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Palomid 529, a Novel Small-Molecule Drug, Is a TORC1/TORC2 Inhibitor That Reduces Tumor Growth, Tumor Angiogenesis, and Vascular Permeability

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Abstract

It has become clear that the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway is central for promoting both tumor and tumor stroma and is therefore a major target for anticancer drug development. First- and second-generation rapalogs (prototypical mTOR inhibitors) have shown promise but, due to the complex nature of mTOR signaling, can result in counterproductive feedback signaling to potentiate upstream Akt signaling. We present a novel PI3K/Akt/mTOR inhibitor, Palomid 529 (P529), which inhibits the TORC1 and TORC2 complexes and shows both inhibition of Akt signaling and mTOR signaling similarly in tumor and vasculature. We show that P529 inhibits tumor growth, angiogenesis, and vascular permeability. It retains the beneficial aspects of tumor vascular normalization that rapamycin boasts. However, P529 has the additional benefit of blocking pAktS473 signaling consistent with blocking TORC2 in all cells and thus bypassing feedback loops that lead to increased Akt signaling in some tumor cells. [*Cancer Res* 2008;68(22):9551–7]

Introduction

In cancer, signaling alterations are evident in multiple components of the microenvironment. For example, we have found that Akt signaling is increased in the tumor endothelium, likely from the constant bombardment of growth factors from the activated tumor and stroma. Importantly, inhibition of this pathway normalizes the vasculature both structurally and, more importantly, from the perspective of vascular function and barrier properties (1). Notably, vascular normalization is proposed to be a key component of the ability of bevacizumab (Avastin) to synergize with chemotherapy and radiation (2). The phosphatidylinositol 3-kinase (PI3K)/Akt/ mammalian target of rapamycin (mTOR) pathway is also a central point of dysregulation in many cancer cells due to direct activating mutations in the kinases or deletion of the PTEN phosphatase that functions to negatively regulate this signaling pathway (3). Because of the link between critical cancer cell receptors such as epidermal growth factor receptor (EGFR) family members, PI3K-Akt signaling can be a complication in resistance to inhibitors of the EGFR pathway in some tumors (4). In glioblastoma, it is not uncommon to

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find both activating mutations in EGFR combined with deletion of PTEN and increased efficacy has been shown by combining EGFR inhibitors with rapamycin (5,6). One of the major downstream parts of the pathway is mTOR pathway, and this pathway has been targeted by the mTOR inhibitors rapamycin and more recent rapalogs (7,8). However, the effects on signaling by rapamycin are complicated by positive and negative feedback loops from mTOR to Akt in different components of the tumor microenvironment (9). TORC1 inhibition of rapamycin can lead to increased Akt signaling due to relief of the S6K suppression of IRS1, causing potentiation of PI3K signaling in many tumor cells (10). On the other hand, stromal Akt signaling is repressed by the same doses of rapamycin that lead to tumor up-regulation of Akt signaling (11). This is possibly due to cell-specific sensitivities that lead to indirect inhibition of TORC2 assembly (12,13). Dual mTOR-PI3K or mTOR-Akt inhibitors may be a solution to these feedback loops; however, in this article, we describe a novel inhibitor that is both a TORC1 and TORC2 inhibitor and consistently down-regulates Akt and mTOR signaling both in a PTEN mutant glioma tumor cells and in endothelial cells. This drug is both antitumor growth and antiangiogenic.

Materials and Methods

Animals and materials

Four- to 6-wk-old female athymic nu/nu mice (National Cancer Institute, Bethesda, MD) were used in our experiments. Nonreplicating adenoviral vector was engineered to express the murine vascular endothelial growth factor (VEGF)-A164 isoform as described previously (14). Recombinant vascular permeability factor/VEGF was obtained from R&D Systems. Palomid 529 (P529) was provided from Paloma Pharmaceuticals, Inc. Wortmannin and okadaic acid (OA) were purchased from Calbiochem, Inc. All antibodies were purchased from Cell Signaling Technology, except anti-h-actin, which was purchased from Sigma. Animal protocols were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee.

Estrogen receptor binding assays

The proteins were produced with rabbit reticulocyte lysates as supplied by Promega Corp. (TNT kit) that couples transcription and translation in a single reaction. The amount of template used in each reaction was determined empirically and expression was monitored in parallel reactions where [³⁵S]methionine was incorporated into the receptor followed by gel electrophoresis and exposure to film. Binding reactions of the estrogen receptors (ER) and P529 were carried out in 100 mL final volumes in TEG buffer [10 mmol/L Tris (pH 7.5), 1.5 mmol/L EDTA, 10% glycerol]. In vitro transcribed-translated receptor (5 AL) was used in each binding reaction in the presence of 0.5 nmol/L [³H]estradiol (E₂). All compounds were routinely tested from 10⁻¹¹ to 10⁻⁶ mol/L and diluted in ethanol. The reactions were incubated at 4°C overnight and bound E₂ was quantified by adding 200 mL dextran-coated charcoal. After a 15-min rotation at 4°C, the tubes were centrifuged for 10 min and 150 mL of the supernatant were added to 5 mL scintillation mixture for determination of cpm by liquid scintillation counting. The maximum binding was determined by competing bound E₂ with only the ethanol vehicle. Controls for background were included in each experiment using 5 mL unprogrammed rabbit reticulocyte lysate. This value, typically 10% to 15% of the maximal counts, was subtracted from all values. The data were plotted and K_i values were calculated using the Prism software. Experiments were conducted at least thrice in duplicate.

Human umbilical vascular endothelial cell proliferation

Human umbilical vascular endothelial cells (HUVEC) and the required medium complements were purchased from Cascade Biologics, and the growth and maintenance of the cultures was as described by the manufacturer. The proliferation assay was carried out by seeding the

HUVECs in 96-well plates at a density of 1,000 per well in complete medium. Following a 24-h plating period, the cells were starved for 24 h in 0.5% serum before being treated with P529 in the presence of 10 ng/mL basic fibroblast growth factor (bFGF) or VEGF in complete medium. After 48 h, cell number was determined using a colorimetric method as described by the supplier (Promega). The results were expressed as the percentage of the maximal bFGF or VEGF response in the absence of P529. Nonproliferating endothelial cells were assayed by growing HUVECs to quiescence in 96-well plates and treating with P529 for 48 h. Initially, 5,000 cells per well were seeded and confluence was achieved the next day. The plates were incubated for another 24 h to ensure growth arrest before treatment with P529. Cell number was determined as outlined above.

HUVEC apoptosis

Cell death was monitored by quantifying the amount of cytoplasmic histone-associated DNA fragments that accumulated in the cell, method as described by the supplier (Roche). The method uses ELISA detection with a monoclonal anti-histone antibody. Briefly, HUVECs were trypsinized, diluted, and aliquoted into microfuge tubes at a concentration of 50,000 per tube. Treatments were made with P529 at 10 mmol/L, camptothecin at 4 mg/mL, or no addition for 6 h at 37°C followed by cell lysis and analysis using the detection kit according to the manufacturer. Apoptosis was quantified calorimetrically at an absorbance of 405 nm.

Cell culture

Human dermal microvascular endothelial cells (HDMECs) were isolated as previously described (15). HDMECs were cultured in EGM-2 medium supplemented with 5% fetal bovine serum (FBS) and growth factors (Clonetics) at 37°C and 5% CO₂. C6V10 cells (rat C6 glioma cells) and U87 cells (human glioma cells) were cultured in DMEM supplemented with 10% FBS. HDMEC and C6V10 cells were serum starved in EGM-2 medium supplemented with 2% FCS and DMEM containing 2% FBS, respectively, and pretreated with P529 (20 Amol/L) for 16 h. Before growth factor stimulation for 15 min, cells were pretreated with wortmannin (50 nmol/L) for 30 min or OA (100 nmol/L) for 2 h.

Immunoblotting and immunoprecipitation

Proteins were extracted from mouse tissue using T-PER Tissue Protein Extraction Reagent Buffer (Pierce) containing 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na₃VO₄, 1 mmol/L EGTA, 1 Ag/mL leupeptin, 0.5% aprotinin, and 2 Ag/mL pepstatin A and from cultured cells using cell lysis buffer [20 mmol/L Tris-HCl (pH 7.5), 0.15 mol/L NaCl, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na₃VO₄, 1 mmol/L EGTA, 1 Ag/mL leupeptin, 0.5% aprotinin, and 2 Ag/mL pepstatin A]. Equal amounts of protein were separated by NuPAGE Novex Bis-Tris Gels (Invitrogen Corp.) and transferred into nitrocellulose membranes (Invitrogen). Membranes were incubated with primary antibodies overnight at 4°C after blocking with 5% milk in TBS-Tween 20 and then detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (Amersham Biosciences UK), respectively, and SuperSignal West Pico Chemiluminescent Substrate system (Pierce). All experiments were carried out independently at least thrice. For immunoprecipitations, C6 glioma cells were serum starved for 24 h. Before insulin-like growth factor (IGF)-I stimulation, cells were pretreated with P529 (20 Amol/L) for 2 or 24 h. Cell lysis buffer consisted of 20 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.3% CHAPS, 25 mmol/L sodium pyrophosphate, 1 mmol/L h-glycerophosphate, 1 mmol/L Na₃VO₄, and 1 Ag/mL leupeptin. Protein (1.5 mg) diluted to 1 mL with cell lysis buffer was centrifuged at 10,000 × g for 10 min at 4°C to remove cellular debris. mTOR (1:100) antibody was added and immune complexes were allowed to form by incubating with rotation for overnight at 4°C. A 50% slurry (25 mL) of protein G-Sepharose was then added and the

incubation was continued for 3 h. Immunoprecipitates were captured with protein G-Sepharose, washed four times with cell lysis buffer, and analyzed by immunoblotting.

Tumor growth

Four- to 6-wk-old female nude mice were pretreated with P529 (200 mg/kg/2 d, i.p.) for 1 wk, and then 1×10^5 C6V10 rat glioma cells were injected s.c. (16). Treatment continued while tumors were allowed to grow for 21 d. U87 cells (3×10^6 /100 AL) were injected s.c. into nude mice. From day 3 after injection of tumor cells, mice were treated by micronized P529 at doses of 50 mg and 25 mg/kg/2 d i.p., respectively. Mice without drug treatment served as controls. U87 tumors were allowed to grow for 24 d. During drug treatment, tumor volumes were measured with a caliper and estimated as length $\times$ width $\times$ width $\times$ 0.53. Animals were euthanized and the tumors were taken for immunohistologic and immunoblotting studies.

Ad-VEGF-induced angiogenesis

Nonreplicating adenoviral vector engineered to express the predominant (164 amino acids) murine isoform of VEGF-A (1×10^7 plaque-forming units) was injected i.d. into the dorsal ears of athymic nu/nu mice as described previously (14). P529 at 200 mg/kg/2 d was administered i.p. into mice (n = 12) 1 wk before adenovirus VEGF-A injection. Mice were harvested at day 5 after adenovirus injection. Ears were mounted flat under the glass slide with immersion oil and photography using a Wild M-400 photomicroscope. For evaluation of permeability in the ears, mice were injected with 0.1 mL of 0.5% Evans blue dye in saline i.v. After 30 min, ears were evaluated by photographed. For quantitative analysis of vascular permeability, six mouse ears from P529-treated and untreated mice were punched by 8-mm punch biopsy 30 min after injection of Evans blue dye. Biopsies were incubated at 56°C water bath for 48 h in 1 mL formamide. Amount of extracted Evans blue dye was detected by absorbance at 620 nm in 96-well plate. For Miles vascular permeability assay, 4- to 6-wk-old female athymic nu/nu mice were pretreated 1 wk with P529 at 200 mg/kg/2 d i.p. before the Miles assay was performed. Briefly, Evans blue dye (100 AL of a 1% solution in 0.9% NaCl) was injected i.v. into mice. After 10 min, 50 AL of human VEGF₁₆₅ (1 ng/AL) or 0.1% bovine serum albumin (BSA) was injected i.d. into the back skin as a control. After 20 min, the animals were euthanized, and an area of skin that included the entire injection sites was removed and photographed.

Immunohistochemistry

Mouse tissues were immersed in freshly prepared 4% paraformaldehyde in 0.02 mol/L phosphate buffer (pH 7.4) for 4 h at room temperature, washed in PBS, and embedded in paraffin. For phosphorylated Akt (pAkt) and phosphorylated S6 (pS6) immunostaining, tissue sections were hydrated and incubated in 3% H₂O₂ in methanol for 20 min, washed in water, and boiled in 1 mmol/L EDTA (pH 8.0) for 15 min. Sections were blocked with 10% normal goat serum (Vector Laboratories) and then incubated with primary antibody to pAkt (1:100 dilution) and pS6 (1:500 dilution) overnight at 4°C. Tissue sections were incubated with secondary antibodies (1:400; Vector Laboratories) for 30 min at room temperature and detected by avidin-biotin complex method (ABC) kit (Vector Laboratories). For CD31 immunostaining, 6-Am frozen sections embedded in OCT were collected. After washing with PBS and blocking with 5% goat serum, tissue sections were incubated with CD31 antibody (1:300 dilution; BD Biosciences) overnight at 4°C. Then, sections were washed by PBS again and incubated with secondary antibodies (1:400; Vector Laboratories) for 30 min at room temperature and detected by ABC kit.

Results

P529 is a novel nonsteroidal small-molecule drug created by modification of dibenzo[c]chromen-6-one antiestrogen derivatives, screened to lack ER binding but retain antiendothelial and antitumor activity.³ To confirm that the Palomid derivative P529 no longer retained ER binding activity, human cDNAs encoding the ERs (ERα and ERβ) were used as templates for in vitro transcription and translation of radioactively labeled receptor proteins for use in in vitro estrogen (E₂) binding assays. The maximal E₂ binding was determined by competing bound E₂ with the ethanol vehicle. Values for percent inhibition were calculated based on the maximal E₂ binding. P529 was unable to bind ERα or ERβ, confirming that P529 no longer retained estrogen antagonism functionality (Fig. 1A). The structure of this P529 is shown in Fig. 1B.

To confirm that P529 retained antiangiogenic activity, however, we used a variety of conventional endothelial assays. P529 inhibited both VEGF-driven (IC₅₀, 20 nmol/L) and bFGF-driven (IC₅₀, 30 nmol/L) endothelial cell proliferation and retained the ability to induce endothelial cell apoptosis (Fig. 1C). The added sensitivity observed in stimulated cells suggests that P529 function is dependent on signaling pathways stimulated by growth factors. Consistent with this hypothesis, we observed that P529 reduced VEGF-A–driven phosphorylation of pAktS473, pGSK3hS9, and pS6. However, P529 inhibited neither phosphorylated mitogen-activated protein kinase (pMAPK) nor pAktT308 as potently as pAktS473. As expected, wortmannin reduced phosphorylation of both pAktS473 and pAktT308 similarly. Furthermore, P529 was able to successfully compete with OA to reduce phosphorylation of pAktS473, pGSKS9, and pS6 but not pAktT308 (Fig. 1D). Together, these data implicate P529 in the inhibition of AktS473 signaling in a relatively specific manner.

We examined the antiangiogenic and antivascular function of P529 in several in vivo assays. One commonly used assay for pathologic angiogenesis is the oxygen-induced retinopathy in the neonatal mouse (17). In this assay, neonatal P7 mice are placed into hyperoxia (70% oxygen) where they experience loss of retinal blood vessels. Following return to ambient oxygen 5 days later, there is insufficient blood oxygen due to vessel loss to meet the demands of the highly metabolically active retina, leading to a “relative” ischemia that triggers a pathologic angiogenic response. The pathologic angiogenesis is characterized by the proliferation of incompletely formed and aberrant vascular structures termed glomeruloid tufts and highlighted by arrows in Fig. 2A. P529 not only reduced the proliferative response in the ischemic retina but also improved the organization and structure of the vessels that did form (Fig. 2B). This result suggested that P529 might have activity in multiple settings of pathologic angiogenesis, including cancer.

The second assay that was performed was the response to VEGF-A in vivo, administered by adenovirus injection to the ear (18). Figure 3A shows a dose-dependent inhibition of the Ad-VEGF-A–driven angiogenesis following P529 treatment. In this assay, we can harvest the ear tissue for analysis of signaling pathways. We found that pAktS473 but not pAktT308 was potently inhibited by P529 when assayed in whole ear lysates (Fig. 3B). Once again, there did not seem to be an alteration in MAPK signaling. Histologic analysis also revealed reductions in pAkt and pS6 antibody staining in blood vessels (Fig. 3C). We also observed an overall reduction in the number and sizes of blood vessels (detected with anti-CD31), and an overall reduction in the thickness of the ear tissue and tissue edema, which can indicate decreased vascular permeability.

Vascular permeability was assayed in two ways. First, a reduction in Evans blue extravasation following drug treatment was visualized in the ears and quantified by extraction of Evans blue

³D. Sherris, Paloma Pharmaceuticals, personal communication.

from ear tissue (Fig. 4A and B). However, in this assay, it is difficult to eliminate the possibility that reduced extravasation was not simply secondary to the changes in vascular density. Therefore, we used the Miles assay to assess the effects of P529 on VEGF-induced acute microvascular permeability in a 30-minute response in wild-type mice. This assay uses a small dermal injection of VEGF-A in mice that have been previously injected with Evans blue i.v. The response is photographed immediately following euthanization and opening of the dorsal skin. The control for nonspecific extravasation or bleeding to the i.d. injection is BSA, and this typically gives a variable but small blue spot (both examples of BSA treatment in this figure are within the reference range). In contrast, VEGF-A (similar to other potent inducers of microvascular permeability) reliably gives a very intense blue staining. After pretreatment with P529, the intense VEGF response was greatly reduced and not much greater than what we can observe with BSA (Fig. 4C). Because there is no opportunity to alter the vasculature density in this short assay, these results strongly implicate P529 in directly reducing VEGF-driven vascular permeability.

Both the inhibition of vascular proliferation and the reduction of permeability are expected to have an effect on cancer. It has been suggested that a significant portion of the efficacy of bevacizumab (Avastin; a VEGF-A antibody) is due to a reduction in permeability in the tumor vessels, which in turn reduces interstitial pressure and improves perfusion and drug delivery to the central parts of the tumor (8). To test the potential of P529 as an anticancer drug, we pretreated C6V10 glioma tumor cell cultures (19) with P529 and stimulated with IGF (Fig. 5A). Similar to what we observed in VEGF-treated endothelial cells, P529 reduced phosphorylation of pAktS473, pGSK3hS9, and pS6 but neither pMAPK nor pAktT308. We also observed consistent results with wortmannin and OA in tumor cells when compared with endothelial cells (compare with Fig. 1). Because of the disconnection between inhibition of pAktS473 and pAktT308, we entertained the possibility that P529 may be a TORC2 inhibitor (13). Based on the structure of P529, it is unlikely that it represents a true ATP-competitive kinase inhibitor, but we hypothesized that it may be an inhibitor of protein-protein interactions within the TORC2 complex that includes mTOR, rictor, mLST8, and SIN1. Previously, TORC2 assembly was quantified by immunoprecipitation of mTOR followed by Western blotting of rictor (12). Therefore, we repeated this assay in the presence and absence of P529 following IGF-I stimulation of C6V10 cells (Fig. 5B). P529 inhibited the association of mTOR with rictor within 2 h. Consistent with the efficient inhibition of S6 phosphorylation of P529, we also observed inhibition in mTOR-raptor association, suggesting that P529 inhibits both the TORC1 and TORC2 complexes. These data predicted that P529 would likely inhibit the growth of C6V10 tumor cells in mice due to its dual inhibition of tumor and endothelial cell signaling. Figure 5C shows that P529 inhibited C6V10 glioma tumor growth in nude mice following i.p. dosing. Analysis of signaling within the tumor lysates revealed that P529 also reduced AktS473 but not AktT308 signaling in vivo (Fig. 5D).

Examination of the tumor histology also revealed some interesting findings that may have implications for therapeutic use of P529. We used the C6 variant C6V10, which is transfected with exogenous mouse VEGF-A164 for these studies, because we have previously found them to have a more accurate vascular pathology to represent high-grade glioblastoma, including glomeruloid vessels, which are not commonly found in mouse tumors. Tumors from animals treated with P529 had reductions in tumor vascular density as seen by staining tumor sections with anti-CD31 (Fig. 6A). Besides reductions in overall vascular density, we observed a specific reduction in the size of glomeruloid vessels in these tumors (Fig. 6B). Glomeruloid vessels are a hallmark of glioblastoma (as they are of oxygen-induced retinopathy; see Fig. 2) and can be partially recapitulated in these C6V10 glioma tumors (note arrows). Glomeruloid vessels are characterized as large disorganized patches of cells, including endothelial cells, pericytes, and leukocytes, and surprisingly have stronger expression of pAkt and pS6 than the

surrounding tumor cells. This expression pattern may explain their hypersensitivity to P529. In treated tumors, the size and staining intensity of pAktS473 and pS6 were reduced.

Due to consistent difficulties with the solubility of P529, recent micronization has led to a formulation with more potent efficacy on C6V10 tumors in both i.v. and p.o. administration (data not shown). We have never observed significant weight loss, lethargy, or other indications of animal toxicity. However, these C6V10 tumors are relatively fast growing and we wanted to test P529 on a slower-growing and independent glioma cell line. Using the micronized formulation, we were able to potently inhibit tumor growth of the human U87 cells at greatly reduced drug concentrations. This is a slower-growing tumor and treatment with 25 and 50 mg/kg/2 days dose dependently reduced tumor growth. Even after 27 days of treatment, the animals still showed no signs of toxicity (Supplementary Fig. S1). Thus, we conclude that P529 is a novel and potent anticancer and antiangiogenic drug whose efficacy in reducing glioma growth warrants additional investigation.

Discussion

In both tumor cells and endothelial cells, P529 similarly reduces signaling from Akt pS473 in a relatively specific manner. Previous studies on the Akt pathway in the tumor vasculature suggest that Akt signaling promotes vascular dysfunction marked by hyperpermeability (1, 16,20). Reduced Akt-mTOR pathway signaling was correlated to vascular normalization, which is expected to enhance drug delivery to the tumor center (2). The observations that signaling from Akt pS473 is more dramatically reduced than Akt pT308 suggested the possibility that P529 is competing directly with TORC2 (the mTOR-ricTOR complex that phosphorylates pAktS473) or promoting activation of a specific pAktS473 phosphatase such as PHLPP1/2 (13,21,22). Our studies favor the former hypothesis because we find that P529 inhibits coimmunoprecipitation of mTOR and rictor similar to what was observed with long-term rapamycin treatment (12). However, different from rapamycin, the disruption of TORC2 that occurs within 2 h of P529 treatment is of similar efficiency as the disruption in TORC1. The mechanism of rapamycin action is via binding FKBP12 and interaction with TORC1 and not TORC2 (23). In the study of rapamycin, it has been observed that inhibition of pAkt occurs only as a secondary effect after prolonged treatment and is less robust than inhibition of pS6K (12). In the case of P529, we observe a comparable blockade of mTOR association with both rictor and raptor, although we also do not have detailed information on the specific molecular interactions that prevent these mTOR interactions. Some possible mechanisms include binding to mTOR to inhibit complex assembly or a disruption of previously assembled complexes. Future studies of structure and function of P529 in combination with TORC1/ TORC2 will be needed to firmly establish how P529 inhibits these complexes. Although further studies are needed to fully elucidate the mechanism of P529 action, it is clear that this inhibitor is a novel tumor therapeutic with efficacy in the inhibition of tumor cell growth, angiogenesis, and vascular permeability and can be considered a TORC1/TORC2 inhibitor. Such a compound has the potential to bypass complications of rapalogs where feedback loops lead to a differential response in tumor and stromal cells, similar to what has been sought by the design of dual mTOR/ PI3K inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Disclosure of Potential Conflicts of Interest

L.E. Benjamin: commercial research support, Paloma Pharmaceuticals. D. Sherris is President and CEO of Paloma Pharmaceuticals.

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Figure 1.

P529 is a derivative of a nonsteroidal estrogen antagonist that maintains antiproliferative activity on endothelial cells while no longer inhibiting ER binding. **A**, the binding of 0.5 nmol/L radiolabeled estradiol to ER α and ER β is not inhibited by P529. Diethylstilbestrol is a synthetic estrogen that inhibits E₂ binding with equal potencies for both ER α and ER β . **B**, structure of P529. **C**, P529 inhibits proliferation and increases apoptosis of endothelial cells. *Left*, proliferation following VEGF and FGF stimulation of HUVECs. The IC₅₀ inhibition was 30 nmol/L for bFGF and 10 nmol/L for VEGF-A. Growth curve differences were statistically significant ($P < 0.05$) from no drug treatments at 10 nmol/L and above. *Right*, P529 has a 4-fold effect on HUVEC apoptosis at 10 Amol/L ($P < 0.05$). Camptothecin-induced apoptosis was used as a positive control. **D**, P529 inhibits VEGF-A-driven Akt and downstream signaling in primary dermal microvascular endothelial cells. P529 is compared with wortmannin and OA. MAPK signaling is shown as an example of an independent VEGF-A-driven signaling pathway not inhibited by P529.

Figure 2.

P529 inhibition of retinal neovascularization. A, the blood vessel proliferation in control retinas and retinas treated for 5 d with 1 mg/d P529 are shown after staining for lectin and flat mounting. The abnormal angiogenesis associated with vitreal invasion can be seen as glomeruloid-like tufts of cells (*arrow*). B, counting the glomeruloid tufts was used to provide a quantitative measure of P529 angiogenesis inhibition. Bars, SD.

Figure 3.

P529 inhibits Ad-VEGF-A– induced angiogenesis and pAktS473 signaling. *A*, dose-dependent inhibition of Ad-VEGF-A in mouse ears. *B*, Western blot analysis from total ear lysates. *C*, staining of sections from Ad-VEGF-A ears for anti-pAkt, anti-pS6 (magnification, $\times 40$), and anti-CD31 (magnification, $\times 10$). The bottom panel also shows the result of edema on ear thickness in cross-sections from ears $\pm$ P529. $n = 12$ ears per group. Representative images are shown.

Figure 4. P529 reduces VEGF-induced permeability. *A*, *top*, Ad-VEGF-A–induced angiogenesis; *bottom*, Evans blue perfusion. *B*, quantification of Evans blue measures a combination of angiogenesis and vascular permeability. *Bars*, SD. *C*, a Miles assay directly measures acute vascular permeability in response to VEGF-A.

Figure 5.

P529 inhibits glioma cell signaling, tumor growth, and tumor angiogenesis. *A*, Western blot analysis of signaling in C6 glioma cells after IGF-I stimulation and in comparison with wortmannin and OA treatment. *B*, coimmunoprecipitation for mTOR with raptor and rictor to assess TORC1 and TORC2 complex inhibition. *C*, tumor growth of C6 glioma cells grown s.c. in nude mice ($n = 12$ per group). *D*, Western blot analysis of tumor tissue lysates. Bars, SD.

Figure 6. P529 inhibited the Akt signaling and size of glomeruloid vessel structures in C6V10 glioma tumors. A, representative tumor tissue sections from untreated and P529-treated C6V10 glioma xenografts were stained with (A) antibody to CD31 to visualize blood vessels (magnification, $\times 10$) and (B) antibodies to pAkt and pS6 to highlight signaling in glomeruloid-like vessels. Arrows, glomeruloid-like structures. Magnification, $\times 20$.