This paper describes the pathophysiology of tumors and reviews past methods of combating cancer by exploiting such pathophysiology. This is followed by a shift to current research on the subject. This discussion of current research leads into the author’s full research project: synthesizing combretastatin analogues with conformational flexibility which can be coupled to bioreductive drugs.

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Exploring Means of Exploiting Tumor Pathophysiology

Taylor Wootton

Cancer is a devastating disease. A silent stalker, its precursors lurk in the air we breathe, the food we eat, and paradoxically, in the very cellular instructions essential to reproducing and sustaining life. As the leading cause of death in persons under 85 years of age, cancer is associated with one out of every four deaths in the United States.¹ In 2006, Americans can expect 1,399,790 new cases and 564,830 deaths.² The most devastating aspect of the disease, however, is not the numbers, but rather cancer’s means of infection and proliferation. Cancer invades its host by exploiting a cell’s natural ability to reproduce life. Something causes the normal cell cycle to go wrong; checkpoints are bypassed; mitosis is hijacked; and if further repair mechanisms fail, a malignant cell is birthed. Further cell replication leads to the formation of a tumor.

However, without a vasculature of its own, a cancer tumor is incapable of causing harm. Limited in size by its dependence on nearby tissues for delivery of oxygen and nutrients and removal of waste, an avascular tumor cannot exceed a diameter of a few millimeters. Upon reaching maximum size, the tumor is trapped in an equilibrium of sorts, during which the amount of cellular generation is equal to the amount of cell death. In this steady state, which can last for years, the malignant cells of the tumor are unable to invade and destroy nearby cells.³

† Definitions of italicized words can be found in the glossary at the end of this paper.
**Tumor Vasculature**

Dramatic change comes, however, when the tumor stimulates the formation of its own vasculature through the release of specific growth factors, of which Vascular Endothelial Growth Factor (VEGF) plays a predominate role. This has been demonstrated by a direct correlation between the production of VEGF and its receptors and the degree of tumor vascularization present. In addition, studies that involve injecting mice with an anti-VEGF antibody demonstrate that the blocking of VEGF almost completely halts the formation of new blood vessels and tumor growth. VEGF may also play a role in metastasis because of its ability to increase vascular permeability and thus contribute to the formation of leaky vessels that can be easily penetrated by malignant cells. Interestingly, VEGF mRNA has been found in tumor cells located in the inner, hypoxic region of the tumor, suggesting that hypoxia may select for cells that can induce vascularization.

The release of VEGF is apparently stimulated by an angiogenic switch, triggered by parameters such as hypoxia, hypoglycemia, and acidosis, all indicative of inadequate vasculature. The stress of host immune response may also play a role. The cycle is perpetuated because as new cells are generated, VEGF is stimulated. The increased vascularization then allows for the further expansion of the tumor by increased amounts of cellular genesis, thus creating an even greater demand for the supply of oxygen and nutrients and stimulating the release of additional growth factor.

**Angiogenesis**

The release of VEGF stimulates the process of angiogenesis in both normal and malignant tissues. The angiogenic process itself is very similar in the two environments. However, the resulting neovascularature differs greatly. In normal tissues, angiogenesis is a highly ordered process, occurring in adults only during ovarian cycles and repair processes such as wound healing. In a simplified view, a vessel composed of a basement membrane, pericytes, and a single layer of endothelial cells is destabilized. In essence, endothelial cells are freed from the tube-like formation of the vessel and made able to proliferate. The cells then migrate, contact each other, and begin to reform a tube-like structure which will serve as the basis of the new vessel. Finally, new vessels
are stabilized with the addition of pericytes and a basement membrane.⁵ Angiogenesis stimulated by normal tissues results in a well-organized vasculature with an established hierarchy of arterioles, capillaries, and venules. Regular branching, vessel size, and vessel spacing adequately supply cells with oxygen, nutrients, and waste removal.⁸

**Abnormalities of tumor angiogenesis.** Tumor angiogenesis follows the same mechanism but the results are sloppy and disordered, most likely attributable to the abnormalities of the tumor environment. The resulting tumor vasculature differs greatly from that of normal tissues. While tumor blood vessels contain the same structural elements as normal vessels—endothelial cells, pericytes, and basement membrane—all three are abnormal and are present in the tumor environment in a tangled array.⁷,⁹ Irregular endothelial cells leave gaps in vessels, a problem exacerbated by abnormal pericytes, which fail to provide support at the junction points of endothelial cells.⁷,¹⁰ Abnormal endothelial cells and abnormal pericytes give rise to an abnormal basement membrane, further contributing to vessel irregularity and leakiness.⁵,⁹,¹⁰ Irregular branching patterns cause an unequal distribution of vessels, contributing to an inadequate supply of oxygen and nutrients to some tumor cells, resulting in hypoxia. Abnormal vessel diameter causes highly variable rates of blood flow, including stagnation in some vessels.⁸,¹⁰

In the past, these structural abnormalities were seen as a downfall to both radiation and chemotherapy cancer treatments. In 1953, Gray and co-workers demonstrated that hypoxic tissue is only one-half to one-third as sensitive to radiation therapy as normal tissue.¹¹ The disorganized pattern of branching and absence of a functional lymphatic system leaves some cells beyond the reach of the vascular delivery of drugs. Variable blood flow, especially flow stagnation, is also a major problem for drug delivery.⁷,¹⁰ However, the very abnormalities that caused problems in the past are now widely seen as the Achilles heel of the tumor.¹² Scientists are now able to inhibit VEGF with anti-angiogenic therapy, selectively target the rapidly proliferating tumor vasculature with vascular disrupting agents, and take advantage of the tumor’s hypoxic environment using bioreductive drugs. This paper will focus on the current understanding of small molecule, tubulin-binding vascular disrupting agents and their application in combination with bioreductive drugs.
Therapies Targeting Tumor Vasculature

The concept of treating cancer by interfering with tumor vasculature is not new. In fact, as early as the nineteenth century, physicians and scientists were exploring this method of treatment. Walsh reported in 1844 that tumors could be cured if their circulation was artificially disrupted. In addition, Goldman (1907) described the chaotic growth of tumor vasculature, and Woglum (1923) discussed the critical role played by tumor vasculature and the possible therapeutic effects of its destruction. This preliminary work laid the foundation for the two branches of vascular targeting research that exist today.

The unique physiology of tumor vasculature presents two distinct methodologies by which to interrupt blood flow and trigger tumor necrosis. The first involves inhibiting the angiogenic pathway and neovascularization, while the second targets and seeks to disrupt existing tumor vasculature.

Anti-angiogenesis

In 1971, Judah Folkman proposed the term “anti-angiogenesis” to describe a therapy that would inhibit tumor angiogenesis. He hypothesized that an antibody against what he termed “Tumor-Angiogenesis Factor” could act as an anti-angiogenic agent and prevent the formation of new capillaries that would support a tumor. Currently, two types of anti-angiogenic therapies are being explored: those that directly target endothelial cell recruitment and proliferation and those indirect inhibitors that interfere with either the production of pro-angiogenic factors or their receptors. The most useful anti-angiogenic agents to date block VEGF or integrin-mediated endothelial cell functions during angiogenesis.

Vascular Disrupting Therapy

The goal of the second method, recently termed vascular disrupting therapy (previously known as vascular targeting), is to selectively target and disrupt the existing, immature tumor vasculature, inhibiting blood flow and thus the delivery of vital oxygen and nutrients to tumor cells. The modern concept of vascular disrupting agents (VDAs) was birthed in the 1980s by Julia Denekamp and co-workers with the discovery of a tremendous differential in the proliferation rates of normal and tumor endothelial cells. The average ratio found for these rates
was fifty—huge in biological terms—and extremes were found to a factor of one thousand. This discovery led to the idea that the rapidly proliferating tumor vasculature could be selectively targeted by cytotoxic agents. One year later, the concept was furthered when it was shown that artificial occlusion of tumor vessels through clamping leads to cell death and the possibility of cure after long periods of ischemia. Based on these findings, Denekamp continued to explore possible methods of exploiting tumor vascular through selective targeting and gained the attention of many other scientists worldwide, who have since contributed to advances in VDAs.

Vascular disruption offers many advantages in cancer treatment. A single vessel supplies thousands of tumor cells with oxygen, nutrients, and waste removal. Thus, damage to only a few endothelial cells that produces occlusion of a vessel could theoretically cause tremendous cell death. Because targeted cells lie in extreme proximity to the bloodstream, drug delivery problems are not an issue. In addition, mutations resulting in treatment resistance are unlikely to arise because endothelial cells are non-transformed.4,6,17

**Types of VDAs**

Two types of VDAs are currently being explored by researchers: biologics or ligand-based agents, which rely on biological agents to deliver effector molecules, and small molecule agents, which exploit differences between normal and tumor vasculature.13,18

**Biologics.** The first group relies on markers on endothelial cells that can be selectively targeted, such as VEGF receptors, cell surface antigens, integrin receptors, and vascular cell adhesion molecules—all of which are upregulated in angiogenesis.6 These VDAs are designed with a targeting portion, usually an antibody, peptide, or growth factor, that will selectively bind to a marker on the endothelium. A toxin, procoagulant or proapoptotic agent is also attached, to be delivered to the endothelium when the targeting portion selectively binds. These agents work by causing vessel occlusion directly or by affecting changes in endothelial cell shape.17,18

**Small molecule.** Small molecule VDAs are further divided into two classes: flavonoids and tubulin-binding agents. Flavonoids are compounds with two aromatic rings linked by a
carbon chain that are designed to induce the synthesis of tumor necrosis factor-alpha (TNF-\(\alpha\)) within tumor tissue.\(^{13}\) TNF-\(\alpha\) initiates necrosis and hemorrhage in tumors due to its vascular disrupting capabilities but is too cytotoxic to be used as a VDA as it is not selective to the vasculature of tumors. In fact, the effective dose of TNF-\(\alpha\) in animals is nearly ten times higher than its maximum tolerated dose (MTD) in humans.\(^{4,17,18,19}\) Flavonoids such as flavone acetic acid (FAA) and dimethylxanthenone acetic acid (DMXAA) seek to increase the selectivity of TNF-\(\alpha\) by stimulating its release locally.\(^4\)

Interestingly, while FAA was shown to induce TNF-\(\alpha\) production in murine cells, only DMXAA has been shown to induce TNF-\(\alpha\) production in human cell lines. DMXAA is restricted by a narrow therapeutic margin due to toxicity, likely caused by TNF-\(\alpha\) escaping into general circulation.\(^{18}\)

Tubulin binding agents, the second class of small molecule VDAs, are inhibitors of tubulin polymerization.\(^4\) Tubulin is the basic component of microtubules, essential cytoskeletal assembled filaments found in all eukaryotes. Microtubules affect several cellular factors, including division and cell shape, and are governed by a dynamic instability of growing and shrinking factors, mediated by the binding and hydrolysis of GTP, a high energy compound.\(^{19}\) Tubulin-binding agents that disrupt the dynamic instability of microtubules can induce anti-mitotic effects and changes in cell shape.

Interest in tubulin-binding factors began in the 1930s when Boyland and Boyland observed hemorrhage in a tumor injected with colchicine and a bacterial filtrate. Soon after, Ludford clearly
demonstrated that colchicine selectively damages newly formed tumor vessels, inducing tumor necrosis.\textsuperscript{4}

\[
\text{Colchicine}
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The activity of colchicine was further explored in many experimental systems, but significant effects were always only achieved at doses approaching the MTD. This apparent dead end led scientists to focus on other newly emerging anti-cancer therapies. Tubulin-binding agents were not explored in depth again until the 1980s and 1990s with studies examining vincristine and vinblastine. Once again, significant damage to tumor vasculature was only achieved at doses near the MTD.\textsuperscript{4}

The limitation of the narrow therapeutic window was overcome with the discovery of a new generation of tubulin-binding agents that selectively induce tumor vascular shutdown at doses less than one-tenth the MTD.\textsuperscript{17} These drugs are much less cytotoxic than their predecessors because they affect vascular shutdown not by anti-mitotic activity but rather by the occluding effects of endothelial cell shape change. The forerunners in this class are CA-4P and ZD6126, with many more compounds nearing clinical trials.

\textit{Combretastatin A-4P.} In 1979, Robert Pettit and co-workers isolated combretastatin from the \textit{Combretum caffrum} tree in South Africa.\textsuperscript{21} From this natural product, several stilbenes were isolated, including combretastatin A-4 (CA-4), which was found to be a particularly potent tubulin-binding inhibitor.\textsuperscript{22} Similar in structure to colchicine, the combretastatins are comprised of two phenyl rings, linked by a carbon bridge, and modified by several methoxy groups.\textsuperscript{19} CA-4P, a prodrug of CA-4, was developed in the mid-nineties to combat water solubility problems. CA-4P is activated when it is dephosphorylated to CA-4, and then binds at or near the colchicine binding site of \(\beta\)-tubulin, inhibiting tubulin polymerization.\textsuperscript{17,19}
Although CA-4P is shown to disrupt mitosis by destabilizing the tubulin cytoskeleton and mitotic spindle, these results take nearly two hours of exposure to the drug and therefore cannot account for the rapid vascular shutdown seen upon administration of CA-4P. Rather, within minutes of exposure, tumor endothelial cells in contact with the drug begin to change shape, leading to a reduction in capillary blood flow, and resulting in tumor cell necrosis and hemorrhage. Abnormalities of the tumor vasculature previously discussed such as high interstitial pressure, variable flow rates, and the tortuous nature of vessels are likely to contribute to the ability of CA-4P to disrupt blood flow. Interestingly, quiescent endothelial cells are relatively unaffected by exposure to the drug. It is postulated that these mature endothelial cells are less sensitive because they possess a highly developed actin cytoskeleton, which resists shape change even after the depolymerization of the tubulin cytoskeleton.

ZD6126. ZD6126 is a colchicine derivative and a prodrug of N-acetylcolchinol, which contains a benzenoid ring in place of a tropolone ring in colchicine. It was designed to achieve potent vascular disrupting effects with minimal toxicity by binding to tubulin and then rapidly eliminating itself from the plasma. Its
mechanism of action is believed to be similar to that of combretastatin and is mainly achieved by an anti-vascular rather than an anti-mitotic effect, enabling the achievement of significant effects on tumor vasculature at 1/30th the MTD.\textsuperscript{23,24}

A recent study provided direct visualization through electron microscopy of the effects of ZD6126 on endothelial cells in the first thirty minutes after exposure.\textsuperscript{24} Specifically, exposure of the basement membrane was seen due to decreasing endothelial cell cytoplasm, indicative of changes in the tubulin cytoskeleton and resulting in endothelial cell shape change within vessels. A pattern of tumor cell death was visualized with cells distant from vessels dying earlier than tumor cells in close proximity to vessels, providing evidence for a mechanism of action due to vascular occlusion rather than cytotoxicity. Within twenty-four hours, the entire tumor became necrotic, with the exception of an outer viable rim. This occurred in all cases with ZD6126 and has also been noted with CA-4P and with ligand-based molecules. It is hypothesized that the outer rim remains viable because it can be supported by the vasculature of adjacent, normal tissues. After treatment ceases, the outer rim proliferates and regenerates the tumor.\textsuperscript{24}

**Combating the Viable Rim**

The viable outer rim has become an obstacle of sorts in vascular disrupting therapy. However, there is hope that it can be overcome by employing combination therapies. The viable outer rim necessitates that VDAs be combined with either radiotherapy or a cytotoxic agent to achieve lasting tumor cell death. This section will focus on bioreductive agents and their potential effects in combination with VDAs.
Bioreductive Agents

Bioreductive drugs form cytotoxic agents capable of causing tumor cell death upon reduction. These drugs are tumor specific because normal tissues are well-oxygenated and lack the hypoxic environment necessary to trigger the activation of the cytotoxic agent. VDAs actually improve the effectiveness of bioreductive agents by increasing the degree and pervasiveness of tumor hypoxia. The activation of the cytotoxic agent is contained in the tumor environment by the production of a transient intermediate, which is susceptible to efficient back-oxidation to the prodrug in the presence of molecular oxygen.

Tirapazamine and RB6145. Tirapazamine is currently the most effective bioreductive drug available. It is reduced by NAD(P)H: cytochrome P450 reductase to form an intermediate oxidizing radical, which induces the formation of DNA radicals, particularly at C4'. The DNA radicals are then converted, in the presence of oxygen, or Tirapazamine itself in the case of hypoxic environments, into DNA strand breaks and base damage. RB6145 is the prodrug of another potent bioreductive agent, RSU 1069. Reduction of the nitro group of the latter yields an alkylating agent capable of creating both mono-adducts and crosslinks in DNA. Clearly, bioreductives possess a cytotoxic property that could be harnessed to attack the viable rim.

Current Research

The goal of this study is to design and synthesize a novel VDA which incorporates the pharmacophores of colchicine and CA-4P and a bioreductive agent capable of combating the outer viable rim. The rationale for the novel VDA involves the
properties of the *cis* and *trans* isomers of CA-4P. Although the *trans* form is most abundant, only the *cis* isomer is active in tubulin-binding. This problem is eliminated by replacing the double-bonded carbon bridge between the phenyl rings with a seven-membered ring, closely resembling that of colchicine. This structural change also keeps the second phenyl ring from spinning on the carbon bond linking it to the bridge. In addition, it is hypothesized that structural changes to CA-4P to more closely resemble colchicine will increase its tubulin-binding activity. After the target molecules are synthesized, a bioreductive agent will be attached via the hydroxyl group of each molecule.

![Colchicine](image1.png)  ![CA-4P](image2.png)

**Target Molecules:**
Researchers and clinicians have made tremendous progress in their understanding of tumor pathophysiology in the last century, and they continue to develop strategies with which to exploit the inherent weaknesses of tumors. Cancer has been cured many times thus far on paper, but the clinic is often a far worse critic than the laboratory bench. A handful of VDAs, including CA-4P, are currently involved in clinical trials, either alone or in combination with radiation or chemotherapy. Several years likely remain, however, before such drugs will be widely approved. One can hope and expect that the next ten to twenty years will bring tremendous progress in the treatment of cancer as advancements in VDAs and bioreductive drugs continue.

**GLOSSARY**

*angiogenesis*—the generation of a network of blood vessels to supply oxygen and nutrients and to remove wastes

*angiogenic switch*—the physiological point at which angiogenesis is turned on, triggered by factors in the tumor environment

*anti-mitotic effects*—inhibiting or interfering with the process of cellular replication (mitosis)

*basement membrane*—surrounds and provides an anchor for endothelial cells within a vessel; made of non-cellular material such as proteins

*cis isomer*—describes a chemical structure with hydrogens on the same side of the double bond

*cytotoxic agents*—drugs or other chemicals that kill cells

*endothelial cells*—cells which line hollow cavities within the body such as blood vessels

*hypoxia*—an oxygen-lacking state
pericytes—cells found within capillaries and venules in close association with endothelial cells; they provide stabilization for the vessel and play a role in producing the basement membrane

trans isomer—describes a chemical structure with hydrogens on opposite sides of the double bond

vasculature—network of arteries, arterioles, capillaries, venules, and veins which supply oxygen, nutrients, and a means of waste disposal to tissues or, abnormally, to tumor cells

REFERENCES


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