavage five times per week, TAX: 5 mg/kg IP every other week, and ZOL: 0.1 mg/kg injected subcutaneously (SC) twice per week. Placebo, given in place of each drug not administered, was 13% ethanol in saline by IP injection every other week in place of TAX, 5% glucose in place of RAD001, and saline SC injection twice per week in place of ZOL. The groups were as follows: (1) control (placebos only), (2) TAX, (3) RAD001, (4) ZOL, (5) TAX + ZOL, (6) RAD001 + ZOL, (7) TAX + RAD001, and (8) TAX + RAD001 + ZOL. Tumor growth was monitored by weekly serum PSA levels. Body weight was measured weekly. Animals were sacrificed 46 days after enrollment. Prior to sacrifice, bone mineral density (BMD) and lean body mass were determined by densitometry (PIXImus Lunar densitometer, GE Healthcare), and tibiae were harvested for μ CT and histomorphometrical analyses.

Analysis of Intra-Osseous Tumors

Histomorphometrical analysis—Five micrometer sections of methacrylate-embedded tibiae stained with Goldner's stain as described [25,26,36] were used for this analysis ($n = 4\text{--}5$ per group). Tumor volume (TuV, mm^2), tissue volume (TV, mm^2), and bone volume (BV, mm^2) were measured in a blinded fashion on longitudinal sections of the tibiae using Bioquant Analysis Software (Bioquant Image Analysis Corporation, Nashville, TN).

Micro-computed tomography (μCT)—A Scanco vivaCT 40 high resolution μCT scanner was used to obtain 10.5- μm voxel resolution images spanning the proximal region and mid-shaft of tumored and non-tumored contralateral tibiae ($n = 5$ per group). Specific regions of analysis included a 0.85-mm section spanning the proximal tibia metaphysis and a 0.55-mm section of mid-diaphyseal cortical bone centered at a distance of 4.35 mm proximal to the tibia–fibula junction. Given the prolific woven bone induction observed in many of the samples, analyses of μCT data were confined to measurements of bone volume (BV; mm^3), tissue volume (TV; mm^3), and the ratio of bone volume to tissue volume in the metaphysis (BV/TV, %), and determinations of cortical volume (mm^3) and periosteal volume (mm^3) at the mid-diaphysis. All imaging and analyses were conducted with the identity of the specimens blinded.

Statistical Analyses

GraphPad Prism 3.0 (GraphPad Software, Inc., San Diego, CA) was used for the statistical analyses. All quantitative data are expressed as mean $\pm$ standard error of the mean (SEM). In initial evaluations of the effects of single agents on tumor growth, the unpaired Student's t -test was used to determine the statistical significance of differences in serum PSA levels. To determine the statistical significance of differences among multiple groups, a one-way analysis of variance (ANOVA) with the Dunnett multiple comparison test was used to compare treatment groups with the placebo group while controlling for multiple testing errors. Results were determined to be significantly different when $P < 0.05$.

RESULTS

Effects of RAD001 on Growth of C4-2 in the Bone Environment

In this pilot study we investigated whether RAD001 inhibits growth of CaP in the bone and sought an optimal dose to use in combination with other agents. Our results show that RAD001 inhibits the growth of C4-2 xenografts in the tibiae of SCID male mice (Fig. 1A). At sacrifice, 5 weeks after the start of treatment, decreases in serum PSA levels of $69.42 \pm 6.18\%$ ($P = 0.026$) and $67.64 \pm 6.18\%$ ($P = 0.029$) compared to control-group levels were observed in the 5 mg/kg and the 10 mg/kg groups, respectively. Differences between the RAD001 groups were not significant. To further examine the effects of treatment on tumor growth we determined the weight of tumored tibiae. Relative to tibial weight in the control group (105.80 ± 7.92 mg), the weight of tumored-tibiae in RAD001-treated groups was significantly decreased in both the 5 mg/kg group (66.80 ± 2.85 mg, $P = 0.0014$) and the 10 mg/kg group (71.75 ± 1.65 mg; $P = 0.0057$). Again there was no significant difference between the RAD001 groups. Additionally, growth of C4-2 in tibiae showed a trend towards decreased overall body weight in control animals. Mean weight at the end of the study in the animals with C4-2 cells in the tibiae was $85.8 \pm 4.6\%$ of that at the initiation of treatment ($P = 0.0047$). The lower dose of RAD001 (5 mg/kg) inhibited this effect of the tumor, with a final weight $101.7 \pm 3.6\%$ of the weight at the start of treatment ($P = 0.71$). Animals treated with 5 mg/kg RAD001 had a final weight $20.9 \pm 4.3\%$ greater than the controls ($P = 0.018$). RAD001 (10 mg/kg) did not have this effect. Evaluation by bone densitometry revealed that C4-2 growth in the tibiae decreased BMD by $\sim 35\%$ versus contralateral non-tumored tibiae; this effect was not altered by RAD001. Because the inhibition of tumor growth with these

two doses of RAD001 was similar, we selected an even lower dose of RAD001, 2.5 mg/kg, for the larger study in order to best elicit differential effects with combination therapy.

Effects of TAX on Growth of C4-2 in the Bone Environment

TAX has been used in preclinical studies with tumors of various histological types. Since the efficacy of this agent depends on the tumor type as well as the growth environment [38], we tested two doses of TAX for inhibition of growth of C4-2 tumors in the bone. Both of the TAX doses inhibited tumor growth, as determined by lower levels of serum PSA in treated versus untreated animals (Fig. 1B). At sacrifice, serum levels of PSA were decreased by $86.9 \pm 5.26\%$ ($P = 0.0007$) and $93.9 \pm 2.6\%$ ($P = 0.0011$) in the 10 and 20 mg/kg TAX groups, respectively, versus the untreated group. No significant differences were observed between the TAX groups ($P = 0.30$). Compared with the control group (97.7 ± 15.3 mg), the weight of tibiae with tumors was significantly decreased in the TAX 20 mg/kg group (59.0 ± 4.0 mg; $P = 0.049$), and decreases that did not reach significance were detected in the TAX 10 mg/kg group (64.0 ± 4.8 mg; $P = 0.053$). The decrease in BMD caused by these cells was not significantly altered by TAX treatment ($P = 0.20$). As with RAD001, based on these results we selected a lower dose of 5 mg/kg every 2 weeks for the combination studies, anticipating that this dose of TAX would not eradicate the whole tumor.

Effects of RAD001, TAX, and ZOL on Growth of C4-2 in the Bone Environment

In comparison with the control group, serum PSA levels at the end of treatment were significantly lower in groups treated with TAX ($67.5 \pm 7.4\%$ decrease, $P < 0.01$), RAD001 ($64.6 \pm 6.5\%$ decrease, $P < 0.01$), RAD001 + ZOL ($75.4 \pm 2.9\%$ decrease, $P < 0.01$), TAX + RAD001 ($67.2 \pm 4.8\%$ decrease, $P < 0.01$), and TAX + RAD001 + ZOL ($52.7 \pm 10.7\%$ decrease, $P < 0.05$), consistent with inhibition of tumor growth (Fig. 2A,B). As we have found previously [38], administration of ZOL alone did not result in decreased levels of serum PSA versus control animals.

Serum PSA levels are frequently used to monitor tumor response to therapy, however serum PSA is not always concordant with tumor volume, particularly in advanced disease. Therefore, to further define the effects of the treatments on tumor growth, we performed histomorphometrical analysis. Our results show that tibiae with untreated C4-2 tumors are larger than those of the treated animals as a result of the tumor growth. This expansion of the tumored tibiae was inhibited in all treatment groups. Compared to untreated mice, tissue volume was decreased by 24–37% with monotherapy ($P < 0.05$), 39–53% with dual therapy ($P < 0.01$), and 59% ($P < 0.01$) with all three agents (Fig. 3A) as determined by ANOVA. These data suggest that RAD001, TAX, ZOL, and their combinations inhibit the bone expansion caused by tumor growth. Further analysis showed that tumor volume decreased by 48% with each single agent treatment ($P < 0.01$ for each; Fig. 3B), and decreases of 47–66% were detected with dual agent treatment ($P < 0.01$ for each). The combination of all three drugs resulted in a 71% decrease in tumor volume versus the control group ($P < 0.01$). Comparing the effect of RAD001 as a monotherapy to its use in combination with TAX and/or ZOL showed a trend toward a greater decrease in tumor volume with combination therapy. There was a decrease of $59.5 \pm 6.1\%$ ($P = 0.09$) with the addition of TAX, $68.7 \pm 3.4\%$ ($P = 0.12$) with ZOL, and $50.1 \pm 8.9\%$ ($P = 0.042$) when all three drugs were used. Our results also revealed a significant positive correlation between TuV and TV ($R = 0.95$, $P < 0.0001$), confirming our conclusion that tumor growth expands the tibiae. To normalize for changes in tissue volume, we also analyzed the ratio of tumor volume to tissue volume (%TuV/TV) in each treatment group. One-way ANOVA showed significant decreases with the treatments ($P = 0.041$) with a significant decrease in %TuV/TV in each of the RAD001 groups: [RAD001 alone: $77.5 \pm 3.2\%$ of the control; RAD001 + ZOL: $74.3 \pm 3.4\%$ of the control; TAX + RAD001: $74.3 \pm 6.8\%$ of the control; and TAX + RAD001 + ZOL: $71.9 \pm$

5.4% of the control ($P < 0.05$ for all)]. These data suggest that the decreases in total TuV are not simply related to the limitation of space caused by increases in BV related to the ZOL treatment, but are due to a direct effect of the drug and their combinations.

Effects of RAD001, TAX, and ZOL on Bone With C4-2 Tumors

Growth of CaP in the bone causes dysregulation of bone remodeling, increasing the rates of both bone formation and osteolysis. We investigated the effects of combination therapy on the bone in the presence of CaP cells, employing multiple modalities, including BMD, BHM, and μ CT analyses. C4-2 tumor growth caused decreases in BMD ($69.4 \pm 2.0\%$ of contralateral tibiae, $P < 0.0001$) that were similar to our published results [26]. Although TAX and RAD001 decreased serum PSA levels and tumor volume, they did not prevent changes in BMD (Fig. 3C). Overall, the normalized BMD of tumored tibiae in groups that did not receive ZOL did not differ significantly from controls ($P = 0.44$). Treatment with ZOL alone or in combination resulted in higher BMD of tumored tibiae versus untreated C4-2 tibiae ($124 \pm 2.1\%$, range 117–132%, $P < 0.05$). No significant differences were observed between the four ZOL groups. Bone volume of tumored tibiae as determined by BHM analysis was increased in the RAD001 group ($210.0 \pm 13.1\%$, $P < 0.01$), the ZOL group ($193.4 \pm 20.2\%$, $P < 0.05$), and the combination groups of TAX + ZOL ($192.1 \pm 22.0\%$, $P < 0.01$) and RAD001 + ZOL ($238.2 \pm 19.3\%$, $P < 0.01$) versus control tibiae with untreated C4-2 tumors. Interestingly, the bone volume of tibiae of animals treated with all three agents was not significantly different from that of untreated C4-2 tibiae (Fig. 3D).

μ CT analysis provides a three-dimensional view of the bone which we used to further evaluate effects of the treatments on the bone and the relationship between tissue and bone volumes. This analysis showed that growth of C4-2 cells expands the bone, increasing TV to $159.6 \pm 6.5\%$ ($P < 0.001$) of that of normal tibia. Treatment with RAD001, TAX + RAD001, or TAX + RAD001 + ZOL inhibited C4-2-induced expansion of tibiae in the metaphyseal region to $67.0 \pm 2.5\%$, $35.8 \pm 7.6\%$, and $35.2 \pm 0.9\%$ of the TV of C4-2 tibiae (Fig. 4A). There were also significant differences between the RAD001-monotherapy group and the RAD001 combinations: RAD001 + ZOL, $39.9 \pm 4.9\%$ of the RAD001 monotherapy; RAD001 + TAX, $49.9 \pm 10.6\%$; and RAD001 + TAX + ZOL, $49.2 \pm 1.2\%$ (all $P < 0.001$). In contrast to TV, C4-2 growth in untreated mice resulted in a decrease in BV ($71.3 \pm 2.6\%$, $P = 0.0024$) versus normal tibiae. Only ZOL alone or in dual combinations resulted in increased BV. The BV/TV ratio was increased in all groups treated with ZOL (Fig. 4B). Analysis of cortical volume at the mid-diaphysis revealed similar findings (Fig. 4C). Periosteal growth occurs in some advanced CaP metastases [37]. In our model C4-2 cells cause increases in the periosteal bone volume relative to normal tibiae ($159.5 \pm 10.1\%$, $P = 0.002$). Treatment with TAX + ZOL and TAX + RAD001 interfered with the periosteal stimulation and decreased periosteal volume to ~80% of the tibiae with untreated C4-2 tumors (Fig. 4E). There is discordance between BHM and μ CT results in evaluating BV in RAD001 monotherapy group. We hypothesize that this is perhaps because μ CT evaluates 3D, while BHM is limited to the mid-tibia section. Moreover, μ CT evaluation of the trabecular bone was used only in the region adjacent to the growth plate, while BHM analysis was performed on whole tibia sections, encompassing more tissue.

Effects of RAD001 on Body Weight

Because metastatic CaP is frequently associated with cachexia, and weight loss is also a side effect of some treatments, we evaluated the effect of the treatments on body weight. C4-2 cells grown in the bone environment caused decreases in body weight. The average weight at the end of the study was $91.0 \pm 2.2\%$ ($P = 0.004$) of the weight of the animals 1 week after the beginning of the treatment—the time point of greatest body weight. Interestingly, our results show that RAD001 as a monotherapy or in combination interfered with loss of

body weight caused by tumor growth in the bone. Animals receiving RAD001 did not lose weight between 1 week after the beginning of treatment and the end of the study ($99.3 \pm 0.8\%$, $P = 0.38$), while animals not receiving RAD001 exhibited weight loss during the same period ($94.1 \pm 1.1\%$, $P < 0.0001$). There was a $10.7 \pm 1.5\%$ difference in sacrifice body weight between groups with and without RAD001 ($P < 0.001$) (Fig. 5). To further investigate this effect of RAD001 we examined total lean body mass as determined from BMD measurements of the whole animals. This analysis showed a significantly greater lean body mass in all animals treated with RAD001 (15.8 ± 0.2 g) versus all animals not receiving RAD001 (14.8 ± 0.3 g, $P = 0.008$).

DISCUSSION

Our studies yielded several novel findings: (1) RAD001 slowed the growth of C4-2 CaP cells in bone; (2) RAD001 inhibition of tumor growth was enhanced by administration of low doses of TAX and ZOL, with a trend toward decreased tumor volume as more agents were added; and (3) RAD001 maintained body weight in tumor-bearing animals.

Our results in this preclinical setting suggest that RAD001 is an effective treatment for advanced CaP in the bone as measured by all parameters related to tumor growth. The growth-inhibitory activity of RAD001 was significant even at the low dose of 2.5 mg/kg, as shown by marked decreases in both serum PSA levels and tumor volume. BHM and μ CT results also indicated inhibition by RAD001 of tumor-related tibial expansion. TAX is currently the only drug that yields a survival benefit in patients with advanced CaP, but in our studies, low doses of RAD001 demonstrated similar inhibitory effects on tumor growth in the bone and serum PSA levels. Inhibition of the mTOR pathway may be of particular importance for CaP bone metastasis, since mTOR is a downstream effector of type I insulin-like growth factor (IGF-1) which is present at high levels in the bone environment. These findings are of special significance because most patients with advanced CaP have bone metastases, and these are associated with significant morbidity and mortality.

It is growing increasingly apparent with the ongoing development of targeted signal transduction therapies that cancer cells readily acquire resistance to these agents when administered as monotherapies. We have recently reported that the combination of ZOL with TAX was more effective than either drug alone in inhibiting growth of CaP in the bone [38], and early clinical studies of this combination have been encouraging [39]. As a logical next step, we have undertaken to add RAD001 to the combination, since this drug affects tumors by an entirely different mechanism. At this point there are few preclinical studies in CaP evaluating mTOR inhibitors in combination with other therapeutics. The mTOR inhibitors rapamycin and CCI-779 in combination with doxorubicin, mitoxantrone, docetaxel, and receptor tyrosine kinase inhibitors have yielded mixed results in studies with CaP cell lines and/or xenografts [9,13,40]. In our studies, we failed to detect synergistic effects with combination therapies, but a trend toward more pronounced inhibition was observed as more agents were added. Administration of all three agents in combination resulted in significantly greater inhibition of tumor growth than treatment with RAD001 alone.

Serum PSA is widely used in the clinical setting to evaluate the response of prostate cancer to treatment. In this study, the drug combinations did not lead to lower PSA levels at sacrifice than treatments with single agents, despite the fact that smaller tumor volumes were observed. While the mechanisms that underlie the decoupling of PSA and tumor volume in bone metastases await elucidation, we and others have previously observed similar discrepancies [26]. These observations coincide with clinical studies involving

patients with bone metastases and illustrate the limitations of PSA as a surrogate marker of response in advanced patients.

Growth of CaP tumors in the bone causes dysregulation of bone remodeling. CaP bone metastases are usually of osteoblastic character, but increased bone lysis is also associated with advanced CaP. Our studies and others have shown that ZOL inhibits growth of CaP tumors in the bone while increasing bone volume. In the present study, only groups treated with ZOL exhibited significant increases in bone volume by BHM and μ CT analyses. Interestingly, despite the observed inhibition of tumor growth by RAD001 and TAX, we did not observe inhibition of the osteolysis caused by the growth of these cells in the bone. We hypothesize that the perturbations in bone remodeling due to the presence of CaP cells occurred early in the course of the experiments, and therefore could not be affected by treatments that began subsequent to tumor establishment in the bone.

Advanced CaP is associated with cachexia, which is a serious complication of many cancers [41]. Our results show that growth of C4-2 tumor cells in the bone results in decreased body weight of tumor-bearing animals, and that treatment with RAD001 results in maintenance of body weight and lean body mass. The Akt/mTOR pathway has been shown to play a role in anorexia, including, possibly, tumor-related cachexia [42]. Leptin and leucine stimulate hypothalamic mTOR and decrease food intake and body weight in rats. These effects can be inhibited by administration of rapamycin. Although no impact of mTOR inhibitors on tumor cachexia has yet been demonstrated, the data presented, coupled with a sound biological rationale, suggest the need to pursue this possibility in further studies.

CONCLUSIONS

In summary, our data show that RAD001 inhibits the growth of CaP in the bone environment at a low dose of 2.5 mg/kg. Moreover, RAD001 combinations with TAX, ZOL, or both exhibit trends toward greater inhibition of tumor growth than any of these agents alone. Inclusion of ZOL in the combination also prevented increased osteolysis and bone loss associated with the growth of these tumors in the bone. Additionally, RAD001 prevented weight loss associated with growth of C4-2 tumors in the bone. Further prospective studies are warranted to determine whether these results are reproducible in other in vivo models. These preclinical observations support the use of RAD001 in the clinical setting, both on its own and in combination with other agents, in the treatment of advanced CaP.

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Fig. 1. Effects of RAD001 and TAX on serum PSA. RAD001 and TAX inhibited tumor growth in bone as demonstrated by decreases in serum PSA levels. Arrows mark start of treatment. **A:** RAD001: 5 mg/kg, $P = 0.026$; and 10 mg/kg, $P = 0.029$. **B:** TAX: 10 mg/kg, $P = 0.0070$; and 20 mg/kg, $P = 0.0011$. Results are plotted as mean $\pm$ SEM.

Fig. 2.

Effects of RAD001, TAX, and ZOL alone and in combination on serum PSA. Treatment of C4-2 tumors in bone resulted in decreases in serum PSA levels. Treatment began at week 4 as indicated by arrow. **A:** Single-agent therapy. **B:** Combination therapy. **C:** Comparison of sacrifice serum PSA levels among groups. Results are plotted as mean $\pm$ SEM. Significant differences versus controls are denoted by: * $P < 0.05$, ** $P < 0.01$.

Fig. 3.

Effects of RAD001, TAX, and ZOL alone and in combination on tumor and bone. Bone histomorphometry was used to evaluate the effects of the treatment on tissue volume (TV) and tumor volume (TuV). Analyses were performed on longitudinal sections of tibiae (n = 5 per group). BMD and BHM analyses were used to evaluate the effects of the treatments on bone. Tissue volume (**A**), and tumor volume (**B**) were significantly decreased in all treatment groups versus the control group. The largest decreases in TV and TuV were observed with all three agents in combination. Comparison of BMD normalized to contralateral (non-tumored) tibiae for each treatment. C4-2 cells cause decreases in BMD. Treatment with low doses of ZOL alone or in combination preserved bone (**C**). Treatments with ZOL generally caused increases in BV. RAD001 treatment increased BV, but the combination of three agents did not (**D**). Results are plotted as mean $\pm$ SEM. Significant differences versus controls are denoted by: * $P < 0.05$, ** $P < 0.01$.

Fig. 4. Micro-CT analyses. Analysis of the response of the bone to treatment was performed for trabecular bone (metaphysis) and cortical bone. **A:** Tissue volume at the metaphysis. **B:** Ratio of bone volume/tissue volume at the metaphysis. **C:** Cortical bone volume. **D:** Periosteal volume. Results are plotted as mean $\pm$ SEM. Significant differences versus untreated C4-2 xenografts are denoted by: * $P < 0.05$, ** $P < 0.01$. Also shown for reference in these figures are the parameters of normal untreated tibiae. **E:** Representative examples of metaphyseal and cortical bone from groups treated with RAD001. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 5.

RAD001 effects on bodyweight. Treatment with RAD001 inhibited weight loss caused by C4-2 growth in bone. For this analysis, animals were grouped based on whether or not they received RAD001. Total bodyweight was maintained in mice receiving RAD001, while animals with C4-2 tumors not treated with RAD001 experienced loss of body weight. There was a $10.7 \pm 1.5\%$ difference in final body weight between groups with and without RAD001 treatment ($P < 0.001$). Results are plotted as mean $\pm$ SEM.