mTOR Inhibitors Block Kaposi Sarcoma Growth by Inhibiting Essential Autocrine Growth Factors and Tumor Angiogenesis

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Abstract

Kaposi sarcoma originates from endothelial cells and it is one of the most overt angiogenic tumors. In Sub-Saharan Africa, where HIV and the Kaposi sarcoma–associated herpesvirus (KSHV) are endemic, Kaposi sarcoma is the most common cancer overall, but model systems for disease study are insufficient. Here, we report the development of a novel mouse model of Kaposi sarcoma, where KSHV is retained stably and tumors are elicited rapidly. Tumor growth was sensitive to specific allosteric inhibitors (rapamycin, CCI-779, and RAD001) of the pivotal cell growth regulator mTOR. Inhibition of tumor growth was durable up to 130 days and reversible. mTOR blockade reduced VEGF secretion and formation of tumor vasculature. Together, the results show that mTOR inhibitors exert a direct anti-Kaposi sarcoma effect by inhibiting angiogenesis and paracrine effectors, suggesting their application as a new treatment modality for Kaposi sarcoma and other cancers of endothelial origin. Cancer Res; 73(7): 2235–46. ©2012 AACR.

Introduction

Kaposi sarcoma (KS) is an aggressive cancer of the endothelial cell origin affecting immunocompromised patients, the elderly, as well as children and adults in Sub-Saharan Africa. Kaposi sarcoma is the most common cancer overall (men and women) in Namibia, Botswana, Zimbabwe, Zambia, Malawi, Mozambique, Uganda, Ethiopia, and parts of South Africa (1). Kaposi sarcoma is an AIDS-defining malignancy. It is regularly seen as a solid organ transplant-associated cancer in Southern Europe, the Mediterranean region, Turkey, and Saudi Arabia (2). Chang and colleagues identified Kaposi sarcoma–associated herpesvirus (KSHV) in any and all forms of Kaposi sarcoma (3). For lack of known tumor-specific target pathways, Kaposi sarcoma is treated with cytotoxic chemotherapy, now predominantly liposomal doxorubicin (Doxil). Although, initial response rates are reasonable, subsequent failures are not uncommon (4). Drug resistance has also been documented. Doxil is associated with toxicity and has a lifetime dose limit. This motivated our experiments to establish a novel animal model for drug evaluation and to explore the PI3K/Akt/mTOR pathway as a new target of intervention in Kaposi sarcoma.

Morphologically, Kaposi sarcoma lesions appear as flat dermal “patches” upon initial presentation. As the lesion progresses, it goes from patch to plaque to nodule and the initial red color takes on a darker, purplish hue. The characteristic color of these lesions is indicative of the high degree of vasculogenesis and angiogenesis associated with Kaposi sarcoma. Angiogenesis in Kaposi sarcoma begins even before the formation of a visible lesion. Hundred percent of the Kaposi sarcoma tumor cells carry KSHV and express the KSHV latency-associated nuclear antigen (LANA) as the defining biomarker for Kaposi sarcoma (5, 6). The tumor cells also express other viral proteins that promote tumor cell growth cell-autonomously or through induction of soluble factors, which act through autocrine and paracrine mechanisms.

vGPCR is the KSHV homolog to human interleukin (IL)-8 receptor; it can signal in the absence of ligand binding, thus resulting in a constitutively active state (7–9). vGPCR can activate phosphoinositide 3-kinase (PI3K) as well as induce VEGF (10). Other viral signaling molecules, which can activate PI3K signaling, include K1 and K15 (11, 12). As multiple (K1, K15, vGPCR, etc.) viral signaling molecules converge on the PI3K pathway, we reasoned that Kaposi sarcoma is addicted to this pathway and that kinases within this pathway represent promising drug targets.

PI3K is activated by membrane receptor signaling and phosphorylates its downstream target Akt. Phosphorylated Akt in turn activates the mTOR kinases, which have multiple targets that support cell growth (reviewed in refs. 13, 14). We, along with other groups, have shown that the PI3K/Akt/mTOR pathway is constitutively activated in Kaposi sarcoma–infected cells as well as in vGPCR- and K1-driven cell culture models (12, 15–18).

Clinical observations also support the importance of the PI3K/Akt/mTOR pathway in Kaposi sarcoma. Stallone and
colleagues (19) reported that switching the immune suppressive regimen of renal transplant patients from cyclosporine A, which inhibits mTOR (20), resulted in regression of Kaposi sarcoma lesions. Others have reported similar cases (21–23); a few exceptions have also been noted (24, 25). Most recently, we completed a pilot study of AIDS-KS and found long-term disease stabilization or partial responses in 6 of 7 (85%) HIV-positive patients (26). These clinical studies allowed for 2 alternative hypotheses about the mechanism of rapamycin and other allosteric mTOR inhibitors (everolimus and temsirolimus), the so-called rapalogs (reviewed in ref. 27). First, rapalogs changed either the type or the level of immune suppression and Kaposi sarcoma tumor regression was secondary. This hypothesis is supported by the sometimes spontaneous regression of Kaposi sarcoma after immune reconstitution of patients with AIDS or after immune suppression tampering of transplant recipients. Second, rapalogs have direct antitumor effects as had been shown for everolimus in renal cell cancer and temsirolimus in mantle cell lymphoma (28–30). These antitumor effects could be impacting the tumor cell directly or the supporting vasculature. Unique to Kaposi sarcoma, both the tumor cell itself and the tumor-feeding blood vessels are of endothelial origin.

To decide among these competing hypotheses, we established a novel mouse model of Kaposi sarcoma, which is dependent on the KSHV virus, and used it to evaluate a battery of structurally different, U.S. Food and Drug Administration-approved mTOR inhibitors: rapamycin/sirolimus, everolimus/ RAD-001, and temsirolimus/CCI-779. As controls, we used cyclosporine A and FK506 (tacrolimus), which is structurally related to rapamycin, but does not inhibit cyclophillin in T cells, to rapamycin (sirolimus), the so-called rapalogs (reviewed in ref. 27). First, rapalogs changed either the type or the level of immune suppression and Kaposi sarcoma tumor regression was secondary. This hypothesis is supported by the sometimes spontaneous regression of Kaposi sarcoma after immune reconstitution of patients with AIDS or after immune suppression tampering of transplant recipients. Second, rapalogs have direct antitumor effects as had been shown for everolimus in renal cell cancer and temsirolimus in mantle cell lymphoma (28–30). These antitumor effects could be impacting the tumor cell directly or the supporting vasculature. Unique to Kaposi sarcoma, both the tumor cell itself and the tumor-feeding blood vessels are of endothelial origin.

Materials and Methods

Cell culture

All cell lines were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 100 μg/mL streptomycin sulfate, 100 U/mL penicillin G (Life Technologies), and 10% FBS at 37°C in 5% CO2. Cell lines were obtained from the American Type Culture Collection (ATCC) or derived in our laboratory. The cells have been tested and authenticated. The method used was whole genome Affymetrix 6.0 SNP array. The cells were last tested by the aforementioned method at submission within receipt or resuscitation from stock. The LIT2 cell line will be submitted to the ATCC.

Quantitative PCR for KSHV genome

Genomic DNA was extracted using Wizard SV Genomic DNA Purification System, as per manufacturer’s protocol (Promega) and quantitative PCR (qPCR) was used to detect the viral genome in the different Kaposi sarcoma cell lines as per our previous publications (32).

CGH analysis

Genomic DNA was extracted as above and hybridized to the 6.0 GeneChip Human Mapping Array that uses more than 906,600 known single-nucleotide polymorphism and 946,000 copy number variation markers (Affymetrix). Data analysis was conducted using Partek Genomics Suite as described previously (33) and normalized to tert-immortalized human umbilical vein endothelial cell (HUVEC).

Immunofluorescence

Cells were cultured overnight on glass coverslips in 6-well plates (Falcon-BD Biosciences Inc.). They were then washed in PBS followed by the protocol for immune fluorescence as described previously (34). Primary mouse anti-LANA (1:600 dil.; Novocastra Laboratories Ltd.), rabbit anti-S6 ribosomal protein, Alexa Fluor 488–conjugated anti-phospho-S6 ribosomal protein for both S235/236 and S240/244 residues (1:200 dil.; Cell Signaling Technology, Inc.) were used for immunofluorescence. Images were taken on a LEICA DM4000B fluorescence microscope (Leica) equipped with a 63/1.4–0.6 numerical aperture objective and a Q-Imaging Retiga 2000R camera. Raw microscopy image stacks were deconvoluted using Simple PCI (Hamamatsu Corp.) 2D Blind Deconvolution that iteratively applies AutoQuant Imaging (Media Cybernetics, Inc.) proprietary algorithm to remove blur and generate high clarity images and stored as tiff files. Images were assembled in Photoshop CS4 under MacOsX 10.7.

Tumor formation

Cells were counted, washed once in ice-cold PBS (Cellgro Mediatech, Inc.), and indicated cell doses were diluted into 100 μL PBS and mixed with 100 μL growth factor–depleted Matrigel (BD Biosciences). A total of 1 × 106 cells were injected subcutaneously into the flank of C.B.17-Prkdcscid mice (Jackson Laboratory) following our previously validated procedures (35). The mice were observed every day for the presence of palpable tumors. Drug or vehicle was injected intraperitoneally at the indicated dosing schedule. Tumor diameters were determined by caliper measurements. Tumor volume was calculated as V = a × b × c, where a, b, and c are the 3 diameters (length, breadth, and width) of the tumor. The tumors were excised from the site of injection and fixed in formalin (Fisher Diagnostics) for subsequent analysis.

Blood rapamycin quantification

Blood was collected from the animals by cardiac puncture 48 hours after the last intraperitoneal injection of rapamycin at either 2.5 or 5 mg/kg dose. Care was taken to ensure that blood was directly collected into EDTA-coated tubes (BD Biosciences) to prevent any coagulation. Rapamycin levels were determined from whole blood by high-performance liquid chromatography/tandem mass spectrometry (HPLC/MS-MS) using Nova_Pak columns (Waters Corp.) offering sensitivity in the 2 to 50 ng/mL range.
Immunohistochemistry

Tumors were removed and processed as per ref. (17) and stained using the appropriate primary antibodies: phospho-S6 ribosomal protein (Ser 235/236) monoclonal antibody (1:100 dil.) and phospho-4EBP1 (Ser 65) antibody (1:50 dil.) from Cell Signaling Inc.. Sections were imaged using a LEICA DM LA histology microscope (Leica) equipped with a ×10/0.25 numerical aperture, a ×40/0.75 numerical aperture N plan objective, or HC PLAN APO ×20/0.70 numerical aperture and Leica DPC 480 camera. Images were stored as tiff files under Mac OS X10.5. Staining intensity was assessed using the FRIDA (Framework for Image Dataset Analysis) as outlined (36). Briefly, FRIDA measures the pixel intensity of the tumor section based on a user defined “positive” stain and we plotted the square root of this total area intensity. To determine the degree of significance, we used ANOVA implemented in the R statistics environment (version 2.14.1).

Tumor vasculature staining

Tumor vasculature was determined using commercially available periodic acid-Schiff (PAS) staining system following manufacturer’s recommendations (Sigma-Aldrich). Sections were imaged, PAS staining quantified using FRIDA, and significance determined as earlier.

VEGF quantification

VEGF levels in the culture supernatants were determined using commercially available VEGF ELISA assay as per manufacturer’s recommendations (PeproTech). The biologic activity of secreted VEGF (Supplementary Fig. S1) was ascertained by sprouting assay as described (37).

Results

A robust, human, KSHV-positive mouse model for Kaposi sarcoma

Despite the identification of Kaposi sarcoma nearly 2 decades ago, there is a dearth of preclinical models for Kaposi sarcoma. Explants from Kaposi sarcoma biopsies have been successful to a limited degree (2 cell lines in 20 years; refs. 38, 39), but rapidly lose the viral episome. Thus, they no longer represent the full spectrum of molecular interactions present in the primary tumor. The best-characterized example until recently was the SLK cell line. It was isolated from an oral Kaposi sarcoma biopsy of an HIV-negative male (38) and at the time of tissue procurement expressed KSHV LANA and exhibited the Kaposi sarcoma–defining spindle cell morphology. KSHV was lost, but the spindle cell morphology was retained and SLK cells form tumors in immunodeficient mice. This year it was shown that the SLK cell line used in laboratories throughout the world has in fact become contaminated with an irrelevant cell line (40). This leaves the field bereft of human tumor models for this cancer.

De novo infection of human endothelial cells in culture by KSHV is possible, but here too the virus is rapidly lost unless maintained under drug selection (37, 41–43). The exceptions are 2 cell lines (TIVE-E1 and TIVE-L1) that were derived by infection of telomerase-immortalized, umbilical cord–derived endothelial cells (44). These cells maintain KSHV in the absence of drug selection, secrete IL-6, IL-8, VEGF, and form tumors in nude mice, albeit slowly.

The L1T2 cell line was established from a subcutaneous tumor that developed upon injecting TIVE-L1 cells into C.B.17-Pkrdc<sup>SCID</sup> mice (Fig. 1A). L1T2 retained the tumor-forming
potential of the parental cell line. They induced uniform, fast growing, subcutaneous tumors in C.B.17-Pkrdc<sup>SCID</sup> (Fig. 1B) and nu/nu mice (data not shown). At the 10<sup>6</sup> cells dose, 100% of the animals developed palpable tumors after only 2 days, which grew exponentially for up to 21 days (Table 1). This phenotype held true at the 10<sup>5</sup> cells dose as well (data not shown). The cells express the viral latent protein LANA in the typical punctate pattern (Fig. 1C). The punctate pattern requires the presence of KSHV terminal repeats, which bind LANA (45), and the so-called "LANA-dots" correlate directly with the number of viral episomes (46). The L1T2-induced tumors were highly proliferative as shown by Ki-67 staining (Fig. 2A and B) and in the tumor-retained expression of the endothelial marker genes CD31 (Fig. 2E and F) and VEGF-R3 (Fig. 2C and D) as well as LANA (Fig. 2G and H). The cells retained the complete viral episome as shown by real-time qPCR with

### Table 1. In vivo efficacy studies

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<th>Drug</th>
<th>Cell</th>
<th>Drug conc., mg/kg&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Progression free, d&lt;sup&gt;b&lt;/sup&gt;</th>
<th>n&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Reduction, %&lt;sup&gt;d&lt;/sup&gt;</th>
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<td></td>
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<td>59&lt;sup&gt;h&lt;/sup&gt;</td>
<td>5</td>
<td>3</td>
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<td>7</td>
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**NOTE:** Treatment started after palpable tumors (2 mm<sup>2</sup>) developed in 50% or more of animals. Each treatment group was compared with a control, vehicle group of similar number of animals that was inoculated simultaneously with the same number of tumor cells. Vehicle-treated animals (control group) require euthanasia between day 20 and 40.

<sup>a</sup>Concentration of drug. All animals were treated on a Monday, Wednesday, Friday schedule intraperitoneally.

<sup>b</sup>Number of days that the animals in the drug-treatment arm remained on study without exponential tumor growth requiring euthanasia or until the experiment was terminated because reduction in tumor volume between matched control and treatment group was significant to P < 0.05.

<sup>c</sup>Number of animals per drug-treated group. In each experiment, these were paired with 5 to 6 vehicle-treated animals that received tumor cells on the same day and from the same passage to allow for direct comparison and calculation of relative reduction in volume.

<sup>d</sup>Percentage reduction in mean tumor volume in the drug-treated group compared with the vehicle-treated group at time point of maximum difference. Typically, this was the day when more than 50% of tumors in the control group grew to 2,000 mm<sup>2</sup> and/or required euthanasia.

<sup>e</sup>P value as determined using t test of tumor volume at each time point (3 times per week). No value reflects no significant difference to control.

<sup>f</sup>Cells were injected without Matrigel support.

<sup>g</sup>Toxicity in the treatment arm.

<sup>h</sup>Progression-free survival >50 days (200% of the untreated group).
primers in each open reading frame across the genome (Fig. 2J). L1T2, for the first time, provides an animal model for Kaposi sarcoma based on human cells, which maintains the virus in the absence of selection, that is fast, economic, robust, and reproducible enough for large scale in vivo drug evaluation.

Rapamycin is efficacious against Kaposi sarcoma xenograft tumors in vivo

To test the hypothesis that rapalogs have direct antitumor activity against Kaposi sarcoma and that this activity is independent of any immune-modulating properties that these agents may also have, we treated established xenografts in...
C.B.17-PkrdcScid mice that lack any immune effector cells with rapamycin and its various analogs. First, we tested TIVE-E1 and TIVE-L1 cells, which had previously been established as model systems to study Kaposi sarcoma pathogenesis in nu/nu mice (44). TIVE E1 and L1 represent 2 independent clones of telomerase-immortalized, KSHV-infected endothelial cells, each of which sustained independent chromosome rearrangements (Fig. 2J). TIVE-L1 (Fig. 3A) and TIVE-E1 (Fig. 3B) cells were injected subcutaneously and upon formation of palpable tumors, animals received rapamycin at 3 mg/kg/d 3 times a week, or vehicle. Figure 3A and B compares tumor growth in rapamycin and mock-treated animals. In each cohort, drug-treated animals showed significantly smaller tumors than mock treatment ($P < 0.05$ by repeated measurements, random effects model). Next, we tested KSHV-negative SLK cells, which thus far were the standard for preclinical drug testing in Kaposi sarcoma (Fig. 3C). Upon formation of palpable tumors, animals received rapamycin at 2.5 mg/kg/d 3 times a week, 5 mg/kg/d 3 times a week, or vehicle. At 5 mg/kg, rapamycin was very effective against tumor progression; however, it adversely affected overall health of the animals (Table 1). At 2.5 mg/kg, rapamycin was equally as effective in tumor inhibition as 5 mg/kg/d dose together with improvement in survival and overall health ($P < 0.05$ by repeated measurements random effects model). Finally, we tested KSHV-positive L1T2 cells (Fig. 3D). Upon formation of palpable tumors, animals received rapamycin at 2.5 mg/kg/d 3 times a week or vehicle. Again, rapamycin effectively inhibited tumor growth compared with vehicle control ($P < 0.05$ by repeated measurements random effects model).

To establish equivalency among the different rapalogs, we also tested temsirolimus and everolimus in the L1T2 system. Each resulted in significant tumor inhibition. We also tested the ATP-competitive mTOR inhibitor PP242 (Table 1). This resulted in tumor reduction but was not superior to allosteric rapalogs. FK506, which is structurally similar to rapamycin and which like rapamycin binds FKBP (but not mTOR), had no activity (Table 1). Table 1 is a summary of all the studies we conducted. These show that rapalogs are efficacious against tumors, irrespective of whether they were isolated from Kaposi sarcoma biopsies (SLK) or generated following de novo infection of endothelial cells (E1, L1, and L1T2). Single agent rapalogs, irrespective of their particular structure, resulted in prolonged (up to 130 days) growth inhibition. Of note, each experiment was carried out in parallel with a similar size group of mock-treated animals, which received the same cell aliquot. The group size $n$ in Table 1 refers only to the size of the experimental group. In total, we observed significant rapalog-dependent tumor inhibition in 85 animals.

Rapalog dosing presents a significant challenge in the clinic, especially considering that rapalogs are metabolized by the cytochrome P450 family members in the liver (47). Even in culture, the IC$_{50}$ for rapamycin may vary more than 1,000-fold between cancer cell lines (48). The reason is unknown and may have as much to do with drug transport as with pathway resistance mutations. P450 is expressed by the tumor endothelial cells within Kaposi sarcoma lesions (Roy and Dittmer; unpublished data). Therefore, we optimized the dosing
regimen required to obtain therapeutic drug trough levels, while minimizing toxicity. Note that our dosing is rather infrequent compared with other experimental designs (16, 49), which require daily dosing. At 5 mg/kg doses of rapamycin, the animals exhibited weight loss, lethargy, and had to be sacrificed despite robust inhibition of tumor progression. At 1.5 mg/kg, the animals remained healthy throughout extended treatment, but antitumor activity was lost (data not shown). On the basis of these data, we established the optimal dose for long-term rapamycin treatment in C.B.17. PKRD<sup>+/−</sup> mice at 2.5 mg/kg 3 times a week. Next, we assessed the actual levels of rapamycin in the blood stream of the animals. Figure 2F shows that at 2.5 mg/kg doses we are able to maintain a steady level of drug in the blood compared with 5 mg/kg (Fig. 2E) where blood levels spike early on and presumably cause drug toxicity. In summary, we established the efficacy of multiple, chemically distinct rapalogs (sirolimus/rapamycin, temsirolimus, everolimus, etc.), and the ATP-competitive mTOR inhibitor PP242 in 4 distinct xenograft models of Kaposi sarcoma. We established 2.5 mg/kg 3 times a week as a low intensity regimen, which resulted in long-term (>60 days) growth suppression and showed that this dosing regimen leads to sustained, systemic trough levels of 20 ng/mL, which was the target trough level in recent clinical trials of this drug family (26).

Rapamycin inhibits phosphorylation of mTOR targets S6 ribosomal protein and 4E-binding protein in vivo

Having extensively validated our tumor model, we used it to elucidate the mechanism of action of allosteric rapalogs in Kaposi sarcoma. Phosphorylation of ribosomal S6 (S6R) protein is considered a robust readout for mTOR activation. We treated L1T2 cells with rapamycin (5 μmol/L corresponding to 4.5 μg/mL) in culture and measured S6 phosphorylation. Figure 4 shows immunofluorescence staining for total (Fig. 4A and D) and phosphorylated S6R [pS6R: Fig. 4B and E for pS6R (Ser235/236) and Fig. 4C and F for pS6R (Ser240/244)]. As expected, S6 is expressed at high levels in L1T2 cells and heavily phosphorylated at both composite sites (Fig. 4A–C). Upon treatment with rapamycin, total S6 expression remained unchanged, whereas S6 phosphorylation was completely abolished (without loss of cell viability). This shows that rapalogs inhibit their molecular targets in our Kaposi sarcoma-tumor model.

Because we were treating established tumors in the mouse model where rapalogs lead to sustained growth inhibition, and only later lead to necrosis due to lack of nutrients and oxygen (see later), we were able to measure molecular markers of mTOR inhibition in early tumors in vivo. The L1T2 tumor morphology of mock-treated tumors was analogous to Kaposi sarcoma biopsies as judged by hematoxylin and eosin stain. The drug-treated tumors were similar, except that the interior of the tumor mainly consisted of necrotic tissue as compared with mock (data not shown). Treatment with rapamycin resulted in dramatically reduced levels of pS6R (Ser235/236) as compared with both mock and FK506 treatment (Fig. 4G–I). In addition to pS6R, a second target of mTOR is 4EBP1 (14, 50). Immunohistochemical staining of tumor sections revealed downregulation of 4EBP1 phosphorylated at Ser65 following rapamycin treatment but no change in mock- and FK506-treatment.
treated tumors (Fig. 4J–L). Rapamycin reduced S6 and 4E-BP1 phosphorylation in vivo (Fig. 4M, with \( P \leq 0.05 \) by two-way ANOVA). This shows that the molecular mechanism of action of rapalogs in Kaposi sarcoma is the same as in other cancers, such as KSHV-associated primary effusion lymphoma (PEL; ref. 17). It validates the use of pS6 and p4E-BP1 as novel biomarkers for rapalog therapy in Kaposi sarcoma.

**Rapamycin inhibits VEGF secretion and associated vasculature development**

Kaposi sarcoma tumors are characterized by their atypical purple color indicative of vastly increased vasculature and angiogenesis. Furthermore, Kaposi sarcoma tumors are rich in VEGF, IL-1\( \beta \), platelet-derived growth factor (PDGF), and other endothelial cell growth factors (51–53). This phenotype is recapitulated in our murine model. Rapalogs are known to inhibit endothelial cell–mediated tumor neoangiogenesis in addition to direct tumor growth (54, 55). We therefore hypothesized that some of the Kaposi sarcoma–specific targets of rapamycin are paracrine growth factors involved in tumor vasculature development. Because of the endothelial cell lineage of Kaposi sarcoma tumors, these same growth factors can also function in an autocrine manner, which may explain the increased sensitivity of Kaposi sarcoma to rapalogs.

VEGF is a key angiogenesis-promoting factor secreted by Kaposi sarcoma and other tumor cells. TIVE-E1, -L1, SLK, and L1T2 cells all secrete VEGF, as detected in the culture supernatant. We measured between 2,000 and 4,000 pg/mL in the supernatant Kaposi sarcoma-like cells over a 48-hour period. In comparison, KSHV-infected lymphoma cell lines secreted 200 to 400 pg/mL in the same, luminex-based, assay. The cells secreted similarly high levels of IL-6 and IL-8 (Roy and Dittmer; unpublished data). Rapamycin downregulated levels of VEGF secreted by TIVE-L1 cells in culture (Fig. 5A). We conducted a time course followed by drug washout experiment to assess both the production and accumulation of VEGF in culture supernatant. We detected levels of secreted VEGF (isoform A) in cells treated with 0.5 \( \mu \)mol/L (0.45 \( \mu \)g/mL) and 5 \( \mu \)mol/L (4.5 \( \mu \)g/mL) rapamycin over 96 hours (Fig. 5A). Upon drug withdrawal, the cells recovered slowly and gradually increased VEGF secretion. Similar results were noted for TIVE-L1, SLK, and L1T2 cells (data not shown). For KSHV-infected BCBL-1 cells, we found that conditioned media from rapamycin-treated cells no longer supported HUVEC tubule formation.
(Supplementary Fig. S1). This shows that rapamycin inhibits biologically active VEGF, which is an autocrine growth factor in Kaposi sarcoma (56).

The xenograft model constitutes a much more demanding setting than in vitro culture and requires cancer cells to establish and maintain an optimal microenvironment for survival. We hypothesized that reduction of growth factors by rapalogs would have a pronounced impact on tumor progression. To address this, we used PAS staining, which marks the vasculature directly, rather than relying on just one particular biomarker protein, thereby detecting the overall physiologic impact (57, 58). Rapamycin resulted in a sustained tumor vasculature defect (Fig. 5B). Rapamycin-treated tumors had less ordered, less mature vessel formation compared with mock and FK506 treatment. The tumor cells appeared in isolated, clumped nests rather than each cell being enmeshed in supportive stroma and vasculature with the Kaposi sarcoma–associated slit-like morphology. Quantification of PAS-positivity showed a statistically significant reduction in staining (Fig. 5C). Rapamycin-treated tumors had less ordered, less mature vessel formation compared with mock and FK506 treatment. The tumor cells appeared in isolated, clumped nests rather than each cell being enmeshed in supportive stroma and vasculature with the Kaposi sarcoma–associated slit-like morphology. Quantification of PAS-positivity showed a statistically significant reduction in staining (Fig. 5C).

**Rapamycin and doxorubicin combination therapy**

Having shown single-agent efficacy of multiple rapalogs against Kaposi sarcoma, we explored the efficacy of rapamycin in the adjuvant setting. Specifically, we wanted to test the hypothesis that adding doxorubicin to rapamycin would result in increased cytotoxicity. We confirmed this hypothesis in the cell culture model of PEL (Supplementary Fig. S2); however, the in vivo studies yielded a surprising result. Preformed L1T2 tumors were treated with 4 different regimens: (i) 2.5 mg/kg rapamycin [intraperitoneally (i.p.) 3× weekly], (ii) 4 mg/kg doxorubicin (i.p. 5× weekly), (iii) 2.5 mg/kg rapamycin (i.p. 3× weekly) + 4 mg/kg doxorubicin (i.p. 5× weekly), or (iv) mock. Individually, both drugs inhibited tumor progression (Table 1). Nonetheless, the combination treatment did not show synergy or an additive effect on tumor growth. We used PAS staining to investigate tumor angiogenesis (Fig. 6). As expected, untreated L1T2 tumors were highly vascularized and individual cells were neatly packed in a supportive, mesh-like vascular microenvironment (Fig. 6B and C). Rapamycin treatment (Fig. 6D and E) destroyed the tumor vasculature ($P < 0.005$ by Wilcoxon sum rank test), which we quantified by counting the number of PAS-positive branches in several tumor sections. Branch points, defined as 2 or more finger-like PAS vessels at a common point, were averaged from 5 distinct regions for each tumor section. Doxorubicin did not affect the tumor vasculature (Fig. 6A). The combination treatment shows a novel phenotype: tumor cells were nested together in relatively few islands separated by extended thicker slit-like structures (Fig. 6H and I). This may explain the absence of synergy between rapamycin and doxorubicin in this model. By restricting angiogenesis, rapamycin restricts at the same time the flow of nutrients and oxygen to the tumor (resulting in growth inhibition) and the flow of the doxorubicin drug, thus limiting the efficacy of the cytotoxic drug. This result has important clinical ramifications. It argues against simultaneous therapy of rapalogs and cytotoxic drugs, which in the extreme may foster the emergence of drug resistant tumor cells due to suboptimal exposure to the cytotoxic drug.

![Figure 6. Combination treatment using rapamycin and doxorubicin. A, the number of vascular branches after treatment with vehicle, doxorubicin (Dox), or rapamycin (Rapa). The line denotes the median, the box denotes 25th and 75th percentile, and the whiskers the range of the data. Significance ($P$ value) is calculated using ANOVA. B–I, PAS staining of the tumor for the different treatment groups at ×100 and ×400 magnifications. The double arrows provide a visual aid to gauge the size of the vessel free tumor cell nests.](cancerres.aacrjournals.org)
Discussion

A large body of clinical evidence suggests that in the solid organ transplant setting switching to a rapamycin-containing immunosuppressive regimen leads to Kaposi sarcoma regression or lowers the risk of Kaposi sarcoma development (21–23, 59, 60). Similar data are missing for AIDS Kaposi sarcoma, endemic Kaposi sarcoma, or classic Kaposi sarcoma or for the use of rapalogs as dedicated, single agent antitumor drugs against Kaposi sarcoma. No data exist for the newer rapamycin analogs everolimus, temsirolimus, or the ATP-competitive inhibitors of mTOR (27). Here, we tested the hypothesis that mTOR inhibitors have antitumor activity against Kaposi sarcoma, and we describe a new mechanism that could explain the extraordinary sensitivity of Kaposi sarcoma to rapamycin. This work was predicated on the development of a new human tumor model of Kaposi sarcoma, which is robust, fast, and mimics all the phenotypes associated with the human tumor (Fig. 1). It was driven by the insights that (i) Kaposi sarcoma is an endothelial cell lineage tumor (42, 61, 62), (ii) Kaposi sarcoma is dependent on autocrine and paracrine growth factors, (iii) extensive neoangiogenesis, and (iv) rapalogs stymy tumor growth in common cancers by inhibiting the host endothelial cells comprising the tumor vasculature and microenvironment as well as through direct inhibition of tumor cell activity (14).

For the purpose of this discussion, we define rapalogs functionally as inhibitors of mTOR, as opposed to FK506, which has a similar chemical structure as rapamycin, but does not bind to or inhibit mTOR. All rapalogs tested induced tumor regression of preformed Kaposi sarcoma-like human tumors in our xenograft model (Fig. 2 and Table 1). Rapalog efficacy did not depend on immune system modulation, as the host animals were deficient for B, T, and natural killer cells and as we achieved long-term, sustained responses with a 3× per week dosing regimen. Rapalog efficacy did not depend on the presence of KSHV, as KSHV-negative SLK cell tumors were also inhibited. This is expected as SLK cells exhibit the same signs of PI3K/Akt/mTOR activation, that is, phosphorylation of key residues, as Kaposi sarcoma tumors (36), and as KSHV-induced Kaposi sarcoma cell lines. Rapalogs decreased phosphorylation of S6 ribosomal protein and 4EBP-1 in vivo (Fig. 4), showing that they inhibit the intended molecular targets downstream of mTOR. The antitumor effect was reversible in vivo: upon withdrawal of the rapalogs the tumors grew back uniformly (Supplementary Fig. S3). In contrast, 2 chemically related drugs cyclosporine A (data not shown) and FK506 had no antitumor effect. This is particularly interesting as FK506 like rapamycin also binds to FKBP12 but cannot bind mTOR (63). It supports the specificity of action of the rapalogs. The ATP-competitive inhibitor PP242 also inhibited tumor progression in vivo, but was not superior to the allosteric mTOR inhibitors. This validates mTOR as a bona fide drug target in Kaposi sarcoma and provides a solid biologic rationale for future clinical trials.

We uncovered a novel mechanism of action for mTOR inhibitors that is specific to Kaposi sarcoma. The rapalogs inhibited VEGF secretion by the Kaposi sarcoma tumor cells (Fig. 3) as well as VEGF secretion in KSHV-dependent lymphomas (Supplementary Fig. S2). We surmise that the translational arrest induced by mTOR inhibition is not universal but preferentially affects autocrine growth factors of endothelial lineage cells and Kaposi sarcoma tumor cells. This is analogous to the situation in PEL, where we showed previously that rapamycin stopped lymphoma growth by inhibiting the essential autocrine growth factors IL-6 and IL-10 (17). This may explain the differential response of different human cancers to rapalogs. Cancers, such as Kaposi sarcoma, that still depend heavily on proangiogenic and growth factors to sustain elevated PI3K signaling are susceptible to mTOR inhibitors. Cancers that evolved growth factor independence such as hormone resistant prostate cancer or estrogen receptor negative breast cancer may be less responsive to mTOR inhibitors.

Finally, we explored the combination therapy of rapamycin with the prototypic cytostatic drug, doxorubicin, which represents the current standard of care for Kaposi sarcoma. We did not find any benefit of combining these 2 drugs in vivo. This represents a cautionary note in regards to combining mTOR inhibitors with other drugs, as mTOR inhibitors may inhibit neovascularization and sprouting of nontransformed endothelial cells (Fig. 6), thereby potentially limiting drug delivery to the tumor.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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mTOR in Kaposi Sarcoma


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