Published in final edited form as:

Integr Biol (Camb). 2011 April 1; 3(4): 375–387. doi:10.1039/c0ib00135j.

A Perspective on Vascular Disrupting Agents that Interact with Tubulin: Preclinical Tumor Imaging and Biological Assessment[#]

Ralph P. Mason[†], Dawen Zhao[†], Li Liu[†], Mary Lynn Trawick[‡], and Kevin G. Pinney^{*,‡}

[†]Department of Radiology, 5323 Harry Hines Boulevard, The University of Texas Southwestern Medical Center, Dallas, Texas, 75390-9058 USA

[‡]Department of Chemistry and Biochemistry, One Bear Place #97348, Baylor University, Waco, Texas 76798-7348, USA

Abstract

The tumor microenvironment provides a rich source of potential targets for selective therapeutic intervention with properly designed anticancer agents. Significant physiological differences exist between the microvessels that nourish tumors and those that supply healthy tissue. Selective drug-mediated damage of these tortuous and chaotic microvessels starves a tumor of necessary nutrients and oxygen and eventually leads to massive tumor necrosis. Vascular targeting strategies in oncology are divided into two separate groups: angiogenesis inhibiting agents (AIAs) and vascular disrupting agents (VDAs). The mechanisms of action between these two classes of compounds are profoundly distinct. The AIAs inhibit the actual formation of new vessels, while the VDAs damage and/or destroy existing tumor vasculature. One subset of small-molecule VDAs functions by inhibiting the assembly of tubulin into microtubules, thus causing morphology changes to the endothelial cells lining the tumor vasculature, triggered by a cascade of cell signaling events. Ultimately this results in catastrophic damage to the vessels feeding the tumor. The rapid emergence and subsequent development of the VDA field over the past decade has led to the establishment of a synergistic combination of preclinical state-of-the-art tumor imaging and biological evaluation strategies that are often indicative of future clinical efficacy for a given VDA. This review focuses on an integration of the appropriate biochemical and biological tools necessary to assess (preclinically) new small-molecule, tubulin active VDAs for their potential to be clinically effective anticancer agents.

Introduction

Tumor growth and metastasis require a functioning vascular network to provide oxygen and other nutrients. While the endothelium of normal, remodeled blood vessels is largely quiescent, the neovasculature of tumors is primitive, distinct in morphology, more responsive to angiogenic cell signaling, and activated in nature.¹⁻³ Consequently, the tumor vasculature offers an excellent, potentially selective target for anticancer therapy. The term “vascular disrupting agents” (VDAs) has been coined to describe a relatively new and rapidly emerging class of anticancer agents that selectively damage established tumor vasculature.⁴⁻⁶ Distinct from angiogenic inhibiting agents (AIAs), such as bevacizumab (AvastinTM)⁷ which halt the formation of new blood vessels, VDAs fall into two general classes referred to as biologics and small-molecules.⁴ The overarching realm of vascular

[#]Dedicated to Professor Mina J. Bissell of Lawrence Berkeley National Laboratory for her lifetime contributions to the overall field of cancer research and specifically the extracellular matrix and the tumor microenvironment.

^{*}To whom Correspondence should be addressed. Tel: (254) 710-4117. Fax: (254) 710-4272. Kevin_Pinney@baylor.edu .

targeting strategies includes both AIAs and VDAs, which are collectively described as vascular targeting agents (VTAs).^{4-6,8} It is important to emphasize that a very clear distinction has developed in the scientific community that defines compounds such as bevacizumab as angiogenic inhibiting agents, which represent a class of anticancer agent that is mechanistically separate and distinct from the compounds known as vascular disrupting agents that are the focus of this perspective. While bevacizumab (Avastin™) has been approved as an antiangiogenic VTA, there are no VDAs, either biologic or small-molecule, that have reached approval by the Food and Drug Administration (FDA) to date. Bevacizumab is a recombinant humanized monoclonal antibody that binds to vascular endothelial cell growth factor (VEGF) and blocks VEGF interaction with its corresponding receptors on the surface of endothelial cells. It is approved for the treatment of colon and lung cancer.^{9,10}

The discovery and development of new small-molecule VDAs has increased significantly over the past decade and today includes approximately a dozen compounds world-wide that are in human clinical trials (Fig. 1).¹¹⁻²⁹

The vast majority of these small-molecule VDAs include an interaction with the tubulin-microtubule protein system as a key component of their mechanism of action. This protein includes two small-molecule binding sites, vinca alkaloid and colchicine, located separately on the $\alpha\beta$ -tubulin heterodimer. In addition, it features a taxoid binding domain located on the microtubule. It is instructive to note that all of the current clinically relevant small-molecule VDAs that include an interaction with tubulin involve a binding event at the colchicine site on β -tubulin.^{30,31} It has been previously observed that the natural product colchicine itself induces vascular damage, but only at doses that are limited by toxicity.^{32,33} In addition, a vascular component has been identified in the mechanism of action attributed to vinblastine and vincristine, as representative vinca alkaloids.^{34,35} Paclitaxel (Taxol™), however, does not induce vascular damage through its interaction at the taxoid binding domain on microtubules although it alters tubulin-microtubule dynamics through stabilization of microtubules.³⁶ One small-molecule VDA known as DMXAA functions through a separate and distinct mechanism involving tumor necrosis factor alpha (TNF- α).³⁷

Typically, VDAs are not administered to humans (in clinical trials) as single agents, but rather are combined with standard chemotherapy, such as carboplatin and paclitaxel. While a small-molecule, tubulin-interactive VDA is capable of selectively starving a tumor of oxygen and nutrients, this, in turn, leaves behind a “viable rim” at the periphery of the necrotic tissue that is capable of supporting tumor regrowth.³⁸ Continued advances in understanding the subtle differences between the tumor microenvironment^{39,40} versus the healthy cell environment, on a molecular level, have been instrumental in providing fundamental support for the conceptualization and realization of VDAs as viable therapeutic agents for the treatment of cancer.

The VDA field has been well-reviewed both in terms of small-molecule agents,⁴¹⁻⁴⁸ currently regarded as the “key players”, and descriptions of the biological mechanism of action^{2,3,49-59} that involve a complex (yet not completely understood) cell signaling pathway that is initiated by rapid microtubule depolymerization in tumor vasculature, but not in normal blood vessels, ultimately leading to selective vascular damage and collapse in the tumor microenvironment. Vascular collapse in turn can result in massive tumor necrosis. Treatment of endothelial cells *in vitro* with potent, tubulin-binding VDAs results, in minutes, in profound cell morphology and cytoskeletal changes that are characterized by microtubule depolymerization leading to cell retraction, rounding and detachment (Fig. 2). The cytoskeletal reorganization includes an increase in actinomyosin contractility, assembly of actin stress fibers, formation of focal adhesions and membrane blebbing in certain cell

sub-populations. Cell-cell junctions and cell-extracellular matrix interactions are disrupted resulting in an increase in permeability. In some cases, apoptosis results.³ While the exact mechanism relating microtubule disassembly to vascular collapse has not been elucidated, a number of enzymes and a cell signaling pathway have been identified. An increase in myosin light chain phosphorylation is observed and the overall effects are largely abolished in the presence of Rho-kinase inhibitors indicating that in addition to RhoA kinase, the intracellular switch RhoA may be involved. RhoA, which hydrolyzes GTP (guanosine triphosphate), cycles between its active GTP binding form, and the inactive form that binds GDP.

Guanine nucleotide exchange factors (GEFs) activate Rho-GTPases by facilitating the exchange of GDP for GTP. In a variety of cells, activated Rho GTPases regulate reorganization of the cellular cytoskeleton in response to multiple signaling pathways via GEFs.⁶⁰⁻⁶² For example, in HeLa cell motility, the actin cytoskeletal rearrangements that occur as a result of microtubule depolymerization are regulated through RhoA.⁶³ GEF-H1 (ARHGEF2) is one of the few GEFs that bind to microtubules thus inhibiting its activity. Upon microtubule depolymerization, GEF-H1 is released and activates the Rho GTPase, RhoA in a number of different cells. In lung endothelial cells, depletion of GEF-H1 attenuated the increase in cell permeability and actin stress formation that results from thrombin treatment of the microtubule depolymerization agent nocodazole.⁶⁴

This review focuses on an integration of the appropriate biochemical and biological tools necessary to preclinically assess new small-molecule, tubulin active VDAs for their potential to be clinically effective anticancer agents.

***In vitro* assessment of tubulin-binding VDAs**

There is a strong correlation between established VDAs and their ability to inhibit tubulin assembly into microtubules, and cytotoxicity against tumor cells lines. The ability of certain VDAs (tubulin-binding) to disrupt microtubule structure is presumed to be the initiating event in the profound morphological changes that occur in vascular disruption.

Inhibition of tubulin assembly into microtubules—To evaluate the effect of the compounds on tubulin assembly *in vitro*, varying concentrations of the compounds are preincubated with 10 μ M tubulin that has been purified from calf brain⁶⁵ in a solution that will promote polymerization (glutamate solution)⁶⁶ at 30 °C and then cooled to 2 °C. After addition of GTP, the mixtures are warmed to 30 °C in a recording spectrophotometer and the assembly of tubulin is observed turbidimetrically at 350 nm followed by depolymerization at 2 °C to determine the baseline (Fig. 3). The IC₅₀ value is defined as the compound concentration that inhibits the extent of tubulin assembly by 50% in a 1500 second incubation. The data obtained for a drug at different concentrations are compared to control samples.⁶⁷⁻⁶⁹ A commercially available fluorogenic variation (cytoskeleton.com) of this assay is also available using a 96-well plate format.⁷⁰ Fluorescence enhancement is measured with the incorporation of a fluorescent reporter into microtubules with neuronal tubulin. Tubulin from sheep, pigs, and recombinant human tubulin isoforms have also been used for assessing inhibition of tubulin polymerization.

Cytotoxicity assay—The antimitotic activity of molecules that bind to tubulin and prevent microtubule assembly is well documented. Inhibition of human cancer growth can be assessed by the standard sulforhodamine B assay,⁷² which measures the total cellular protein as a means to determine cellular growth. Cells are distributed into 96-well plates, followed by treatment with study compounds and controls, at varying concentrations at 37 °C for 48 h. A growth inhibition of 50% in comparison to untreated controls (GI₅₀ or the

drug concentrations causing a 50% reduction in net protein increase) is calculated by nonlinear regression analysis. Alternatively, the MTT assay,⁷³ which is based on the reduction of the yellow tetrazole, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, to a purple formazan in living cells, is used to assess the effect of the compound on cell growth.

IC₅₀ values for the inhibition of tubulin assembly into microtubules for excellent VDAs are usually in the low micromolar range, while the GI₅₀ values for the cytotoxicity assay are often in the nanomolar range or lower. This amplification is consistent with the involvement of the RhoA-GTP signaling system and activation of the enzyme RhoA kinase.

For the direct *in vitro* assessment of the VDA on endothelial cells, tube disruption, cell adherence (rounding up), and cell permeability assays are carried out.

Endothelial tube disruption and reorganization assay—Human primary umbilical vein endothelial cells (HUVECs) can be induced to form three-dimensional, capillary-like tubular structures by growing these cells on a substitute for the extracellular matrix such as Matrigel™,⁷⁴ basement membrane extract (BME), or laminin-rich extracellular matrix,⁷⁵ and using a growth factor rich medium, which is added to each well of a 24-well or 96-cell plate. A suspension of HUVEC cells in EGM-2 (endothelial cell growth media) without antibiotics is seeded into each well and allowed to incubate at 37 °C from 4 to 24 h to determine the amount of time required for significant tube formation.⁷⁶ Tubule formation is determined by microscopy and documented by a photographic record. In preliminary experiments, the cells are treated with different concentrations of the compound to be assayed that has been dissolved in DMSO and diluted with medium, added to cells and allowed to incubate at 37 °C for 1-3 h to determine their effect. The compound is then removed and fresh media added. The disruption of tube structure is evaluated by light microscopy after a further 24 h of incubation. Cells can be conveniently stained with Calcein AM for fluorescence imaging⁷⁷ including confocal microscopy. An estimated IC₅₀ value is obtained through visual inspection of the images.

Cell adherence (rounding up) assay—The endothelial layer of the tumor vasculature undergoes cell retraction, rounding and detachment upon treatment with VDAs. To assess cell adherence, HUVECs are cultured and seeded onto fibronectin coated 96-well plates at high density (deactivated model) and grown with serum with no added growth factors versus low density seeding and growth with VEGF containing (activated) medium.⁷⁸ VDAs preferentially affect rapidly growing HUVECs.⁷⁹ A comparison of IC₅₀ values under the two conditions provides information on the vascular disrupting ability of compounds on tumor vasculature relative to normal vessels. Cell viability is assessed by the trypan blue exclusion assay.

Endothelial Cell Permeability Assay—The endothelial cell lining of vasculature defines a permeability barrier between the blood and the interstitial spaces. VDAs cause a reorganization of tumor endothelial cytoskeleton and an increase in vascular permeability.⁸⁰⁻⁸² To assay endothelial cell permeability, HUVECs are seeded onto gelatin coated membrane (0.45 µm pore) inserts and incubated with growth medium in a modified Boyden chamber until confluent. Varying concentrations of VDA are incubated with the HUVECs. After treatment, FITC-Dextran is added on top of the cells. The permeation of FITC-Dextran through the cell monolayer indicates a change in the tight junctions between cells. The extent of permeability is determined by measuring the fluorescence of the plate well solution.⁸³

Apoptosis Assay—Inhibition of tubulin assembly into microtubules and the antiproliferative effects of tubulin-binding VDAs are characterized by cell-cycle arrest in the G₂/M phase, which can be followed by flow cytometry. G₂/M blockade is often followed by cell apoptosis.^{84,85} Apoptosis can be measured by a variety of assays including activated caspases 3 and 7 to assess enzyme activity. One cell-based assay uses proluminescent caspase-3/7 DEVD-aminoluciferin substrate and luciferase after cell lysis.⁸⁶ Caspase cleavage of the substrate liberates free aminoluciferin, which is consumed by the luciferase, generating a luminescent signal. The signal is proportional to caspase-3/7 activity and apoptosis. IC₅₀ values for endothelial tube disruption are often much lower than for apoptosis, for a given VDA.

Preclinical Tumor Imaging

The nature and applications of VDAs have been the subject of many earlier reviews^{4,38,87-89} and here we provide an update, particularly featuring the relevance of imaging techniques in assessing VDA activity. There is hope and promise that Radiology can move beyond an anatomical approach to provide effective biomarkers of potential response to a therapy and early indication of therapeutic efficacy.⁹⁰

Tumor vasculature is recognized to be highly disorganized and inefficient. Many investigators have demonstrated the tortuous mesh of microvessels characterized by blind ends, constrictions and loops causing non-linear flow, as elegantly revealed in vascular casts, such as the classic studies of Konerding.⁹¹ We show a typical corrosion cast in Figure 4, achieved by infusing a liquid monomer into a rat breast tumor growing in the kidney of a rat. Once the material had polymerized, tissue was removed by caustic maceration, the cast coated with a sputtering of palladium-gold and scanning electron microscopy (SEM) performed. Many reports show such corrosion casts in some cases encompassing whole animals.⁹² Such casts provide an indication of vasculature in three dimensions, though quantitative analysis is not trivial potentially requiring micro CT.⁹³⁻⁹⁵ The polymer filaments are fragile and sometimes the finest capillaries may be lost. The cast provides no dynamic information and polymer may be forced into vessels otherwise occluded by temporary thromboses.

More commonly, vascular extent is assessed using histological specimens with immunohistochemistry, *e.g.*, Figure 4b reveals blood vessels based on anti-CD31 monoclonal antibody binding. Vascular perfusion may also be observed by infusion of a vascular reporter prior to sacrifice. Figure 4c shows distribution of Hoechst 33342 dye, which had been infused intravenously 60 seconds prior to sacrifice. Overlay on the CD31 image reveals the fraction of vessels that were perfused (Figure 4d). We have used this approach to show the change in vascular extent and perfusion in tumors with respect to administration of typical VDAs.^{96,97} Two hours after administration of combretastatin A-4P vasculature was detected based on CD31 (similar to baseline), but essentially all perfusion had ceased. Of course, such measurements generally require separate specimens (tumors) for each time point relying on the similarity of matched tumor-pairs. Hoechst dye extravagates from the vasculature, whereas other markers may reveal perfusion based on endothelial binding (*e.g.*, tomato lectins) or trapping of fluorescent or radioactive microspheres.⁹⁸ Indeed, sequential administration of stains of different colors before and after an intervention in a pulse chase fashion can reveal dynamic changes *post facto*⁹⁹ as demonstrated by Chaplin *et al.* with respect to vascular collapse following administration of the vasodilator hydralazine.¹⁰⁰

Superficial vasculature may also be assessed *in vivo* using intra vital microscopy particularly as applied to window chamber models.¹⁰¹ Vascular development may be examined

repeatedly over a period of days and, with respect to drug interventions and video loops, has revealed passage of individual erythrocytes sometimes revealing fluctuations and reversal of flow within individual vessels.^{102,103} Addition of fluorescent markers of pH and hypoxia has allowed correlation of multiple physiological parameters in small regions at microscopic resolution.¹⁰⁴ While microscopy is often limited to a surface depth of about 100 μ m, multi-photon approaches have revealed three-dimensional structure and multimodality interactions as represented by photoacoustic tomography allow deeper penetration.¹⁰⁵ Laser Doppler flowmetry has provided non-invasive evidence for acute response to CA4P.¹⁰⁶

Pre-clinical studies allow sacrifice and high resolution post-mortem analysis of tissues, but a requirement for biopsies to define vascular extent is less satisfactory in patients. It is particularly unsatisfactory for evaluating vascular dynamics since samples may not be representative of the whole tumor and the same tissue cannot be examined twice precluding dynamic studies. Thus, there is a need for non-invasive approaches to reliably, reproducibly, and simply examine changes in tumor vasculature at depth. Today, this is most commonly achieved using dynamic contrast enhanced (DCE) proton MRI (magnetic resonance imaging).¹⁰⁷ MRI is widely available, measurements are non-invasive and semi-quantitative measurement is facile based simply on changes in tissue water signal in response to distribution of paramagnetic contrast agent. Indeed, DCE MRI is now included in the clinical development of many VDAs.¹⁰⁸⁻¹¹¹ Dynamic computer tomography (CT) has also been used,¹¹² but it is often considered to be less attractive due to the radiation dose of repeated CT scans and potential for anaphylactic response to iodinated contrast agents or other adverse side effects.^{112,113} Paramagnetic gadolinium based contrast agents are recognized to be much safer. Recently, a few instances of NSF (nephrogenic systemic fibrosis) have been reported, but these appear to be associated with poor renal function.¹¹⁴ DCE does require a bolus IV (intravenous) infusion of contrast agent and there is extensive discussion of the optimal procedures regarding data acquisition (temporal and spatial resolution) and interpretation (analytical approaches).¹¹⁵⁻¹²⁰ Quantitative analysis is generally more relevant to subtle investigations of angiogenesis and vascular leakiness, while the beauty of many VDAs is the massive acute vascular response, which is readily detected even with simple semi-quantitative analyses.

Pre-clinical studies allow much greater versatility in imaging methodology and we show techniques in our laboratory both emulating the successes of other investigators and introducing novel paradigms.

DCE MRI has been most widely applied in the development of the VDAs. Measurements are non-invasive though they do require the IV infusion of a contrast agent. Essentially all imaging approaches require that animals be anaesthetized, but modern fluorinated gaseous anesthetics, such as isoflurane and sevoflurane, appear to be much less vasoactive, toxic and perturbing than earlier agents such as halothane or pentobarbital or ketamine.¹²¹ MRI can provide high temporal resolution and may generate 3D data sets for whole tumor coverage. More typically, a single slice through the center of a tumor is examined, since this reveals heterogeneity (*e.g.*, differential response of tumor center and periphery) with high temporal resolution. Assessment of vascular dynamics requires administration of sequential doses of contrast agent and measurements could be perturbed by wash-out of residual material from prior measurements. This can be overcome by increasing the dose of successive injections or simply allowing a sufficient interval for wash-out (generally, greater than 30 mins.) and most reports have used intervals of two hours or more between examinations. Since effective VDAs generally cause massive acute effects, experimental protocols and interpretation are quite facile. Even if an animal is removed from the magnet, precluding precise correlation of individual voxels, large regions tend to behave similarly and data are readily compared based on histograms or spatial consideration of regions of interest.

Animals may be allowed to wake up between scans, but it may generally be assumed that subtle physiological changes attributable to tumor development are minimal over a few hours. Thus, observed changes due to VDAs are readily identified. This is very different from antiangiogenesis agents, which normally act over days, and thus any changes in vasculature must be separated between “normal” tumor progression and response to drug.¹²²

There are extensive reports of DCE MRI applied to many VDAs including combretastatin A-4P (ZybreStatTM)^{96,97,106,123-126} and combretastatin A-1P (Oxi4503),¹²⁷ 5,6-dimethylxanthene 4-acetic acid (DMXAA, also called ASA404 (vadimezan),^{128,129} ZD6126 (N-acetylcoichinol-O-phosphate),¹³⁰⁻¹³² ABT-751,¹³³ protamine¹³⁴ and CYT997.¹³⁵ In many cases simple DCE used small paramagnetic contrast agents, but in other cases larger materials designed to be retained in the vasculature such as macromolecular contrast agent albumin-gadolinium diethylenetriaminepentaacetate (albumin-GdDTPA)¹³⁶ or SPIOs were used. Diverse tumors have been examined for research in animals (mice and rats) and as part of clinical trials in patients.¹³⁵ Several investigators have taken the opportunity to use MRI to compare the efficacy of different VDAs.^{124,137,138} Such measurements have accelerated development of agents providing insight into efficiency, dosing, timing and heterogeneity of activity. An example from our laboratory is in Figure 5 for a 13762NF rat breast tumor with respect to a single dose of combretastatin A-4P administered intraperitoneally (IP). At 2 h, vascular perfusion was severely reduced and delayed, but substantial recovery was observed at 24 h, notably in the tumor periphery. We have presented more extensive data in this tumor system and in a mouse tumor previously.^{96,97} Analyses of DCE MRI with respect to VDAs have used various levels of complexity ranging from changes in relative signal intensity following infusion of contrast agent (semi-quantitative) to rigorous calculation of perfusion fraction, vascular leakage and transit times. Ultimately, parameters are required to reflect efficiency and data reduction may provide averaged values such as mean and median or perfused fractions.

Alternate vascular dependent contrast mechanisms might be exploited including vascular spin labeling, though often tumors have such small blood vessels with sluggish flow that measurements are impractical. Oxygen may be considered as a contrast agent with changes in BOLD (Blood Oxygen Level Dependent) or TOLD (Tissue Oxygen Level Dependent) contrast response accompanying oxygen breathing challenge before and after the VDA administration. Certainly, tumor vascular extent has been correlated with BOLD response^{139,140} and flow must be considered, as noted in the FLOOD concept.¹⁴¹ Dynamic response to a hyperoxic gas challenge may reveal vascular shut down, but direct response to drug alone may be confused by coincidental changes in local hematocrit (blood volume), fraction of deoxyhemoglobin, and flow and Thomas *et al.* reported a complex pattern in response to carbogen challenge following CA4P treatment in rat bladder tumors growing in nude mice.¹⁴² Indeed, Howe *et al.*,¹⁴³ have observed apparently contradictory results whereby BOLD signal increased following death, attributable to vascular collapse and deoxyhemoglobin clearance rather than improved oxygenation.

In this regard, vascular volume and oxygenation may be monitored directly using near infrared spectroscopy (NIRS) noting the differential absorption coefficients of oxy- and deoxyhemoglobin.¹⁴⁴⁻¹⁴⁶ To date, NIRS has generally lacked spatial resolution, but multi-exponential behavior implies heterogeneity.

Increasingly, it is recognized that combined therapy approaches are needed to successfully treat patients, particularly in the case of VDAs, which often leave a surviving peripheral tumor rim causing rapid tumor reoccurrence. Vascular shut down has implications for concomitant chemotherapy based on effective drug delivery and retention. It is also crucial

for combination with radiotherapy, where vascular occlusion is expected to cause regional hypoxia, and hence, radio resistance. Indeed, several studies have shown that the combination of irradiation and VDA is crucially dependent on timing.¹⁴⁷⁻¹⁴⁸ We recently examined tumor oxygen dynamics directly based on ¹⁹F MRI oximetry with respect to VDA. Using FREDOM (Fluorocarbon Relaxometry Using Echo Planar Imaging for Dynamic Oxygen Mapping)¹⁴⁰ we found significant acute hypoxiation in the 13762NF rat breast tumor within 30 min of administering combretastatin A-4P.⁹⁶ Heterogeneous regional re-oxygenation was observed 24 h later. An example of such a measurement is shown in Figure 6, although here the hypoxiation was a little slower. Crucially, sequential pO₂ measurements are non-invasive and can be repeated every 6½ min. In comparison, DCE approaches require repeated administration of the contrast agent requiring *a priori* choice of measurement times. Such pO₂ measurements may be accelerated further by using a Look-Locker approach (90 s) as presented recently by Gallez, *et al.*¹⁴⁹ or based on a partial saturation measurement (1 s in a perfused heart¹⁵⁰). Most significantly, such measurements allowed us to optimize timing of combined irradiation and combretastatin to enhance tumor growth delay.¹⁵¹

Vascular imaging may also be achieved using ultrasound,¹⁵² notably, with the availability of the new small animal VisualSonics systems, which can provide microscopic resolution or exploit micro bubble contrast agents. Doppler approaches are attractive since they require no contrast agent, hence avoiding the associated costs and technical challenge of IV administration. However, sluggish perfusion of small vessels may handicap observations in some tumors. In Figure 7, we show vascular changes based on Power Doppler in a rat breast tumor, but the effect is quite subtle. In other tumors, we have seen much more extensive vasculature (manuscript in preparation). Vascular shutdown was readily apparent in this tumor based on infusion of contrast micro bubbles (Figure 7b and d). More extensive ultrasound studies have been reported by others, notably with respect to vascular disrupting agents or vascular flare following irradiation.¹⁵³⁻¹⁵⁹ As with MRI, such measurements may be applied clinically.

We recently introduced a novel approach exploiting dynamic bioluminescent imaging (dBLI) to investigate the acute effects of vascular disrupting agents.⁹⁷ Various reports have considered the dynamics of light emission for luciferase expressing cells growing in tumors in animals following the administration of luciferin substrates.¹⁶⁰⁻¹⁶² Most reports have focused on magnitude and duration of light emission together with reproducibility, *e.g.*, intravenous administration gives most rapid and intense, yet highly transient, light emission kinetics, while intraperitoneal administration is technically easier and gives a longer signal plateau, so that the timing of imaging acquisition is less critical.¹⁶³ However, we and others have noted a substantial failure rate with no (or minimal) light emission being observed. On the other hand, we find that subcutaneous (SC) administration of luciferin in the back/neck region provides highly reproducible light emission kinetics.¹⁶⁴ Noting that light emission requires delivery of luciferin substrate to the tumors by the vasculature this provides an effective assay of vascular patency. We have shown that following administration of CA4P to nude mice with human breast tumor xenografts consistent results were achieved using dynamic BLI or dynamic contrast enhanced MRI.⁹⁷ MRI does of course provide spatial information including potentially 3D representations. An example of dBLI is shown in Figure 8 for human prostate PC3-Luc tumors growing in two nude mice. Each mouse shows intense BLI signal prior to CA4P with diminished signal at 2 h and significant recovery at 24 h. Kinetic curves of light emission for one of the tumors are shown in the graph. We have now applied this procedure to several VDA drugs, disease sites and tumor types. The method is particularly simple to implement, cheap and offers high throughput. The primary drawback of this approach is the need for luciferase expressing cells.

In essence, any technology providing signal sensitive to vascular extent and flow may be applied to investigate VDA activity. In other cases, radionuclides have been used in conjunction with autoradiography, biodistribution, PET and SPECT.¹⁶⁵⁻¹⁶⁷

Concluding Remarks

In summary, new, innovative biological assays, modifications of existing techniques, and tumor imaging strategies are combined to assess the preclinical effectiveness of promising tubulin-binding VDAs as single agents and in combination therapy. In regard to cell-based technologies, the application of three-dimensional cell culture using components of the extracellular matrix provides a more physiologically relevant model for following the disruption of tubular networks. In the realm of tumor imaging, there now exists a diverse portfolio of imaging methods to evaluate VDAs. The ultimate choice of a particular approach may depend on the imaging needs for spatial and temporal resolution, costs, study size and nature of investigation, *e.g.*, high throughput preliminary survey studies versus detailed mechanisms and translatability to patients. dBLI is an economical and rapid technique to screen new VDAs. Collectively, these preclinical assessment tools should prove valuable in the identification of new drug candidates for human clinical trials.

Insight, innovation, integration

New small-molecule therapeutic intervention in the treatment of cancer relies heavily on insightful and innovative strategies that integrate molecular design and chemical synthesis with clearly defined biological evaluation techniques. The discovery and development of vascular disrupting agents (VDAs) provides such an example. Recent advances in preclinical tumor imaging such as bioluminescence imaging (BLI) coupled with DCE MRI (dynamic contrast enhanced magnetic resonance imaging) rapidly assess the selectivity of tumor blood-flow shutdown. Advanced biological assessment expands the standard inhibition of tubulin assembly and cytotoxicity assays to include endothelial tube disruption and reorganization, cell adherence, and endothelial cell permeability studies. In a highly integrative sense, these assays and tumor imaging strategies collectively provide information that is potentially predictive of clinical efficacy for VDAs.

Acknowledgments

Studies reported here were facilitated by grants from the NIH NCI (R01 CA139043-01A1 (to R.P.M.); 1R01 CA140674-01A1 (to K.G.P. and M.L.T.)), the Cancer Prevention and Research Institute of Texas (CPRIT (RP100406), to K.G.P. and M.L.T.), Oxigene Inc. (to K.G. P. and M.L.T.), and Department of Defense Congressionally Directed Breast Cancer Research Program, Department of the Army (DAMD17-03-1-0363 and DAMD#17-00-1-0437 (to R.P.M.)), together with Shared Instrumentation Grants from NIH (1S10RR024757-01 and 1S10RR025648-01) and the infrastructure provided by the Southwestern Small Animal Imaging Research Program (SW-SAIRP) supported in part by 1U24 CA126608 and Simmons Cancer Center (P30 CA142543-01) as well as AIRC (P41 RR02584). The authors are appreciative to Dr. James Karban and Dr. Michelle Nemecek (Director) for use of the shared Molecular Biosciences Center at Baylor University. In addition, we are indebted to Dr. Peter Peschke of the German Cancer Center Heidelberg for advice on tumor pathophysiology and Dr. David J. Chaplin of Oxigene Inc. for helpful discussions and for providing CA1P and CA4P. Finally, the authors are grateful to Dr. Tracy Strecker and Ms. Amanda K. Charlton-Sevcik for assistance with biochemical and biological assay descriptions, and Ms. Cristin McAnear and Ms. Tracy A. Pinney for expert assistance with the final manuscript drafts. As a potential conflict of interest statement, two of the authors (KGP and MLT) have current funding support from Oxigene Inc., and one author (KGP) wishes to disclose a consulting relationship with Oxigene Inc.

References

1. Siemann DW. Cancer Treatment Rev. 2011; 37:63–74.
2. McKeage MJ, Baguley BC. Cancer. 2010; 116:1859–1871. [PubMed: 20166210]

3. Tozer GM, Kanthou C, Baguley BC. *Nat. Rev. Cancer*. 2005; 5:423–435. [PubMed: 15928673]
4. Siemann DW, Bibby MC, Dark GG, Dicker AP, Eskens FA, Horsman MR, Marme D, Lorusso PM. *Clin. Cancer Res*. 2005; 11:416–420. [PubMed: 15701823]
5. Dougherty, GJ.; Chaplin, DJ. *Vascular Disruptive Agents for the Treatment of Cancer*. Meyer, T., editor. Springer; New York: 2010. p. 1-27. Chapter 1
6. Siemann, DW. *Vascular-Targeted Therapies in Oncology*. Siemann, D., editor. John Wiley & Sons; London, UK: 2006. p. 1-8. Chapter 1
7. Ferrara N, Hilian KL, Novotry W. *Biochem. Biophys. Res. Commun*. 2005; 333:328–335. [PubMed: 15961063]
8. Thorpe PE, Chaplin DJ, Blakey DC. *Cancer Research*. 2003; 63:1144–1147. [PubMed: 12615734]
9. Rhee J, Hoff PM. *Expert Opinion on Pharmacotherapy*. 2005; 6:1701–1711. [PubMed: 16086656]
10. Cohen MH, Gootenberg J, Keegan P, Pazdur R. *Oncologist*. 2007; 12:713–718. [PubMed: 17602060]
11. Pettit GR, Temple C Jr, Narayanan VL, Varma R, Simpson MJ, Boyd MR, Renner GA, Bansal N. *Anticancer Drug Des*. 1995; 10:299–309. [PubMed: 7786396]
12. Siemann DW, Chaplin DJ, Walicke PA. *Expert Opin. Invest. Drugs*. 2009; 18:189–197.
13. Nagaiah G, Remick SC. *Future Oncol*. 2010; 6:1219–1228. [PubMed: 20799867]
14. Delmonte A, Sessa C. *Expert Opin. Invest. Drugs*. 2009; 18:1541–1548.
15. Pettit GR, Lippert JW 3rd. *Anticancer Drug Des*. 2000; 15:203–216. [PubMed: 11049088]
16. Mainwaring PN, Lickliter J, Brown MP, Millward M, Chaplin DJ, Goldberg Z. *J. Clin. Oncol*. 2010; 28:15s. 2010 ASCO Annual Meeting. abstract TPS164.
17. Zweifel M, Patterson DM, Padhani AR, Middleton MR, Price P, Stratford MR, Ross P, Halford S, Godberg Z, Rustin GJ. *J. Clin. Oncol*. 2010; 28:15s. 2010 ASCO Annual Meeting. abstract 2594.
18. Kasibhatla S, Baichwal V, Cai SX, Roth B, Skvortsova I, Skvortsov S, Lucas P, English NM, Sirisoma N, Drewe J, Pervin A, Tseng B, Carlson RO, Pleiman CM. *Cancer Res*. 2007; 67:5865–5871. [PubMed: 17575155]
19. Mauer AM, Cohen EE, Ma PC, Kozloff MF, Schwartzberg L, Coates AI, Qian J, Hagey AE, Gordon GB. *J. Thorac. Oncol*. 2008; 3:631–636. [PubMed: 18520803]
20. Natsume T, Nakamura T, Koh Y, Kobayashi M, Saijo N, Nishio K. *Invest. New Drugs*. 2001; 19:293–302. [PubMed: 11561688]
21. Burns CJ, Fantino E, Phillips ID, Su S, Harte MF, Bukczynska PE, Frazzetto M, Joffe M, Kruszelnicki I, Wang B, Wang Y, Wilson N, Dilley RJ, Wan SS, Charman SA, Shackelford DM, Fida R, Malcontenti-Wilson C, Wilks AF. *Mol. Cancer Ther*. 2009; 8:3036–3045. [PubMed: 19887548]
22. Lickliter JD, Francesconi AB, Smith G, Burge M, Coulthard A, Rose S, Griffin M, Milne R, McCarron J, Yeadon T, Wilks A, Cubitt A, Wyld DK, Vasey PA. *British Journal of Cancer*. 2010; 103:597–606. [PubMed: 20733579]
23. Traynor AM, Gordon MS, Alberti D, Mendelson DS, Munsey MS, Wilding G, Gammans RE, Reid WL. *Invest. New Drugs*. 2010; 28:509–515. [PubMed: 19434370]
24. Nicholson B, Lloyd GK, Miller BR, Palladino MA, Kiso Y, Hayashi Y, Neuteboom STC. *Anti-Cancer Drugs*. 2006; 17:25–31. [PubMed: 16317287]
25. Kremmidiotis G, Leske AF, Lavranos TC, Beaumont D, Gasic J, Hall A, O'Callaghan M, Matthews CA, Flynn B. *Mol. Cancer Ther*. 2010; 9:1562–1573. [PubMed: 20515948]
26. Lee J, Kim SJ, Choi H, Kim YH, Lim IT, Yang H, Lee CS, Kang HR, Ahn SK, Moon SK, Kim D-H, Lee S, Choi NS, Lee KJ. *J. Med. Chem*. 2010; 53:6337–6354. [PubMed: 20690624]
27. Cai SX, Drewe J, Kemnitzer W. *Anti-Cancer Agents in Med. Chem*. 2009; 9:437–456.
28. Anthony SP, Read W, Rosen PJ, Tibes R, Park D, Everton D, Tseng B, Whisnant J, Von Hoff DD. *J. Clin. Oncol*. 2008; 26. 2008 ASCO Annual Meeting. abstract 2531. [PubMed: 18165637]
29. Lee J, Bae S, Lee S-h, Choi H, Kim YH, Kim SJ, Park GT, Moon SK, Kim D-H, Lee S, Ahn SK, Choi NS, Lee KJ. *Bioorg. Med. Chem. Lett*. 2010; 20:6327–6330. [PubMed: 20850313]
30. Lee RM, Gewirtz DA. *Drug Dev. Res*. 2008; 69:352–358.

31. Pinney, KG. Vascular-Targeted Therapies in Oncology. Siemann, D., editor. John Wiley & Sons; London, UK: 2006. p. 95-121. Chapter 6
32. Boyland E, Boyland ME. *Biochem. J.* 1937; 31:454–460. [PubMed: 16746357]
33. Seed L, Slaughter DP, Limarzi LR. *Surgery.* 1940; 7:696–709.
34. Hill SA, Lonergan SJ, Denekamp J, Chaplin DJ. *Eur. J. Cancer.* 1993; 29:1320–1324. [PubMed: 8343277]
35. Baguley BC, Holdaway KM, Thomsen LL, Zhuang L, Zwi LJ. *Eur. J. Cancer.* 1991; 27:482–487. [PubMed: 1827725]
36. Personal Communication. Dr. David J. Chaplin, Oxigene Inc.; Paclitaxel, unlike other tubulin-microtubule interacting VDAs, does not induce rapid and extensive blood flow reductions.
37. McKeage MJ, Fong P, Jeffery M, Baguley BC, Kestell P, Ravic M, Jameson MB. *Cancer Res.* 2006; 12:1776–1784.
38. Horsman MR, Siemann DW. *Cancer Res.* 2006; 66:11520–11539. [PubMed: 17178843]
39. Park CC, Bissell MJ, Barcellos-Hoff MH. *Molecular Medicine Today.* 2000; 6:324–329. [PubMed: 10904250]
40. Kenny PA, Bissell MJ. *Int. J. Cancer.* 2003; 107:688–695. [PubMed: 14566816]
41. Cai SX. Recent Patents on Anti-Cancer Drug Discovery. 2007; 2:79–101. [PubMed: 18221055]
42. Daenen LGM, Roodhart JML, Shaked Y, Voest EE. *Current Clinical Pharmacology.* 2010; 5:178–185. [PubMed: 20406172]
43. Sebahar PR, Willardsen JA, Anderson MB. *Current Bioactive Compounds.* 2009; 5:79–97.
44. Pilat MJ, LoRusso PM. *Journal of Cellular Biochemistry.* 2006; 99:1021–1039. [PubMed: 16927308]
45. Kingston DGI. *J. Nat. Prod.* 2009; 72:507–515. [PubMed: 19125622]
46. Cai Y-C, Zou Y, Xian L-J. *Acta Pharmaceutica Sinica.* 2010; 45:283–288. [PubMed: 21351502]
47. Siemann DW, Horsman MR. *Cell Tissue Res.* 2009; 335:241–248. [PubMed: 18752004]
48. Schwartz EL. *Clin. Cancer Res.* 2009; 15:2594–2601. [PubMed: 19351751]
49. Kanthou C, Tozer GM. *Int. J. Exp. Path.* 2009; 90:284–294. [PubMed: 19563611]
50. Fens MHAM, Storm G, Schiffelers RM. *Expert Opin. Invest. Drugs.* 2010; 19:1321–1338.
51. Heath VL, Bicknell R. *Nat. Rev. Clin. Oncol.* 2009; 6:395–404. [PubMed: 19424102]
52. Tozer GM, Kanthou C, Lewis G, Prise VE, Vojnovic B, Hill SA. *Br. J. Radiol.* 2008; 81:S12–S20. [PubMed: 18819993]
53. Neri D, Bicknell R. *Nat. Rev. Cancer.* 2005; 5:436–446. [PubMed: 15928674]
54. Gridelli C, Rossi A, Maione P, Rossi E, Castaldo V, Sacco PC, Colantuoni G. *Oncologist.* 2009; 14:612–620. [PubMed: 19474164]
55. Horsman MR, Bohn AB, Busk M. *Exp. Oncol.* 2010; 32:143–148. [PubMed: 21403608]
56. Madlambayan GJ, Meacham AM, Hosaka K, Mir S, Jorgensen M, Scott EW, Siemann DW, Cogle CR. *Blood.* 2010; 116:1539–1547. [PubMed: 20472832]
57. Shaked Y, Tang T, Woloszynek J, Daenan LG, Man S, Xu P, Cai S-R, Arbeit JM, Voest EE, Chaplin DJ, Smythe J, Harris A, Nathan P, Judson I, Rustin G, Bertolini F, Link DC, Kerbel RS. *Cancer Res.* 2009; 69:7524–7528. [PubMed: 19738066]
58. Daenan LG, Shaked Y, Man S, Xu P, Voest EE, Hoffman RM, Chaplin DJ, Kerbel RS. *Mol. Cancer Ther.* 2009; 8:2872–2881. [PubMed: 19825805]
59. Nagy JA, Chang S-H, Dvorak AM, Dvorak HF. *Br. J. Cancer.* 2009; 100:865–869. [PubMed: 19240721]
60. Guillemot L, Paschoud S, Jond J, Foglia A, Citi S. *Mol. Biol. Cell.* 2008; 19:4442–4453. [PubMed: 18653465]
61. Krendel M, Zenke FT, Bokoch GM. *Nat. Cell Biol.* 2002; 4:294–301. [PubMed: 11912491]
62. Birkenfeld J, Nalbant P, Yoon S-H, Bokoch GM. *Trends Cell Biol.* 2008; 18:210–219. [PubMed: 18394899]
63. Ren Y, Li R, Zheng Y, Busch H. *J. Biol. Chem.* 1998; 273:34954–34960. [PubMed: 9857026]

64. Birukova AA, Adyshev D, Gorshkov B, Bokoch GM, Birukov KG, Verin AD. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 2006; 290:L540–548. [PubMed: 16257999]
65. Hamel E, Lin CM. *Biochemistry.* 1984; 23:4173–4184. [PubMed: 6487596]
66. Hamel E, Lin CM. *Biochimica et Biophysica Acta.* 1981; 675:226–231. [PubMed: 6115675]
67. Verdier-Pinard P, Lai J-Y, Yoo H-D, Yu J, Marquez B, Nagle DG, Nambu M, White JD, Falck JR, Gerwick WH, Day BW, Hamel E. *Mol. Pharmacol.* 1998; 53:62–76. [PubMed: 9443933]
68. Monk KA, Siles R, Hadimani MB, Mugabe BE, Ackley JF, Studerus SW, Edvardsen K, Trawick ML, Garner CM, Rhoads MR, Pettit GR, Pinney KG. *Bioorg. Med. Chem.* 2006; 14:23–46.
69. Hamel E. *Cell Biochem. Biophys.* 2003; 38:1–21. [PubMed: 12663938]
70. Bonne D, Heusele C, Simon C, Pantaloni D. *J. Biol. Chem.* 1985; 260:2819–2825. [PubMed: 3972806]
71. Mugabe, BE. Ph.D. Dissertation. Baylor University; Waco, TX: 2005.
72. Monks A, Scudiero D, Skehan P, Shoemaker R, Paull K, Vistica D, Hose C, Langley J, Cronise P, Vaigro-Wolf A. *J. Natl. Cancer Inst.* 1991; 83:757–766. [PubMed: 2041050]
73. Mosmann T. *J. Immunol. Methods.* 1983; 65:55–63. [PubMed: 6606682]
74. Kubota Y, Kleinman HK, Martin GR, Lawley TJ. *J. Cell Biol.* 1988; 107:1589–1598. [PubMed: 3049626]
75. Lee GY, Kenny PA, Lee EH, Bissell MJ. *Nature Methods.* 2007; 4:359–365. [PubMed: 17396127]
76. Davis G, Senger D. *Circ Res.* 2005; 97:1093–1107. [PubMed: 16306453]
77. Braut-Boucher F, Pichon J, Rat P, Adolphe M, Aubery M, Font J. *J. Immunol. Methods.* 1995; 178:41–51. [PubMed: 7829864]
78. Kremmidiotis G, Leske AF, Lavranos TC, Beaumont D, Gasic J, Hall A, O'Callaghan M, Matthews CA, Flynn B. *Mol. Cancer Ther.* 2010; 9:1562–1573. [PubMed: 20515948]
79. Smith RA, Rooney MM, Lord ST, Mosesson MW, Gartner TK. *Thrombosis and Haemostasis.* 2000; 84:819–825. [PubMed: 11127863]
80. Bates DO, Hillman NJ, Williams B, Neal CR, Pocock TM. *Journal of Anatomy.* 2002; 200:581–597. [PubMed: 12162726]
81. Dejana E. *J. Clin. Invest.* 1996; 98:1949–1953. [PubMed: 8903311]
82. Lal BK, Varma S, Pappas PJ, Hobson RW, Duran WN. *Microvascular Research.* 2001; 62:252–62. [PubMed: 11678628]
83. Corada M, Liao F, Lindgren M, Lampugnani MG, Breviario F, Frank R, Muller WA, Hicklin DJ, Bohlen P, Dejana E. *Blood.* 2001; 97:1679–1684. [PubMed: 11238107]
84. Preaudat M, Ouled-Diaf J, Alpha-Bazin B, Mathis G, Mitsugi T, Aono Y, Takahashi K, Takemoto H. *J. Biomol. Screen.* 2002; 7:267–74. [PubMed: 12097189]
85. Larson B, Worzella T. *Cell Notes.* 2005; 12:13–6.
86. Liu JJ, Wang W, Dicker DT, El-Deiry WS. *Cancer Biol. Ther.* 2005; 4:885–92. [PubMed: 16177559]
87. Hinnen P, Eskens F. *Br. J. Cancer.* 2007; 96:1159–1165. [PubMed: 17375046]
88. Thorpe PE. *Clin. Cancer Res.* 2004; 10:415–27. [PubMed: 14760060]
89. Tozer GM, Kanthou C, Baguley BC. *Nat. Rev. Cancer.* 2005; 5:423–435. [PubMed: 15928673]
90. Desar IME, van Herpen CML, van Laarhoven HWM, Barentsz JO, Oyen WJG, van der Graaf WTA. *Cancer Treatment Rev.* 2009; 35:309–321.
91. Konerding MA, Fait E, Gaumann A. *Br. J. Cancer.* 2001; 84:1354–1362. [PubMed: 11355947]
92. Krucker T, Lang A, Meyer EP. *Microscopy Res. Tech.* 2006; 69:138–147.
93. Folarin AA, Konerding MA, Timonen J, Nagl S, Pedley RB. *Microvascular Res.* 2010; 80:89–98.
94. Schneider P, Krucker T, Meyer E, Ulmann-Schuler A, Weber B, Stampanoni M, Muller R. *Microscopy Res. Tech.* 2009; 72:690–701.
95. Heinzer S, Kuhn G, Krucker T, Meyer E, Ulmann-Schuler A, Stampanoni M, Gassmann M, Marti HH, Müller R, Vogel J. *NeuroImage.* 2008; 39:1549–1558. [PubMed: 18077185]
96. Zhao D, Jiang L, Hahn EW, Mason RP. *Int. J. Radiat. Oncol. Biol. Phys.* 2005; 62:872–880. [PubMed: 15936572]

97. Zhao D, Richer E, Antich PP, Mason RP. *FASEB J.* 2008; 22:2445–2451. [PubMed: 18263704]
98. Raman V, Artemov D, Pathak AP, Winnard PT, McNutt S, Yudina A, Bogdanov A, Bhujwalla ZM. *Cancer Res.* 2006; 66:9929–9936. [PubMed: 17047055]
99. Trotter MJ, Chaplin DJ, Durand RE, Olive PL. *Int. J. Radiat. Oncol. Biol. Phys.* 1989; 16:931–934. [PubMed: 2703399]
100. Trotter MJ, Acker BD, Chaplin DJ. *Int. J. Radiat. Oncol. Biol. Phys.* 1989; 17:785–789. [PubMed: 2777668]
101. MacDonald IC, Chambers AF. *Methods Enzymol.* 2008; 444:201–230. [PubMed: 19007666]
102. Dreher MR, Liu WG, Michelich CR, Dewhirst MW, Yuan F, Chilkoti A. *J. Natl. Cancer Inst.* 2006; 98:335–344. [PubMed: 16507830]
103. Monsky WL, Fukumura D, Gohongi T, Ancukiewicz M, Weich HA, Torchilin VP, Yuan F, Jain RK. *Cancer Res.* 1999; 59:4129–4135. [PubMed: 10463618]
104. Cardenas-Navia LI, Mace D, Richardson RA, Wilson DF, Shan S, Dewhirst MW. *Cancer Res.* 2008; 68:5812–5819. [PubMed: 18632635]
105. Ntziachristos V. *Nat. Methods.* 2010; 7:603–614. [PubMed: 20676081]
106. Ley CD, Horsman MR, Kristjansen PEG. *Neoplasia.* 2007; 9:108–112. [PubMed: 17356706]
107. Choyke PL, Dwyer AJ, Knopp MV. *J. Magn. Reson. Imaging.* 2003; 17:509–520. [PubMed: 12720260]
108. Evelhoch JL. *J. Magn. Reson. Imaging.* 1999; 10:254–259. [PubMed: 10508284]
109. Padhani AR. *Br. J. Radiol.* 2003; 76:S60–S80. [PubMed: 15456715]
110. Padhani AR, Miles KA. *Radiology.* 2010; 256:348–364. [PubMed: 20656830]
111. Ashton E. *J. Magn. Reson. Imaging.* 2010; 31:279–288. [PubMed: 20099339]
112. Ng Q-S, Goh V, Carnell D, Meer K, Padhani AR, Saunders MI, Hoskin PJ. *Int. J. Radiat. Oncol. Biol. Phys.* 2007; 67:1375–1380. [PubMed: 17275203]
113. Mortelet KJ, Oliva MR, Ondategui S, Ros PR, Silverman SG. *Am. J. Roentgenol.* 2005; 184:31–34. [PubMed: 15615946]
114. Rofsky NM, Sherry AD, Lenkinski RE. *Radiology.* 2008; 247:608–612. [PubMed: 18487530]
115. Heilmann M, Walczak C, Vautier J, Dimicoli J-L, Thomas C, Lupu M, Mispelter J, Volk A. *Magn. Reson. Mater. Phys. Biol. Med.* 2007; 20:193–203.
116. Yankeelov TE, Luci JJ, DeBusk LM, Lin PC, Gore JC. *Magn. Reson. Med.* 2008; 59:326–335. [PubMed: 18228592]
117. Yang C, Karczmar GS, Medved M, Stadler WM. *Magn. Reson. Med.* 2007; 58:1266–1275. [PubMed: 17969061]
118. Eyal E, Degani H. *NMR in Biomed.* 2009; 22:40–53.
119. Cao Y, Brown SL, Knight RA, Fenstermacher JD, Ewing JR. *Magn. Reson. Med.* 2005; 53:282–293. [PubMed: 15678542]
120. Strijkers GJ, Hak S, Kok MB, Springer CS, Nicolay K. *Magn. Reson. Med.* 2009; 61:1049–1058. [PubMed: 19215042]
121. Menke H, Vaupel P. *Radiat. Res.* 1988; 114:64–76. [PubMed: 2895486]
122. Zhao D, Jiang L, Hahn EW, Mason RP. *Neoplasia.* 2005; 7:678–87. [PubMed: 16026647]
123. Maxwell RJ, Wilson J, Prise VE, Vojnovic B, Rustin GJ, Lodge MA, Tozer GM. *NMR in Biomed.* 2002; 15:89–98.
124. Beauregard DA, Pedley RB, Hill SA, Brindle KM. *NMR in Biomed.* 2002; 15:99–105.
125. Galbraith SM, Maxwell RJ, Lodge MA, Tozer GM, Wilson J, Taylor NJ, Stirling JJ, Sena L, Padhani AR, Rustin GJS. *J. Clin. Oncol.* 2003; 21:2831–2842. [PubMed: 12807936]
126. Stevenson JP, Rosen M, Sun W, Gallagher M, Haller DG, Vaughn D, Gantonio B, Zimmer R, Petros WP, Stratford M, Chaplin D, Young SL, Schnall M, O'Dwyer PJ. *J. Clin. Oncol.* 2003; 21:4428–38. [PubMed: 14645433]
127. Salmon HW, Siemann DW. *Clin. Cancer Res.* 2006; 12:4090–4094. [PubMed: 16818709]
128. Howe FA, McPhail LD, Griffiths JR, McIntyre DJO, Robinson SP. *Int. J. Radiat. Oncol. Biol. Phys.* 2008; 71:1470–1476. [PubMed: 18538948]

129. Seshadri M, Ciesielski MJ. *Journal of Cerebral Blood Flow and Metabolism*. 2009; 29:1373–1382. [PubMed: 19458603]
130. McIntyre DJO, Robinson SP, Howe FA, Griffiths JR, Ryan AJ, Blakey DC, Peers IS, Waterton JC. *Neoplasia*. 2004; 6:150–157. [PubMed: 15140404]
131. McPhail LD, McIntyre DJO, Ludwig C, Kestell P, Griffiths JR, Kelland LR, Robinson SP. *Neoplasia*. 2006; 8:199–206. [PubMed: 16611413]
132. Robinson SP, McIntyre DJ, Checkley D, Tessier JJ, Howe FA, Griffiths JR, Ashton SE, Ryan AJ, Blakey DC, Waterton JC. *Br. J. Cancer*. 2003; 88:1592–1597. [PubMed: 12771928]
133. Luo YP, Hradil VP, Frost DJ, Rosenberg SH, Gordon GB, Morgan SJ, Gagne GD, Cox BF, Tahir SK, Fox GB. *Anti-Cancer Drugs*. 2009; 20:483–492. [PubMed: 19398903]
134. Su MY, Samoszuk MK, Wang J, Nalcioğlu O. *NMR in Biomed*. 2002; 15:106–113.
135. Lickliter JD, Francesconi AB, Smith G, Burge M, Coulthard A, Rose S, Griffin M, Milne R, McCarron J, Yeadon T, Wilks A, Cubitt A, Wyld DK, Vasey PA. *Br. J. Cancer*. 2010; 103:597–606. [PubMed: 20733579]
136. Vogel-Claussen J, Gimi B, Artemov D, Bhuiwalla ZM. *Cancer Biol. Ther*. 2007; 6:1469–1475. [PubMed: 17881899]
137. Breidahl T, Nielsen FU, Stodkilde-Jorgensen H, Maxwell RJ, Horsman MR. *Acta Oncologica*. 2006; 45:306–316. [PubMed: 16644574]
138. McPhail LD, Griffiths JR, Robinson SP. *Int. J. Radiat. Oncol. Biol. Phys*. 2007; 69:1238–1245. [PubMed: 17967313]
139. Robinson SP, Rijken PF, Howe FA, McSheehy PM, van der Sanden BP, Heerschap A, Stubbs M, van der Kogel AJ, Griffiths JR. *J. Magn. Reson. Imaging*. 2003; 17:445–454. [PubMed: 12655584]
140. McPhail LD, Robinson SP. *Radiology*. 2010; 254:110–118. [PubMed: 20032145]
141. Howe FA, Robinson SP, Rodrigues LM, Griffiths JR. *Magn. Reson. Imaging*. 1999; 17:1307–1318. [PubMed: 10576716]
142. Thomas CD, Walczak C, Kaffy J, Pontikis R, Jouanneau J, Volk A. *Neoplasia*. 2006; 8:587–595. [PubMed: 16867221]
143. Howe FA, Robinson SP, McIntyre DJ, Stubbs M, Griffiths JR. *NMR in Biomed*. 2001; 14:497–506.
144. Liu H, Song Y, Worden KL, Jiang X, Constantinescu A, Mason RP. *Appl. Opt*. 2000; 39:5231–5243. [PubMed: 18354520]
145. Liu H, Gu Y, Kim JG, Mason RP. *Methods Enzymol*. 2004; 386:349–378. [PubMed: 15120261]
146. Zhao D, Jiang L, Mason RP. *Methods Enzymol*. 2004; 386:378–418. [PubMed: 15120262]
147. Murata R, Siemann DW, Overgaard J, et al. *Radiother. Oncol*. 2001; 60:155–161. [PubMed: 11439210]
148. Siemann DW, Rojiani AM. *Int. J. Radiat. Oncol. Biol. Phys*. 2002; 53:164–171. [PubMed: 12007956]
149. Jordan BF, Cron GO, Gallez B. *Magn. Reson. Med*. 2009; 61:634–638. [PubMed: 19097235]
150. Mason RP, Jeffrey FMH, Malloy CR, Babcock EE, Antich PP. *Magn. Reson. Med*. 1992; 27:310–317. [PubMed: 1334204]
151. Zhao D, Chang C-H, Kim JG, Liu H, Mason RP. *Int. J. Radiat. Oncol. Biol. Phys*. 2010 accepted.
152. Goertz DE, Yu JL, Kerbel RS, Burns PN, Foster FS. *Cancer Res*. 2002; 62:6371–6375. [PubMed: 12438217]
153. Gee MS, et al. *Cancer Res*. 2001; 61:2974–2982. [PubMed: 11306476]
154. Goertz DE, et al. *Cancer Res*. 2002; 62:6371–6375. [PubMed: 12438217]
155. Pinter SZ, Lacefield JC. *Ultrasound in Medicine & Biology*. 2009; 35:1217–1228. [PubMed: 19394752]
156. Alcázar JL, Jurado M, López-García G. *International Journal of Gynecological Cancer*. 2010; 20:393–397. [PubMed: 20375803]
157. Moon HJ, et al. *Radiology*. 2010; 255:260–269. [PubMed: 20308462]
158. Zackova T, et al. *Reproductive Biology and Endocrinology*. 2009; 7:151. [PubMed: 20035622]

159. Palmowski M, Peschke P, Huppert J, Hauff P, Reinhardt M, Maurer M, Karger CP, Scholz M, Semmler W, Huber PE, Kiessling FM. *Neoplasia*. 2009; 11:856–863. [PubMed: 19724679]
160. Paroo Z, Bollinger RA, Braasch DA, Richer E, Corey DR, Antich PP, Mason RP. *Mol. Imaging*. 2004; 3:117–124. [PubMed: 15296676]
161. Baba S, Cho SY, Ye Z, Cheng L, Engles JM, Wahl RL. *Mol. Imaging*. 2007; 6:315–322. [PubMed: 18092516]
162. Keyaerts M, Verschueren J, Bos TJ, Tchouate-Gaikam LO, Peleman C, Breckpot K, Vanhove C, Caveliers V, Bossuyt A, Lahoutte T. *Eur. J. Nucl. Med. Mol. Imaging*. 2008; 35:999–1007. [PubMed: 18180921]
163. Bollinger, RA. Ph.D. Thesis. UT Southwestern: 2006.
164. Contero A, Richer E, Gondim A, Mason RP. *Methods Mol. Biol.* 2009; 574:37–45. [PubMed: 19685298]
165. Lew YS, Brown SL, Griffin RJ, Song CW, Kim JH. *Cancer Res.* 1999; 59:6033–6037. [PubMed: 10626785]
166. Brown SL, Hunt JW, Hill RP. *Nucl. Med. Biol.* 1988; 15:381–390.
167. Kötz B, West C, Saleem A, Jones T, Price P. *Mol. Cancer Ther.* 2009; 8:303–309. [PubMed: 19208824]

Fig. 1. Tubulin Binding Small-Molecule Vascular Disrupting Agents (VDAs)

A compilation of VDAs currently in human clinical trials that function, in part, through a tubulin mechanism include: CA4P¹¹⁻¹³, AVE8062¹⁴, CA1P¹⁵⁻¹⁷, MPC-6827¹⁸, ABT-751¹⁹, TZT-1027²⁰, CYT997^{21,22}, MN-029²³, NPI-2358²⁴, BNC105P²⁵, EPC2407²⁶⁻²⁸, CKD-516²⁹.

Fig. 2. Model for downstream effects of treatment with tubulin-binding VDAs in tumor endothelium. Microtubule disassembly leads to cell rounding and detachment that can result in a rapid collapse in tumor blood flow.

Fig. 3. (A) Inhibition of tubulin polymerization as a function of increasing concentration of VDA (CA1) from the top two traces, 0 μ M (control), to the lowest curve at 5 μ M CA1. (B) IC₅₀ determination for CA1 (1.9 μ M).⁷¹

Fig. 4. Vascular heterogeneity in tumors identified *ex vivo*

These panels provide evidence for vascular heterogeneity based on *ex vivo* analysis:

- A)** Scanning Electron Microscopy reveals disorganized tumor vasculature with many tortuous microvessels and blind ends. A 13762NF mammary adenocarcinoma was grown using a kidney tissue-isolated preparation in a syngeneic adult female Fischer 344 rat. Prior to sacrifice the abdominal aorta proximal to the renal artery was perfused with Batson's #17 polymer generating a vascular cast. The vascular corrosion cast was sputtered with gold-palladium prior to SEM (data obtained in collaboration with Andrew Abbott and Dr. C. Gilpin).
- B)** Immunohistochemistry reveals vascular extent in a Dunning prostate R3327-HI tumor based on anti-CD31 mAb. The tumor was grown in a syngeneic male Copenhagen rat and Hoechst 33342 dye (seen in blue in C) was infused IV followed by tumor excision 60 s later. The green stain shows vascular extent based on anti-CD31 mAb in a frozen 6 μ m-thick section. Original magnification x100.
- C)** Extravasated Hoechst dye (blue) reveals tumor perfusion in same slice as B.
- D)** Overlay of the fluorescent images in B and C reveals that essentially all blood vessels were perfused in this tumor.

Fig. 5. Evaluating VDAs non-invasively by MRI

Dynamic contrast enhanced (DCE) MRI was performed pre, and 2 h and 24 h after treatment of a 13762NF rat breast tumor with CA4P (30 mg/kg, IP). A series of T_1 -weighted images was acquired with respect to infusion of a bolus injection of contrast agent (Gd-DTPA-BMA (Omniscan); 0.1 mmol/kg). A) Normalized images of signal enhancement 25 s after contrast agent administration are shown for a representative tumor. Significantly less signal enhancement was observed for the whole tumor region 2 h after treatment with CA4P. A recovery was apparent in the tumor rim 24 h later. B) Time course curves showed rapid increase in signal intensity after infusion of contrast agent prior to CA4P, whereas much slower increase and significantly less enhancement was observed 2 h after CA4P. Twenty-four hours later signal enhancement recovered to ~90% of the baseline.

Fig. 6. Assessment of hypoxia accompanying vascular disruption

A) pO₂ maps obtained using FREDOM (¹⁹F MRI) with respect to hyperoxic gas breathing challenge and CA4P treatment (30 mg/kg, IP). Following administration of hexafluorobenzene (HFB) directly into the 13762NF tumor ten individual locations (voxels) could be examined sequentially with respect to an oxygen breathing and CA4P administration over a period up to 2 h. The next day, residual HFB allowed further interrogation of the tumor using FREDOM showing thirteen voxels, which could be assessed with respect to oxygen challenge. At baseline, there was a significant increase in pO₂ with respect to breathing oxygen. A significant decrease in pO₂ was evident for all the individual voxels after CA4P, and pO₂ no longer responded to oxygen inhalation after 2 h. The 24 h maps showed that pO₂ again responded to oxygen breathing. B) Mean tumor pO₂ with respect to interventions. * p < 0.05 from baseline air, + p < 0.05 from 24 h air.

Fig. 7. Assessment of acute changes in tumor vasculature using ultrasound

High resolution ultrasound using a VisualSonics Vevo770 small animal ultrasound device of an 8 mm diameter syngeneic MTLn3 mammary tumor growing subcutaneously in a Fisher rat with respect to infusion of the new VDA CA1P (30 mg/kg, IP) A) Doppler mode using 40 MHz transducer shows a few major blood vessels in tumor and significant vasculature in skin. B) Infusion of commercial contrast microbubbles revealed vasculature more clearly, particularly revealing small blood vessels using a 40 MHz transducer. C) 30 mins after infusion of CA1P the Color Doppler indicated minor reduction in blood flow. D) Infusion of fresh contrast agent clearly showed substantial decrease in perfusion.

Fig. 8. Assessment of acute changes in tumor vasculature using dynamic bioluminescent imaging (dBLI)

- A)** Human prostate PC3-Luc cells were implanted subcutaneously in the right thighs of two nude mice and observed by BLI following administration of luciferin 80 μ l (40 mg/ml) SC in the shoulder neck region. Images were acquired every 60 s for 30 mins to observe the dynamic evolution of the bioluminescent signal (dynamic BLI). Following a baseline time course, CA4P was administered IP (100 μ l; 150 mg/kg) and 2 h later dBLI was repeated with fresh luciferin. DBLI was repeated the following day.
- B)** Mean light intensity indicated a maximum at 25 min under control conditions. Following CA4P light emission was decreased at least 90%. Twenty-four hours later there was substantial recovery in light emission reaching about 30% of baseline.