The Microtubule Depolymerizing Agent CYT997 Causes Extensive Ablation of Tumor Vasculature In Vivo

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ABSTRACT

The orally active microtubule-disrupting agent (S)-1-ethyl-3-(2-methoxy-4-(5-methyl-4-((1-(pyridin-3-yl)butyl)(amino)pyrimidin-2-yl)phenyl)urea (CYT997), reported previously by us (Bioorg Med Chem Lett 19:4639–4642, 2009; Mol Cancer Ther 8:3036–3045, 2009), is potently cytotoxic to a variety of cancer cell lines in vitro and shows antitumor activity in vivo. In addition to its cytotoxic activity, CYT997 possesses antivascular effects on tumor vasculature. To further characterize the vascular disrupting activity of CYT997 in terms of dose and temporal effects, we studied the activity of the compound on endothelial cells in vitro and on tumor blood flow in vivo by using a variety of techniques. In vitro, CYT997 is shown to potentely inhibit the proliferation of vascular endothelial growth factor-stimulated human umbilical vein endothelial cells (IC_{50} 3.7 ± 1.8 nM) and cause significant morphological changes at 100 nM, including membrane blebbing. Using the method of corrosion casting visualized with scanning electron microscopy, a single dose of CYT997 (7.5 mg/kg i.p.) in a metastatic cancer model was shown to cause destruction of tumor microvasculature in metastatic lesions. Furthermore, repeat dosing of CYT997 at 10 mg/kg and above (intraperitoneally, b.i.d.) was shown to effectively inhibit development of liver metastases. The time and dose dependence of the antivascular effects were studied in a DLD-1 colon adenocarcinoma xenograft model using the fluorescent dye Hoechst 33342. CYT997 demonstrated rapid and dose-dependent vascular shutdown, which persists for more than 24 h after a single oral dose. Together, the data demonstrate that CYT997 possesses potent antivascular activity and support continuing development of this promising compound.

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ABBREVIATIONS: CA4P, combetastatin A-4P; VDA, vascular disrupting agent; HUVEC, human umbilical vein endothelial cells; VEGF, vascular endothelial growth factor; DMH, dimethyl hydrazine; MTD, maximum tolerated dose; CYT997, (S)-1-ethyl-3-(2-methoxy-4-(5-methyl-4-((1-(pyridin-3-yl)butyl)(amino)pyrimidin-2-yl)phenyl)urea; ABT-751, N-[2-[(4-hydroxyphenyl)amino]-3-pyridinyl]-4-methoxybenzenesulfonamide; AVE8062, (2S)-2-amino-3-hydroxy-N-[2-methoxy-5-[[2]-2-(3,4,5-trimethoxyphenyl)ethenyl]phenyl]propanamide hydrochloride; NPI-2358, (3E,6E)-3-benzylidene-6-[(5-[tert-butyl]-1H-imidazol-4-yl)methylene]piperazine-2,5-dione; Y-27632, trans-4-[(1R)-1-aminoethyl]-N-(4-pyridinyl)cyclohexanecarboxamide.
currently first-line therapy for many cancers (Hamel, 1996). Nonetheless, these agents have drawbacks, including the development of resistance during the course of their use (van Ark-Otte et al., 1998; Yin et al., 2007) and the necessity for intravenous administration because of their limited oral bioavailability. As such, there has been considerable effort in recent years toward the discovery and development of novel tubulin binding drugs that possess improved physicochemical and pharmacokinetic properties, thereby allowing oral administration (Hearn et al., 2007).

Of the numerous examples of novel small-molecule tubulin binding compounds, a selected few have been shown to possess effects on tumor vasculature in in vivo models and clinical trials (Schwartz, 2009). For example, the phospho-ester prodrug of combretastatin A-4P (CA4P; Zybrestat) was found to induce a rapid reduction in tumor blood flow and a concomitant increase of cellular necrosis in mouse tumor models (Malcontenti-Wilson et al., 2001). Now known as vascular disrupting agents (VDAs), several drug candidates with this mechanism of action have entered clinical development, and there is encouraging evidence that the approach may be useful in the treatment of solid tumors (Tozer et al., 2005a). Other tubulin binding agents that have been tested in the clinic with reported vascular disrupting activity include N-[2-[(4-hydroxyphenyl)amino]-3-pyridinyl]-4-methoxybenzenesulfonamide (ABT-751) (Luo et al., 2009), (2S)-2-amino-3-hydroxy-N-[2-methoxy-5-[(Z)-2-(3,4,5-trimethoxy-phenyl)ethenyl]phenyl]propanamide hydrochloride (AVE8062) (Hori and Saito, 2004), and (3E,6E)-3-benzylidene-6-[(5-(tert-butyl)-1H-imidazol-4-yl)methylene]piperazine-2,5-dione (NPI-2358) (Nicholson et al., 2006). All of these drugs inhibit tubulin polymerization and have been shown to possess antivascular effects in various in vitro and in vivo models.

We have reported previously the discovery (Burns et al., 2009b) and preliminary biological characterization (Burns et al., 2009a) of (S)-1-ethyl-3-(2-methoxy-4-(5-methyl-4-((1-(pyridin-3-yl)butyl)amino)pyrimidin-2-yl)phenyl)urea (CYT997), a novel synthetic microtubule depolymerizing compound first synthesized in our laboratories. The compound inhibits the polymerization of tubulin with an IC_{50} of ~3 μM and possesses potent cytotoxic activity against a panel of cancer cell lines. It is noteworthy that CYT997 is orally bioavailable and shows significant activity in tumor xenograft models when dosed orally. Antivascular activity of the compound was demonstrated in vitro against human umbilical vein endothelial cells (HUVEC) and in vivo, where a significant reduction in tumor blood flow was observed after both intraperitoneal and oral dosing. In this article, we further characterize the antivascular effects of CYT997 in vitro and in vivo as well as report the dose effects and pharmacodynamic effect of the compound in a xenograft model.

Materials and Methods

CYT997 was prepared as described previously (Burns et al., 2009b) as was CA4P [phosphate prodrug of (Z)-2-methoxy-5-(3,4,5-trimethoxyaryl)phenol] (Petit et al., 1995). Vincristine, paclitaxel, and doxorubicin were purchased from Sigma (St. Louis, MO). HUVEC were obtained from Invitrogen (Mulgrave, Victoria, Australia) and cultured in Media 200 (Invitrogen) supplemented with L-glutamine and 100 U/ml penicillin/streptomycin and endothelial cell growth factor (Roche Diagnostics, Indianapolis, IN) at 200 ng/ml on gelatin-coated plates.

HUVEC were cultivated in M199 basic medium with HEPES buffer (Invitrogen), with 20% fetal bovine serum supplemented with 1-glutamine and 100 U/ml penicillin/streptomycin and endothelial cell growth factor (Roche Diagnostics, Indianapolis, IN) at 200 ng/ml on gelatin-coated plates.

HUVEC Morphology

HUVEC were seeded at two different densities onto 24-well tissue culture plates (20,000 cells/well), cultured for 4 days, and monitored until they reached confluence. Representative fields of view for both confluent and nonconfluent cells were photographed for photographic recording. Both confluent and nonconfluent cells were photographed under two different magnifications (10 and 40×). Cells were then treated with two concentrations of CYT997 (100 and 1000 nM) for 1 h. The changes in treated cells were monitored under a microscope and photographed in the same manner as untreated cells.

HUVEC Proliferation Assay

Cells (10^4) were plated in flat-bottomed 96-well plates and allowed to adhere overnight in M199 medium + 1% fetal bovine serum. Compounds were added at the concentrations indicated, in fresh medium containing 0.1% dimethyl sulfoxide. After 1-h incubation at 37°C, VEGF (recombinant human VEGF121; R&D Systems, Minneapolis, MN) was added (10 ng/ml final concentration). After 5 days at 37°C, 5% CO₂, Alamar Blue (10 μl) was added for the final 4 to 6 h of the culture, and fluorescence was read at an excitation wavelength of 544 nM and an emission wavelength of 590 nM with a Polarstar plate reader (BMG Labtech GmbH, Offenburg, Germany).

In Vivo Tumor Models

Metastatic Colon Cancer Model. The colon cancer liver metastases model was used as described previously (Malcontenti-Wilson et al., 2001). Male CBA mice (Laboratory Animal Services, Adelaide, Australia) were inoculated intrasplenic with 5 x 10⁶ mouse DMH colon cancer cells followed by splenectomy. Mice were allocated at random to treatment groups on day 9. CYT997 tartrate salt in 0.1 M Captisol (equivalent to 5, 10, or 15 mg of free base/kg/day; n = 14 mice/group) or 0.1 M Captisol (10 ml/kg, n = 12 mice) and were dosed intraperitoneally twice daily, 12 h apart. Mice were killed after 11 days of treatment, and wet liver weight was measured. A dose of 15 mg/kg/day was tolerated by mice; however, two mice were assessed as requiring euthanasia before endpoint (2/14 or 14%). The percentage of liver volume occupied by metastases was calculated by using quantitative stereological assessment as described below.

Determination of Metastatic Burden. Mice were weighed at the endpoint, and livers and other organs were placed in formalin. After 2 days, livers were placed in a solution of 50% ethanol, and after removing excess fluid, the wet weights of all livers were determined. Stereological assessment of liver metastases was performed as follows. Each liver (after being fixed in formalin for 4 days) was cut into 1.5-mm slices by using a multiblade fractionator, and alternate slices were used for measurement. Images of the slices were captured by using digital imaging, and an Image Analysis software program (Image Pro Plus; Media Cybernetics, Perth, Australia) was used to determine tumor load by using stereological principles.

Microvascular Corrosion Casting. Following the procedure described previously (Malcontenti-Wilson et al., 2001) the thorax of
tumor-bearing animals from the DMH colon cancer model described above was opened to expose the heart and thoracic vessels. The aorta was cannulated with a 24-gauge catheter, and the vasculature was flushed with warm saline solution containing heparin (10 IU/ml), papaverine (12 mg/ml), and 6% polyvinyl pyrrolidinone (PVP40) (Sigma). The effluent was discharged through a right atrial puncture, and once clear, followed by the infusion of an acrylic resin of Mercox CL-2B (Okenshoji Co, Tokyo, Japan), methyl methacrylate (Sigma), and catalyst MA (Vilene Med Co., Japan) infused at a pressure of 160 mm Hg to allow microvascular filling. After polymerization of the resin overnight, the liver was excised and tissue was digested in 20% potassium hydroxide at 37°C for a minimum of 2 days. Once the digestion process was complete the resulting resin casts were then frozen and cut into sections with a high-speed cutting tool. The casts were washed in distilled water, dried at 37°C, and mounted on aluminum stubs with Electrodag 415 (Acheson Colloids, Port Huron, MI). Specimens were gold-coated with a Baltec SCD005 sputter coater (Leica Microsystems, Inc., Bannockburn, IL) and viewed on a scanning electron microscope (Hitachi 570 SEM; Hitachi, Tokyo, Japan). Scanning electron micrograph digital images from each specimen were captured at various magnifications using Spectrum Imaging (Monterey, CA) software. Several tumors and normal liver were examined from every animal in each group.

Colon Adenocarcinoma Model. Female BALB/c immunodeficient nude mice aged 6 to 12 weeks (Harlan, Loughborough, UK) were used. Tumors were excised from a donor animal, placed in sterile physiological saline containing penicillin and streptomycin, and cut into small fragments of approximately 2 mm. Under brief pressure of 160 mm Hg to allow microvascular filling. After polymerization of the resin overnight, the liver was excised and tissue was digested in 20% potassium hydroxide at 37°C for a minimum of 2 days. Once the digestion process was complete the resulting resin casts were then frozen and cut into sections with a high-speed cutting tool. The casts were washed in distilled water, dried at 37°C, and mounted on aluminum stubs with Electrodag 415 (Acheson Colloids, Port Huron, MI). Specimens were gold-coated with a Baltec SCD005 sputter coater (Leica Microsystems, Inc., Bannockburn, IL) and viewed on a scanning electron microscope (Hitachi 570 SEM; Hitachi, Tokyo, Japan). Scanning electron micrograph digital images from each specimen were captured at various magnifications using Spectrum Imaging (Monterey, CA) software. Several tumors and normal liver were examined from every animal in each group.

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Determination of Vascular Shutdown. Mice were treated with a single dose of CYT997 (40 mg/kg, as dihydrochlochloride) by oral gavage (three groups) and a vehicle (saline)-treated group was maintained as a control. At 1, 6, or 24 h after CYT997 treatment, vascular shutdown was assessed as follows: Hoechst 33342 dye was dissolved in sterile saline and injected intravenously by the tail vein at 40 mg/kg. One minute after injection the mice in the relevant treatment group were sacrificed by cervical dislocation, and the tumors were carefully and rapidly excised. One tumor from each mouse was then wrapped in aluminum foil, immediately immersed in liquid nitrogen, and stored at −80°C until ready for ultracryotomy. The other tumor was immersion-fixed in 10% neutral buffered formalin for 24 h and processed for paraffin embedding. The control group were processed at the same time as the 24-h cohort.

Frozen sections of 10-μm thickness were taken at approximately 100-μm intervals through the tumor. Up to five fields from each of 10 random sections were examined for each tumor under UV illumination by using a Leica DMRB microscope, with images captured digitally through a JVC (Yokohama, Japan) 3-CDD camera and processed using AcQuis software (Synoptics, Cambridge, UK). Functional vasculature was assessed by placing a cm² grid over the captured digital image and counting the number of points on the grid overlaying fluorescently stained cells. The percentage of functional vasculature was calculated by taking the total number of fluorescence-positive points for each field and dividing by the total number of points. An average percentage for each animal was calculated. Comparisons were made between percentage vasculature in control and treated tumors. Statistical analysis of shutdown was carried out by using a one-way analysis of variance with a post hoc pairwise comparison.

Dose Effect on Vascular Shutdown. Tumor-bearing mice obtained as described above (n = 3 per group) were treated with a single oral dose of either 10, 20, or 40 mg/kg CYT997 or vehicle control by oral gavage. At 1 h after treatment, vascular shutdown was assessed as described above.

Results

CYT997 Affects HUVEC Morphology In Vitro. To elucidate activity of CYT997 upon tumor endothelium, the effects of the compound on HUVEC in culture were studied. The morphological changes wrought on the HUVEC monolayer by CYT997 were visualized by using microscopy and are depicted in Fig. 1. There was minimal effect on cells exposed to 10 nM CYT997 after 1 h; however, there was a profound alteration in cell shape and clear disruption of

Fig. 1. CYT997 disrupts endothelial cell monolayers and causes morphological changes. HUVEC grown in monolayers were treated with 10 or 100 nM CYT997 for 1 h, and the cell morphology was visualized by light microscopy at 10× (A) or 40× (B) magnification. A, CYT997 (100 nM) caused disruption of the endothelial monolayer compared with cells treated with CYT997 (10 nM). B, most cells treated with CYT997 (100 nM) adopted a retracted, rounded morphology with a granular surface, compared with a typical spread-out endothelial cell in the same field.
monolayer confluence for cells exposed to 100 nM CYT997. At this dose and time, the cells were clearly rounded up with significant membrane blebbing apparent.

**CYT997 Is Cytotoxic to Proliferating HUVEC In Vitro.** To determine the effect of longer-term exposure of CYT997 to HUVEC, increasing concentrations of CYT997 and a selection of other tubulin binding agents were added to VEGF-stimulated HUVEC grown in culture, and proliferation was determined by Alamar Blue assay after 72 h. As shown in Fig. 2, CYT997 inhibited the proliferation of HUVEC with an IC_{50} of 3.7 ± 1.8 nM (S.D.), and at doses above 10 nM it diminished cell viability. Similar data were obtained for CA4P and vinblastine (IC_{50} 3.1 ± 2.7 and 0.9 ± 1.0 nM, respectively) in agreement with published results (Vacca et al., 1999; Vincent et al., 2005). Doxorubicin also caused complete destruction of viable cells, although at much higher concentrations (IC_{50} > 2 μM). In contrast, at the concentrations tested, paclitaxel inhibited VEGF-stimulated growth, but did not cause loss of viable cells, demonstrating a cytostatic rather than cytotoxic effect, as reported previously (Pasquier et al., 2004).

**CYT997 Is Active in the DMH Metastatic Colon Cancer Model and Disrupts the Vasculature of Hepatic Metastases.** The DMH metastatic colon cancer liver metastasis model is a well validated model that has been used to study the antimesetastatic effects and vascular-disrupting properties of CA4P and other VDAs (Malcontenti-Wilson et al., 2001, 2008; Chan et al., 2007). CYT997 was therefore profiled in this model with CA4P as positive control.

Quantitative stereological assessment of the number and volume of metastatic nodules in livers of tumor-bearing mice were determined after 11 days of treatment with CYT997 (intraperitoneally, b.i.d.) Doses of both 10 and 15 mg/kg/day of CYT997 significantly reduced the total wet weight of the liver compared with control (Fig. 3A). The percentage of liver volume occupied by metastases was also dramatically reduced in a dose-dependent manner (Fig. 3B). In addition, macroscopic examination of the liver clearly showed a dose-dependent reduction in tumor burden with some livers being essentially tumor-free in the 10 and 15 mg/kg cohorts (Supplemental Fig. 1).

The effect of CYT997 on tumor endothelium in vivo was determined by corrosion casting of tumor microvasculature in the metastatic lesions and imaged by using scanning electron microscopy. Figure 4A depicts typical images from untreated liver metastases, where a dense, tortuous microvascular network is observed and heterogeneous vasculature with variations in tumor vessels diameter is also evident. A single dose of CYT997 (7.5 mg/kg; <30% of the MTD) was shown to cause extensive disruption to the tumor vasculature in vivo (Fig. 4B). Tumors demonstrated large areas with absence of vessels, indicating vessel occlusion or damage (Fig. 4B, images A and C). An obvious reduction in the density of patent vessels was seen. Extravasation of resin is apparent in some areas where damaged vessels may allow escape of casting material through gaps in the endothelial wall (Fig. 4B, images B and D). Normal liver vasculature, however, seemed unaffected by treatment with CYT997. The data obtained for CYT997 show similarities to that obtained for CA4P dosed at 100 mg/kg, which was investigated concurrently as a positive control (data not shown), and with data reported previously (Malcontenti-Wilson et al., 2001).

**Single-Dose CYT997 Causes Time- and Dose-Dependent Vascular Shutdown.** Having established that CYT997 causes profound disruption to tumor vasculature, a separate study of the dose response and pharmacodynamics of this effect with oral administration was undertaken. Athymic nude mice bearing subcutaneous DLD-1 human colon adenocarcinoma xenografts were treated with single daily doses of CYT997 at 30 mg/kg, 10 mg/kg, or 5 mg/kg for 5 days, starting 1 day after tumor induction. Liver weights in normal mice (no tumor) were 0.09 g (n = 7). B, percentage of liver metastases was significantly reduced with all three doses of CYT997 in a dose-dependent manner (mean ± S.D.). Statistical analysis was performed using Student’s t test, two samples assuming unequal variances.

![Fig. 3. Assessment of tumor burden. Percentage of liver metastases was significantly reduced with all three doses of CYT997 in a dose-dependent manner.](https://example.com/fig3.png)

![Fig. 2. CYT997 inhibits proliferation of endothelial cells. HUVEC were stimulated with VEGF and treated with compounds as indicated. Viability was measured after 5 days using the metabolic indicator Alamar Blue.](https://example.com/fig2.png)
cancer xenografts (~150 mm³ in size) were dosed with CYT997 (40 mg/kg p.o., ~80% of MTD), and at 1, 6, or 24 h post-CYT997 treatment vascular shutdown was assessed by injection of Hoechst 33342 dye (40 mg/kg) and determination of dye fluorescence in tumor cross-sections (Fig. 5, A and B). Vascular shutdown was evident over the study period (approximately 93, 77, and 70% shutdown, respectively; *p* < 0.01) with a nonstatistical trend to apparent recovery ob-

Fig. 4. CYT997 disrupts tumor microvascular architecture in a mouse model of colorectal liver metastases. Scanning electron micrographs of microvascular resin casts of liver metastases are shown. A, representative micrographs (A–D) of typical microvascular architecture of untreated tumors showing dense tortuous tumor microvasculature, flattened dilated vessels, and connections of tumor vessels with normal liver sinusoids. B, representative micrographs (A–D) of liver metastases 6 h after treatment with a single dose of CYT997 (7.5 mg/kg i.p.), depicting disruption of tumor blood vessels. Areas of incomplete filling, spindly branches of vessels tapering toward area of occlusion, and masses of extravasated resin are seen. Some vessels remaining at the tumor margin are dilated and flattened, then end abruptly.

Fig. 5. CYT997 reduces functional vasculature in a xenograft tumor model. A, time-dependent vascular shutdown of DLD-1 human colon adenocarcinoma xenografts show functional vasculature using Hoechst 33342 staining, after a single oral dose of CYT997 (40 mg/kg) at 1, 6, and 24 h postdose. C, dose-dependent vascular shutdown of DLD-1 human colon adenocarcinoma xenografts were assessed 1 h after a single oral dose of CYT997 at 10, 20, or 40 mg/kg relative to vehicle (mean ± S.D.).
served at 6 and 24 h. Histological evaluation of hematoxylin and eosin-stained sections showed a notable increase in the amount of necrosis in the DLD-1 tumors at 24 h after CYT997 (data not shown). The delay in the appearance of necrosis presumably reflects the delay in tumor cell response post-treatment, after loss of blood supply.

In a separate experiment, the dose dependence of vascular shutdown was determined. Animals received a single oral dose of 10, 20, or 40 mg/kg CYT997 (or vehicle control) 1 h before assessment of vascular shutdown, which was performed as described above. Doses of 40 and 20 mg/kg resulted in shutdown of approximately 98.6 and 94.4%, respectively, both of which were highly significant relative to the untreated control tumors (p < 0.001; Fig. 5C). The lowest dose tested, 10 mg/kg, led to a less profound, but still significant, effect (p = 0.03), with approximately 60.6% shutdown in tumor vasculature observed 1 h postdose.

**Discussion**

The development of anticancer drugs with an improved therapeutic window through the selective targeting of drugs to cancerous tissue has become a major focus in oncology drug development. Compounds that selectively affect the vasculature of tumors have been the focus of considerable attention in recent years, and antiangiogenesis agents that inhibit tumor neovascularization, such as bevacizumab, sunitinib, and sorafenib, are now marketed for the treatment of certain vascularized solid tumors (Markland et al., 2010). Vascular disrupting agents are a related, but separate, class of compounds that disrupt the existing vasculature in tumors, and promising data have been reported from clinical trials of the more advanced compounds in this class (Anderson et al., 2003; Hande et al., 2006).

CYT997 is a structurally novel, orally active VDA discovered in our laboratories (Burns et al., 2009b), which has successfully completed two phase I clinical trials (Franceseoni et al., 2009; Lickleter et al., 2010). Preliminary antiangiogenic activity of the compound has been reported (Burns et al., 2009a), and in this article we have described further studies to better understand the vascular disrupting effects of the compound with particular regard to dose and functional activity in vivo tumor models.

Thus, the well characterized DMH colon cancer liver metastases model was used to assess the antimetastatic and VDA activity of CYT997. In this model metastatic lesions develop in the liver of CBA mice 21 days after inoculation of cells from a DMH-induced primary colon cancer cell line maintained in donor mice. Mice treated with CYT997 (intraperitoneally, b.i.d.) for 11 days from day 9 after tumor cell inoculation showed a dose-dependent reduction in liver weight, which was used as a surrogate measure of tumor burden, compared with vehicle control (Fig. 3A). The percentage of liver volume occupied by metastases was also dramatically reduced in a dose-dependent manner (Fig. 3B). In addition, macroscopic examination of the liver clearly showed a dose-dependent reduction in tumor burden with some livers being essentially tumor-free in the 10 and 15 mg/kg/day cohorts (Supplemental Fig. 1).

In a separate study using this model, CYT997 showed a dramatic impact on the distribution and penetration of a polymer into the vascular bed of the liver metastases in corrosion cast experiments. Thus, a single dose of CYT997 resulted in dramatic reductions in the density of tumor blood vessels both centrally and at the tumor-liver margin in the vasculature of hepatic metastases. The tumor microvasculature of these metastases in control animals was shown to be continuous with normal liver sinusoids. In contrast, CYT997-treated tumor-bearing livers demonstrated loss of continuity with the liver sinusoids, most likely as a result of the lack of resin penetration at sites of blocked vasculature. Significant extravasation of the resin was also apparent, manifesting as plaques of resin material at the margin of the metastasis. It is noteworthy that the profound differences in the intratumoral microvasculature of CYT997-treated tumors were not accompanied by significant changes in the microvasculature of the surrounding normal liver, as the fine, evenly distributed liver sinusoidal pattern can clearly be seen adjacent to the areas of disrupted tumor vasculature.

The catastrophic effect CYT997 has on tumor microvasculature was also measured by using the fluorescent dye Hoechst 33342 in a xenograft model using the human DLD-1 colon adenocarcinoma cell line. In this model a single oral dose of CYT997 (40 mg/kg) reduced functional vasculature in tumors with a maximal effect at 1 h post-CYT997 dose, and with minimal recovery over 24 h. The disruption to tumor blood flow at 1 h after CYT997 dosing was shown to be dose-dependent with a dose of 10 mg/kg (~20% of MTD) showing ~60% reduction in tumor blood flow compared with control-treated animals. In these studies it is apparent that tumor cells at the periphery of the tumor are resistant to the antiangiogenic effects of drug, an effect that has also been observed with other VDAs (Dark et al., 1997; Chan et al., 2008). These peripheral cells define the viable rim of the tumor and are recognized as a resistance mechanism to VDAs in cancer treatment, possibly leading to tumor revascularization and regrowth (see below). Preclinical studies have shown that combinations of VDAs and cytotoxic agents or angiogenesis inhibitors can overcome this resistance in certain instances (Shi and Siemann, 2005; Horsman and Siemann 2006), which has led to clinical studies of VDAs in combination with either cytotoxic or antiangiogenesis agents.

The detailed mechanisms by which VDAs cause such profound vascular damage in tumors are not fully understood and are the subject of ongoing research in a number of laboratories. Most VDAs inhibit microtubule polymerization, and it seems that the immature endothelial cells of tumor microvasculature are more susceptible to the effects of tubulin-binding agents because these cells rely more on the microtubule network for cellular integrity (Davis et al., 2002). The precise biochemical processes occurring within endothelial cells leading to the reported effects, however, have not been fully elucidated (Kanthou and Tozer, 2009). Activation of Rho kinase and mitogen-activated protein kinase signaling pathways have been observed in endothelial cells treated with VDAs as have effects on vascular endothelial-cadherin (Kanthou and Tozer 2002; Vincent et al., 2005). Indeed, the Rho-kinase inhibitor trans-4-[(1R)-1-aminomethyl]-N-4-pyridinylcyclohexanecarboxamide (Y-27632) has been shown to antagonize the vascular disruption caused by CA4P in an in vivo xenograft model (Kanthou and Tozer 2002), whereas neutralizing monoclonal antibodies to vascular endothelial-cadherin have been shown to synergize with CA4P in vivo (Vincent et al., 2005).
Morphological changes to endothelial cells after treatment with VDAs, including membrane blebbing, have been observed in both in vitro and in vivo studies (Kanthou and Tozer, 2002; Yeung et al., 2007; Bonezzi et al., 2009), and the rounding-up and blebbing clearly apparent in HUVEC exposed to CYT997 (Fig. 1) are thus fully consistent with these literature data. Clearly such morphological changes would be expected to have a significant impact on tumor microvessel integrity, leading to an increase in tumor vascular permeability, as has been reported for other VDAs (Tozer et al., 2005b; Reyes-Aldasoro et al., 2008).

Although the ablative effects on tumor vasculature for tumors treated with CYT997 and other VDAs are significant, it is clear from preclinical studies with other VDAs that tumor microvasculature is rapidly re-established on cessation of drug dosing. The process seems to arise from a drug-induced spike in bone marrow-derived circulating endothelial progenitor cells that locate to the viable rim remaining after VDA treatment (Shaked et al., 2006; Farace et al., 2007), most likely driven by cytokines such as granulocyte colony-stimulating factor, VEGF, and stromal cell-derived factor-1, levels of which are elevated after VDA treatment (Shaked et al., 2009). Whereas most VDAs studied to date are given intravenously, thereby limiting dosing frequency in the clinical setting, the oral activity of CYT997 allows for frequent (e.g., daily) dosing that would presumably lead to repeated vascular insult, even at the sub-MTDs demonstrated herein. Whether such a dosing regimen is more effective in targeting the revascularization and tumor regrowth processes discussed above remains to be determined and will be the subject of further studies.

The collected data for CYT997 reported in this article and its earlier work (Burns et al., 2009a) clearly demonstrate that the compound has significant effects on endothelial cells grown in culture and profound vascular disrupting activity in vivo. The effects parallel those reported for CA4P and other VDAs, and it is likely that the biochemical mechanisms underlying the antivasculogenic effects are similar. In the clinic, the oral bioavailability of CYT997, allowing for more frequent administration, may prove beneficial in minimizing the tumor revascularization observed with other intravenously dosed VDAs. Taken together, we believe the studies reported herein support the ongoing development of this promising new VDA.

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Participated in research design: Burns, Fantino, Powell, Shnyder, Phillips, Wilks, and Smith.
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Conducted new reagents or analytic tools: Christophi and Harte.
Performed data analysis: Burns, Fantino, Powell, Shnyder, Malcontenti-Wilson, Phillips, and Wilks.
Wrote or contributed to the writing of the manuscript: Burns, Shnyder, Malcontenti-Wilson, Segal, Wilks, and Smith.

References
Yeung M, Cooper, Nelson, Malcontenti-Wilson, Du-


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Supplementary Figure

*The microtubule depolymerising agent CYT997 causes extensive ablation of tumor vasculature in vivo*

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**CYT997 prevents metastatic tumor formation in a mouse model of Colorectal Liver Metastases**

**Macroscopic images of livers.** Livers of animals treated with 5 mg/kg/day, 10 mg/kg/day or 15 mg/kg/day of CYT997 or control vehicle (0.1M Captisol). Liver metastases are visible on the surface of the liver lobes in controls as white, translucent masses. All doses of CYT997 administered from Day 11-21 post tumor induction caused a decrease in number and size of metastatic lesions.