Chapter 2
Identification and Development of Vascular Disrupting Agents: Natural Products That Interfere with Tumor Growth

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Abstract Despite rapid progress made in understanding the biological mechanism regulating developmental and tumor vasculature, the clinical success of targeted therapeutics that interfere with key molecular and cellular pathways of angiogenesis has been limited by a lack of improved patient survival when these agents were administered as single agents. These findings indicate a redundancy of mechanisms regulating blood vessel formation, leading to tumor refractoriness to anti-angiogenic treatment. In contrast, vascular disruptive agents (VDAs) induced rapid regression of established tumors in preclinical studies. For several classes of VDAs, including the natural products flavonoids, colchicines and vincas, it was demonstrated that the effects on the vascular compartment diverge from that on the tumor parenchyma and are both anti-angiogenic (vascular stasis/prevention) and vascular disrupting (vascular regression)—resulting in catastrophic insults to the nascent vasculature. Vascular endothelial cells (ECs) are exquisitely dependent on the tubulin cytoskeleton for essential functions, including invasion, proliferation, migration, tube formation, and cell signaling—hallmarks of tumor angiogenesis. However, the clinical development of VDAs was frequently hampered by their narrow therapeutic index, limiting the use of these compounds due to side effects in the normal vasculature and/or other normal tissues. In this chapter we review the progress made to identify VDAs in

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oncology, with focus on preclinical and clinical studies. The identification of novel natural products and therapeutic compounds with improved potency and safety characteristics continue to keep great promise to overcome the limitations of first generation VDAs.

2.1 Introduction

2.1.1 Identification of Natural Products Targeting Tumor Vasculature

The prerequisite for the success of anti-angiogenic paradigm is the identification of pathophysiological differences between ECs of normal vasculature and tumor vessels, which can be harnessed therapeutically. A large variety of molecular and cellular differences between normal and tumor vasculature have been described, including the remodeling of tumor vessels, their reliance on a tubulin cytoskeletal network for functional integrity, the lack of pericytes, and increased vascular permeability in tumor vs. normal vasculature (Pluda 1997; Darland and D'Amore 1999; Jain 2003, 2005; Neri and Bicknell 2005; Carmeliet and Jain 2011a, b). Tumor associated ECs proliferate faster compared to ECs within the vasculature of normal tissues, which display an average doubling time of several months (Denekamp 1982; Denekamp and Hobson 1982). Natural products targeting tubulin formation display various degrees of selectivity for tumor vasculature. Among the most promising tubulin interfering agents are derivatives of combretastatins, colchicines, and dolastatin-10 (Tozer et al. 2005).

In addition to the increased proliferation of ECs in the tumor vasculature, a variety of changes between normal, resting vasculature and tumor vasculature, were reported. Tumor vasculature is abnormal in a variety of structural and functional aspects. Blood vessels within tumors are heterogeneous, tortuous, with irregular branching patterns and vascular lumens. There is increased interstitial fluid pressure within tumor due to the leakiness of tumor vessels and escaping fluid (Jain 2005). As a consequence, blood flow is heterogeneous and blood cells, hematopoietic cells and nutrients are distributed unevenly, causing a milieu of stress, including hypoxia, hypoglycemia, and low pH. In summary, the magnitude and the variety of the molecular and cellular vascular changes in tumor vs. normal tissues provide a wealth of opportunities for therapeutic interference, including the identification of compounds targeting metabolic and cell signaling pathways or structural components such as tubulin. Ideally, novel therapeutic compounds selectively interfere with mechanisms critical for the development of pathologic tumor vasculature, but not with the maintenance of established, quiescent vasculature in normal tissues. Harnessing the differences between contact inhibited, resting ECs and tumor associated, rapidly proliferating ECs, yields great promise to identify a new generation of natural products interfering selectively with tumor vasculature.

2.1.2   Pharmacological Advantages of Compounds Targeting Tumor Vasculature

The recent FDA approvals of targeted therapeutics, or vascular targeting agents (VTAs) interfering with vascular endothelial growth factor (VEGF) induced angiogenesis for the treatment of solid tumors has validated the anti-angiogenic approach as a viable therapeutic strategy for the treatment of solid tumors (Ferrara et al. 2007). Given these clinical successes, the focus of future clinical and preclinical oncology research is likely to include vascular targeting strategies. One of the key advantages of VTAs, including VDAs, is that they do not depend on penetration of the many cellular and membrane layers within a tumor mass to exert their pharmacological effects. Vascular ECs are in direct contact with the circulatory system and therapeutic agents, thus the frequently described limited tumor perfusion of high-molecular-weight compounds into the tumor mass does not limit the exposure to VTAs to their respective targets. Such favorable exposure/efficacy relationship of VTAs may ultimately provide an important advantage for drug development by potentially limiting undesired on- and/or off-target toxicities. In addition, the vascular targeting approach should be applicable to most solid tumor types, based on their common dependence on angiogenesis for tumor progression (Folkman 1985).

2.2   Flavonoids

2.2.1   Identification and Mechanisms of Action

Flavonoids, which belong to a large family of polyphenolic compounds, are compromised of more than 5,000 compounds that can be classified into ten chemical groups. These compounds are found in diverse botanical sources, including a variety of fruits and vegetables, as well as green tea and red wine. They have been reported to exhibit a variety of pharmacological properties including inhibition of tumor cell proliferation and anti-angiogenic activities (Wahl et al. 2011).

Early in the developmental program of the National Cancer Institute (NCI), more than 200 flavonoids were evaluated for their antitumor activities in vivo. At the time none of these compounds demonstrated activity. However, the plant-derived flavonoid quercetin was eventually rescreened in 1971 using different in vivo models and demonstrated moderate antitumor activity (Folkman 1985) (Fig. 2.1). The results from these observations led the NCI to screen a series of flavones from Lyonnaise Industrielle Pharmaceutique (Venditti et al. 1984). Flavone acetic acid (FAA) ester (LM985) emerged as a lead compound from this screen after demonstrating modest antitumor activity (Plowman et al. 1986) (Fig. 2.1). LM985 was selected for a Phase I clinical trial in the UK, however it did not progress due to the negative results of the Phase I data. It was reported during this time that LM985 appeared to act as a prodrug for FAA, the compound which was thought to be responsible for the antitumor activity in mice (Double et al. 1986; Bibby et al. 1987) (Fig. 2.1).
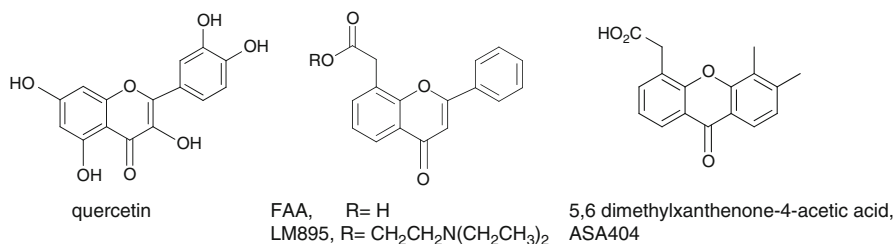
Fig. 2.1 Flavonoids

Evidence from follow-up studies suggested FAA inhibited tumor growth by reducing blood flow to the tumor as a result of vascular disruption (Bibby et al. 1989; Zwi et al. 1989) without effecting systemic blood flow (Bibby and Double 1993). FAA induced the release of cytokines from inflammatory cells, including tumor necrosis factor (TNF), and these factors were proposed to play an important role in the antitumor activity of FAA (Chabot et al. 1993). Further data suggested that FAA-mediated nitric oxide (NO) production within the tumor also contributed to the observed cytotoxic activity (Thomsen et al. 1990, 1991, 1992; Harris and Thorgeirsson 1997).

2.2.2 *Clinical Development of Flavonoids as Vascular Disrupting Agents*

A number of unsuccessful Phase I and II clinical trials were carried out with FAA (Havlin et al. 1991; Pratt et al. 1991; Siegenthaler et al. 1992). The negative results led to a search for more active drugs with structures related to FAA. This effort led to the identification of a tricyclic analog xanthenone-4-acetic acid (XAA), and eventual synthesis of the highly potent XAA derivative 5,6-dimethylxanthenone-4-acetic acid (DMXAA, ASA404), which displayed a significant increase in activity as compared to FAA (Rewcastle et al. 1989) (Fig. 2.1).

While the precise mechanism of action of ASA404 is largely unknown, the results of preclinical studies suggest ASA404-mediated vascular disruption may occur by direct and indirect anti-vascular activities. Experimental data from murine studies have reported a number of responses following ASA404 administration, namely, increased tumor-vascular permeability (Zhao et al. 2005; Chung et al. 2008), increased tumor endothelial cell apoptosis (Ching et al. 2002, 2004), and decreased tumor blood flow (Zwi et al. 1994; Lash et al. 1998). Similar to what was observed with FAA, evidence suggests vascular disruption may be occurring by increasing intra-tumoral concentrations of TNF and NO following administration (Joseph et al. 1999; Ching et al. 2002). TNF appears to be a major

contributor to the induction of vascular collapse. This was demonstrated by a significant reduction in anti-vascular activity in TNF or TNF-receptor (TNFR) knockout mice following treatment with ASA404 (Ching et al. 1999; Zhao et al. 2002). Likewise, induction of NO after administration of ASA404 is considered an important factor in mediating increased vascular permeability and inhibition of blood flow (Thomsen et al. 1990). In response to tumor hypoxia resulting from the disruption of tumor blood flow ASA404 has also been reported to increase VEGF production (Baguley and Siemann 2010). TNF has been shown to enhance VEGF-mediated endothelial cell permeability (Clauss et al. 2001), therefore the activity of ASA404 may involve the combined effects of all these factors. The effects of ASA404 culminate in the breakdown of vasculature and hemorrhagic tumor necrosis. ASA404 has also shown to have either additive or synergistic antitumor effects when combined with several cytotoxic chemotherapeutic agents (Siim et al. 2003). The preclinical demonstration of ASA404 activity in combination with taxanes and platinum-based anticancer drugs provided a rationale for combination clinical trials. Three Phase II trials were designed for patients with non-small cell lung cancer NSCLC, ovarian cancer, and prostate cancer.

In the Phase II trial of carboplatin and paclitaxel with or without ASA404 conducted in patients with NSCLC, ASA404 plus chemotherapy appeared to improve efficacy over chemotherapy alone in terms of overall response rate (ORR), median time to progression (TTP), and median overall survival (OS) (McKeage et al. 2008). To further verify those results a single-arm phase II trial of patients with advanced NSCLC was performed to evaluate ASA404 at a higher dose, again in combination with carboplatin and paclitaxel. Again efficacy appeared to be improved with ASA404 in ORR, TTP, and OS. However, these increases did not reach statistical significance (McKeage et al. 2009). Both Phase II trials designed for patients with ovarian cancer and prostate cancer demonstrated increased response rates when treatment was combined with ASA404, but did not prolong survival (Pili et al. 2010).

The Phase II results led to the randomized, double blind, placebo-controlled Phase III trials in advanced NSCLC, ATTRACT-1 (Antivascular Targeted Therapy: Researching ASA404 in Cancer Treatment) as first-line therapy, and ATTRACT-2 as second-line therapy. ATTRACT-1 included patients with stage IIIB/IV NSCLC who had not previously received systemic therapy for metastatic disease. These patients were treated with ASA404 plus carboplatin and paclitaxel vs. placebo plus carboplatin and paclitaxel (Lara et al. 2011).

In 2010 independent data monitoring committees reviewed the interim results from the ATTRACT-1 and ATTRACT-2 Phase III trials. The committees recommended halting both trials when interim data analyses demonstrated ASA404 when used in combination with chemotherapy would not likely meet their primary endpoints of significantly extending overall survival (Lara et al. 2011) and the pharmaceutical company leading this effort announced that it would discontinue further development of the tumor-vascular disrupting agent ASA404.

2.3   Colchicines

2.3.1   *Colchicines as Vascular Disrupting Agents*

Of the three major classes of microtubule-binding agents, those that bind to the colchicine binding site are probably the least identified as traditional anticancer therapies; however, the colchicines may have the most promise as VDAs—resulting in severe interruption of tumor blood flow and necrosis to the tumor cell compartment. As a class, colchicinoid microtubule-destabilizing agents are structurally related to the well-studied tubulin binding agent colchicine and bind to the colchicine binding site of tubulin, located at the interface between α and β subunits of the tubulin dimer, thereby inhibiting microtubule polymerization. So well is the colchicine site characterized, that previously tubulin was referred to as a "colchicine binding protein." Members of this class have been coined as mitotic or spindle poisons due to their rapid and deleterious effects on the cytoskeleton. A significant advantage of colchicinoid VDAs, is dual targeting of both the stromal and parenchymal populations within a given tumor microenvironment, due to the concomitant EC and tumor cell sensitivity to this class. The downstream effects of colchicinoids occur just a few minutes after binding, starting with disruption in blood flow and intravascular thrombosis, followed by direct assault on the ECs (Dark et al. 1997). Events after drug administration include morphological changes and disruption of interphase microtubules, cytoskeleton collapse, mitotic catastrophe—EC death, subsequent vascular injury, platelet activation, thrombosis, vascular blockage, plasma protein leakage into interstitial space, vasoconstriction, neutrophil recruitment, reduction in blood flow, avascularization, and hemorrhagic necrosis (Parkins et al. 2000; Galbraith et al. 2001; Tozer et al. 2001; Kanthou and Tozer 2002; Prise et al. 2002; Brooks et al. 2003).

The ancient natural product colchicine, for which the class is named, is one of the oldest known microtubule inhibitors and was noted for its spindle poisoning properties by Pernice, a Sicilian pathologist in 1889 (Stafford et al. 2005) (Fig. 2.2). Colchicine is a highly soluble tricyclic alkaloid isolated from *Colchicum autumnale*, also known as autumn crocus or meadow saffron. Colchicine has long been demonstrated to have anti-vascular effects (Ludford 1948; Baguley et al. 1991), and has recently been demonstrated to be more precisely anti-angiogenic in an endothelial sprouting assay; however, significant inhibitory effect blocking the initiation of angiogenesis was only seen at higher concentrations of colchicine, whereas lower, clinically more relevant dose levels were ineffective (Stafford et al. 2005). Therefore these data and others indicate that, although colchicine induces vascular damage, it is only at doses that are limited by toxicity—in fact, there is no clear-cut distinction between nontoxic, toxic and lethal doses (Finkelstein et al. 2010). As a result, colchicine is FDA approved exclusively for its anti-inflammatory activities in the prevention and treatment of gout and familial Mediterranean fever. Notwithstanding, several groups have shown that at least some of the anti-inflammatory properties of colchicine
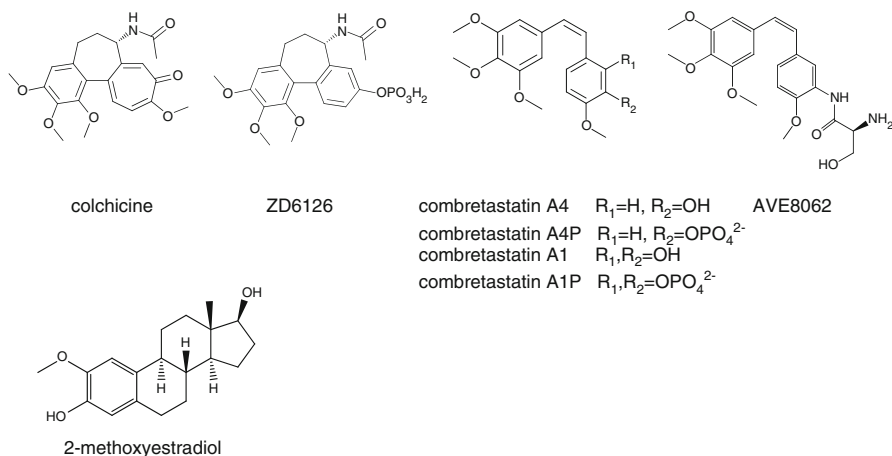
Fig. 2.2 Colchicines and 2-methoxyestradiol

are due to the regulation of adhesion molecules, such as selectins and VCAM, on the EC surface, preventing neutrophil rolling, attachment, and extravasation (Cronstein et al. 1995; Asahina et al. 2001). Interestingly, a prodrug of an N-acetylcolchinol, related to colchicine, ZD6126 (also known as ANG453) (Fig. 2.2), has been in clinical trials as a treatment for cancer as a VDA resulting in vascular occlusion and tumor necrosis. ZD6126 had shown promising results in vitro and in vivo in tumor-bearing mice although due to dosing concerns and limited dose related cardio-cytotoxicity in human patients further development was halted (Micheletti et al. 2003; LoRusso et al. 2008). Notably, cardiotoxicity related to tubulin inhibitors has been demonstrated to be a direct effect on myocardial ECs (Mikaelian et al. 2010).

Combretastatins, a class of natural stilbenoid phenols, were the first microtubule-binding agents identified to have vascular disrupting properties and are the leading agents in the colchicinoid class, if not the entire category of VDAs. They are competitive inhibitors of the binding of colchicine to tubulin (Lin et al. 1988). In particular, combretastatin A-4 (CA4) was first isolated from the bark of the South African bush willow, *Combretum caffrum* (Pettit et al. 1987, 1989) (Fig. 2.2). Indeed, over a dozen combretastatins have been isolated and reported from *C. caffrum* and related plants. CA4 displayed potent cytotoxic activities against a wide range of human cancer cell lines, including multidrug resistant (MDR) cancer cell lines, with IC_{50} values consistently in low nanomolar to subnanomolar range. CA4 is not recognized by P-glycoprotein, often cited as responsible for MDR, and therefore CA4 is less apt to result in drug resistance (Atalay et al. 2006). Besides their effects on tumor cells themselves, CA4 and derivatives target ECs and cause disruption of the endothelial cytoskeleton by binding to the colchicine binding site of the β- subunit of tubulin, thereby inhibiting the polymerization of tubulin to microtubules. CA4 has been demonstrated to be more potent, anti-proliferative and pro-

cytotoxic to ECs than would be expected for a tubulin inhibitor and at doses significantly lower than the maximum tolerated dose (MTD) in mice. In fact, in vitro studies using human umbilical vein endothelial cells (HUVEC) by several groups demonstrated that CA4 is cytotoxic if the cells are proliferating, resulting in cell rounding, retraction, membrane blebbing, and substratum detachment, but not if they are quiescent (Dark et al. 1997; Galbraith et al. 2001). CA4 has also been shown to inhibit active angiogenesis in a HUVEC tube formation assay (Grosios et al. 1999). The perhaps unexpected safety profile in mice may be due to the resistance of quiescent endothelia to the effects of combretastatin—likely due to the discriminating dependence of nascent endothelia on the tubulin cytoskeleton (Galbraith et al. 2001; Young and Chaplin 2004). In support of this, a major change that occurs after EC exposure to CA4 is an alteration in shape and morphology, which is profoundly less than that which occurs in quiescent ECs, offering combretastatin, and tubulin inhibitors with similar mechanism of action, an exquisite level of selectivity for ECs in the pathological milieu (Galbraith et al. 2001).

Significant selectivity of colchicinoid compounds for vascular targeting and disruption has been demonstrated in preclinical and clinical studies. Of the combretastatin derivatives, CA4P a simple water soluble prodrug derivative (active when dephosphorylated to CA4 which can then bind tubulin), was the first and perhaps most extensively to be studied (Banerjee et al. 2008) (Fig. 2.2). CA4P induces microtubule disruption by targeting neovascular ECs in the highly proliferative tumor environment which denude from the vessel wall, thereby disrupting tumor blood flow and increasing vascular permeability, resulting in central hemorrhagic necrosis (Galbraith et al. 2001; Tozer et al. 2001). CA4P effects in vivo are rapid and severe, resulting in an acute reduction in tumor blood flow within minutes of drug administration. Significantly, the precise mechanism of action has been defined, in that CA4P interrupts VE-cadherin signaling and induces the regression of susceptible emerging tumor neovessels thereby selectively disrupting tumor vasculature. CA4P actions on VE-cadherin and associated beta-catenin/AKT signaling pathways, critical for endothelial cell survival, results in increased vascular permeability and endothelial cell death (Vincent et al. 2005). However, reduction in tumor growth is not attainable with a single dose and requires repeated administration of the combretastatin over time. Notably synergism is attainable by combination treatments with anti-VE-cadherin neutralizing antibodies (Vincent et al. 2005).

Although CA4P was shown in Phase I studies to selectively reduce tumor blood flow at well-tolerated doses, it unfortunately does not result in tumor regressions or substantial tumor growth delay as a single agent—so more selective targeting, combination therapies (if toxicities can be mediated), and/or improved colchicinoids may be required (Banerjee et al. 2008). In fact, CA4P was recently shown to improve overall survival in a phase II/III trial in anaplastic thyroid cancer in combination with paclitaxel and carboplatin. Currently, CA4P is in studies for NSCLC and platinum resistant ovarian carcinoma, and in combination with anti-VEGF bevacizumab for persistent ovarian epithelial, fallopian tube, or primary peritoneal carcinoma.

This is particularly interesting as combining VTAs with already established angiogenesis inhibitory agents may provide the one-two punch necessary to prevent tumor regrowth.

Naturally found congeners of CA4 include combretastatin A-1 (CA1), a simple hydroxyl derivative of CA4 which binds to the colchicine binding site at high affinity (Fig. 2.2). The diphosphate prodrug of CA1, referred to as CA1P or Oxi4503, is currently in clinical development (Fig. 2.2). Preclinical studies with CA1P in mice demonstrated profound damage to tumor microvasculature with minimal uninvolved organ vascular bed damage, however complete tumor eradication was not achieved—suggesting that combination therapies with other chemotherapeutic modalities might achieve complete tumor eradication (Chan et al. 2007). CA1P is currently in Phase I clinical trials for relapsed and refractory acute myelogenous leukemia (AML) and myelodysplastic syndrome (MDS). Furthermore, several synthesized derivatives of combretastatin have been pursued and are in advanced clinical development. In particular the combretastatin-serine AVE8062 is currently being evaluated in clinical cancer trials (Fig. 2.2). AVE8062 is a water soluble synthetic combretastatin analog with nanomolar potency inducing G2/M arrest and apotosis in mouse ECs in vitro and in vivo, which is enhanced by combination therapy with docetaxel (Kim et al. 2007). Phase I and II studies of CA1P and CA4P (and several other colchinoids—both natural products and synthetics) reported cardiovascular toxicities. Cardiac events were dose-limiting in phase I trials with VDA monotherapy and combination therapy (Subbiah et al. 2011). These studies suggest that monitoring of cardiac biomarkers could play a valuable role in future trials of VDAs.

In 1994, Judah Folkman's laboratory demonstrated that a previously identified anti-angiogenic metabolite of estradiol, 2-methoxyestradiol (2MeO-E2, Panzem) (Fig. 2.2), inhibited angiogenesis in the chick chorioallantoic membrane (CAM) via binding to the colchicine site of tubulin (D'Amato et al. 1994). 2-MeO-E2's anti-angiogenic activity lies within its ability to reduce endothelial cell proliferation, induce EC apoptosis, and reduce the transcription and expression, nuclear accumulation, and transcriptional activity of HIF-1α—thereby demonstrating that 2MeO-E2 is a true anti-neoangiogenic compound (Mabjeesh et al. 2003; Verenich and Gerk 2010). This agent also inhibits tumor cell growth by binding to tubulin, resulting in antimitotic activity, and by inducing caspase activation, resulting in cell cycle arrest in the G2 phase, DNA fragmentation, and apoptosis. Phase I and II clinical trials revealed that orally administered 2MeO-E2 is well tolerated by patients with only grade 2 and 3 toxicities observed (Verenich and Gerk 2010). However, 2MeO-E2 faces many challenges due to poor solubility, no MTD determined in early clinical studies, only nanogram per milliliter levels detected in plasma due to presystemic metabolism despite large doses, and in vitro cytotoxicity effects on tumor cells in the micromolar to submicromolar range (Verenich and Gerk 2010).

In addition to toxicities outlined above, one of the main concerns with using colchinoids/combretastatins for vascular targeting as single agents is the potential for reactive angiogenesis from the residual viable rim left after the central necrosis

events have taken place (Horsman and Siemann 2006). There are two working non-mutually exclusive hypotheses for what may contribute to the remaining viable rim after colchinoid/combretastatin treatment: (1) functional and structural tumor endothelial heterogeneity (interstitial pressure, vessel caliber, vascular compensation) resulting in drug susceptibility of the inner vasculature vs. outer vasculature; (2) sustenance of the outer rim tumor cells from the surrounding normal vasculature, largely resistant to VDAs (Tozer et al. 2005). One could argue that neither colchinoids nor any tubulin inhibitors were designed to be single therapy agents and are much more likely to be effective when administered in intermittent doses and in synergy with other therapeutics (Young and Chaplin 2004). To combat the reactive rim rebound angiogenesis, several groups suggested and evaluated the efficacy of combination therapies, including nonconventional therapies, to inhibit the regrowth of the tumor from the reactive angiogenic rim—including markedly successful combinations with cisplatin, cyclophosphamide, radiation and a host of other standards of care (Dark et al. 1997; Li et al. 1998; Chaplin et al. 1999; Horsman et al. 2000; Siemann et al. 2002; Young and Chaplin 2004; Madlambayan et al. 2010). The differential sensitivity for alternative mechanism of action chemotherapies and radiation vs. combretastatins allows for a unique opportunity for dual population targeting (central hypoxic tumor cells and tubulin inhibitor resistant tumor rim), resulting in additive or synergistic long-term outcomes. Notably, other groups have successfully used combretastatin-mediated vascular targeting to enhance the retention of therapeutic antibodies within the tumor microenvironment (Lankester et al. 2007). In addition, because therapeutic antibody targeting strategies often have greatest affect at the tumor rim due to tumor penetration, it makes sense to combine these strategies with VDAs, such as colchinoids. It is of note that vascular and tumoral heterogeneity can confound the response of the patient or preclinical animal to CA4P—not the least of which are tumor size, vascular perfusion, interstitial fluid pressure, and vascular permeability. Using biomarker and clinical strategies for appropriate drug administration should improve the long-term benefits of colchinoid class tubulin inhibitors in the clinic, resulting in personally designed dosing regimens with less toxicity and improved efficacy indices—ultimately saving patient lives.

2.4 *Vinca* Alkaloids and Peptide Tubulin Inhibitors as Vascular Disrupting Agents

Vinca alkaloids and peptide tubulin inhibitors are distinct classes of natural products which share similar function as microtubule depolymerizing agents. The earliest reported *Vinca* alkaloids were natural products derived from the periwinkle *Vinca rosea*, later renamed *Catharanthus roseus*. Vinblastine and later vincristine were the first natural alkaloids discovered from extracts of these plants (Kruczynski and Hill 2001) (Fig. 2.3). Medicinal chemistry efforts successfully generated multiple analogs via semisynthetic approaches, several of which underwent clinical evaluation, including vinorelbine, vindesine, and vinflunine (Fig. 2.3). The peptide tubulin
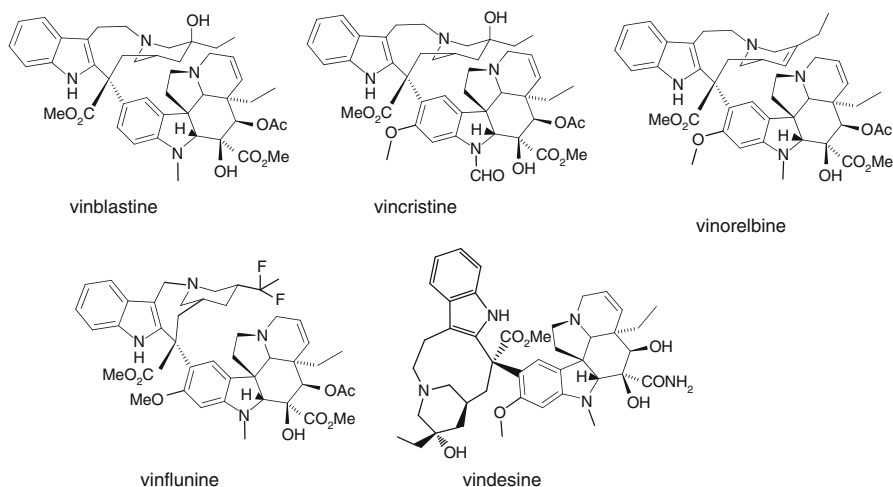
Fig. 2.3 Vinca alkaloids

inhibitors are a highly diverse class of natural products, most of which are composed of nonnatural amino acids. Representatives of this class include dolastatins, auristatins, hemiasterlins, cryptophycins, and others. Synthetic efforts have also produced multiple analogs with modified tubulin binding, cellular potency, and in vivo efficacy. *Vinca* alkaloids and peptide tubulin inhibitors share an overlapping binding site on β-tubulin that is distinct from the colchicine and taxane binding sites. In general, *Vincas* noncompetitively inhibit the binding of peptides such as dolastatin and analogs to tubulin (Bai et al. 1990). Both classes of tubulin inhibitors destabilize microtubules and shift tubulin into the soluble, heterodimeric form. During normal physiological processes, microtubules undergo treadmilling, the net addition of subunits at the growing "+" end of the microtubule and loss at the "−" end, and dynamic instability, the switching at microtubule ends between phases of slow and rapid growth. *Vincas* and peptides bind to microtubule ends and suppress both dynamic instability and treadmilling. Several members of both *Vinca* and peptide classes have demonstrated activity against both epithelial and endothelial cells. We highlight vinflunine and soblidotin, one member of each class with enhanced anti-vascular activity compared to their related analogs.

2.4.1 Vinflunine

In order to identify potentially more active *Vinca* analogs with an improved safety profile, chemists and biologists at the Pierre Fabre research group designed, synthesized, and analyzed novel *Vinca* analogs, ultimately advancing vinflunine (Kruczynski and Hill 2001) (Fig. 2.3). Vinflunine represents a modification of

vinorelbine at the 20′ position with the addition of two fluorine atoms and reduction of the 3′,4′ double bond in the catharanthine moiety. Despite the structural similarities among the four major *Vinca* analogs, there are notable differences in their tubulin binding and cellular properties. Data from multiple groups suggest that the binding of vinflunine to tubulin is actually weaker than other *Vincas*, on the order of vincristine > vinblastine > vinorelbine > vinflunine. Moreover, drug competition studies demonstrated that vinflunine only weakly inhibited the binding of radiolabeled vinblastine or vinorelbine and did not inhibit vincristine (Kruczynski et al. 1998). Vinflunine also showed unique effects on microtubule dynamics compared with vinblastine (Ngan et al. 2000). These distinct differences in vinflunine's interaction with tubulin compared with other *Vincas* may contribute to the reported antivascular effects of vinflunine at concentrations that are lower than cytotoxic doses.

Tubulin mediates cell division by forming the organized spindle network of microtubules that control chromosomal segregation into daughter cells. However, microtubules also play critical roles during interphase, including cytoskeletal structure, cell migration, adhesion site dynamics, and protein transport. These activities are particularly important during neovascularization. Therefore, drugs interfering with tubulin function have pleiotropic effects on cell physiology, in addition to the cytotoxicity promoted by mitotic catastrophe. ECs may be more sensitive to the non-mitotic effects of tubulin inhibitors due to their dependence on microtubules for a dynamic cytoskeleton, cell migration, and other functions.

The Braguer lab elegantly observed the effects of vinflunine on microtubule dynamics in human dermal microvascular endothelial cells (HMVEC), allowing direct assessment of vinflunine's properties in an endothelial cell system (Honore et al. 2008). In general, tubulin depolymerizing agents cause a decrease in the amount of stabilized or "paused" microtubules. Using time-lapse video microscopy, the number of microtubule pauses were measured in HMVEC and found to be approximately 50% in untreated cells. Vinflunine doses as low as 10 pM reduced microtubule pauses to ~20%, and also shortened the duration of the pauses. These effects were apparent between 0.01 and 2 nM. Therefore, at very low concentrations, even below the doses typically causing cytotoxicity, vinflunine destabilizes microtubules and prevents them from participating in normal interphase functions. A direct comparison among *Vincas* on microtubule dynamics conducted by another group confirmed that vinflunine neither reduces the rate of microtubule shortening nor the duration of the "pause" state, in contrast to vinblastine (Ngan et al. 2000). This reduced affinity of vinflunine with tubulin likely results in the observed 4–7-fold reduced microtubule treadmilling rates compared with vinorelbine and vinblastine.

In HMVEC, vinflunine also decreased the duration of microtubule end interaction with adhesion sites from 2.5 min in untreated cells to 38 min in the presence of 1 nM vinflunine (Honore et al. 2008). In addition, vinflunine altered actin dynamics by increasing the size and duration of stress fibers between focal adhesions. Microtubule-associated proteins such as EB1 typically mediate tubulin dynamics by binding to the growing tubule at the (+) end. Vinflunine at 1 nM eliminated the proper localization of transfected GFP-EB1 from microtubules in HMVEC. A direct

correlation was also observed between vinflunine concentrations that alter microtubule dynamics and the phenotype of cell migration. HMVEC speed was decreased from a normal rate of 0.4 to 0.2 µm/min in the presence of 0.01–1 nM vinflunine, doses which are commensurate with the destabilization of microtubules.

The anti-proliferative effects of vinflunine were compared with other *Vinca* analogs in a panel of tumor cell lines after 2 or 3 day treatment (Kruczynski et al. 1998). In general, vinflunine was 2–44-fold less potent than vinblastine, vincristine, or vinorelbine in eight cell lines, and >10- to >1,000-fold less potent in two cell lines. The Kruczynski lab later reported the effects of vinflunine on ECs (Kruczynski et al. 2006). One hour treatment with vinflunine caused the dissociation of newly formed endothelial cell tubes at doses of 100 nM and greater. Statistically significant inhibition of HUVEC migration was observed at 100 nM vinflunine. Much higher concentrations of vinflunine were required to induce endothelial cell death during acute exposure, ranging from 8 µM upon 4 h exposure and >100 µM upon 1 h exposure. Similar results were reported for HMVECs (Pourroy et al. 2006). Low concentrations of vinflunine increased microtubule dynamics in HMVEC and were sufficient to inhibit Matrigel tube formation and cell speed without altering mitotic spindle function. This suggests that vinflunine selectively affects interphase microtubule functions that are necessary for anti-vascular effects at concentrations that do not induce mitotic arrest or cytotoxicity.

The effects of vinflunine on tumor vasculature were assessed in the poorly differentiated and rapidly growing colon adenocarcinoma model, MAC15A (Holwell et al. 2001). At the single-administration maximally tolerated dose of 50 mg/kg, vinflunine caused >50% tumor growth inhibition 1 week following a single injection. Assessment of vascular damage in tumors was conducted by infusion with Hoechst dye following drug treatment. Microscopy revealed staining only of ECs and confirmed >50% vascular shutdown in tumors 4 h after treatment with vinflunine at 10–40 mg/kg. Approximately 80% inhibition of functional vasculature was evident 24 h after a single dose at concentrations below the MTD.

Vinflunine also inhibited growth factor induced angiogenesis when bFGF-embedded Matrigel plugs were implanted subcutaneously in mice (Kruczynski et al. 2006). At vinflunine doses of 1.25 mg/kg and greater, 2–3-fold decreases in hemoglobin content were observed in the plugs, suggesting impaired vascularization. In the same study, LS174T tumor cells were injected into mice spleens to assess the effect of vinflunine on experimentally induced metastasis. Vinflunine treatment reduced the number of liver metastases at 1.25 mg/kg, or 16-fold lower than the MTD. In contrast, colchicine or vinblastine produced similar effects only at doses near MTD. Vinflunine was also efficacious across multiple studies in xenografts derived from a variety of human tumor cell lines, including lung NCI-H69 and LX1, renal RXF944LX, colon TC37, prostate PC3, breast MX1, and pancreas PAXF546 (Hill et al. 1999).

Several clinical studies were conducted to evaluate the safety and anticancer efficacy of vinflunine. The predominate adverse events for vinflunine therapy across all trials were grade 3 or 4 neutropenia (approximately 50% of patients) and constipation, fatigue, and/or anemia (approximately 10–20%). As a result of an acceptable

safety profile in various phase I trials, vinflunine was advanced to proof-of-concept studies. Phase II trials were conducted in patients with multiple tumor types including renal cell carcinoma, ovarian cancer, and melanoma, with the most impressive responses observed in lung, breast, and bladder cancers (Yun-San Yip et al. 2008). In a phase II study conducted in 63 patients with NSCLC, an 8.3% partial response rate was observed and responses persisted for an average of 7 months. In another phase II study, metastatic breast cancer patients who failed taxane and anthracycline therapies showed a 30% ORR and 14.3 month median overall survival. Another study with 51 bladder cancer patients who previously failed platinum therapies showed 18% partial response rate and 67% disease control; 14% of patients previously treated with a vinblastine-containing cocktail responded (Culine et al. 2006). Overall, single-agent vinflunine showed higher response rates in clinical trials compared with trials evaluating paclitaxel, docetaxel, oxaliplatin, lapatinib, bortezomib, or pemetrexed (Yun-San Yip et al. 2008). Several trials were also conducted to evaluate combination of vinflunine with platins, gemcitabine, or capecitabine. Combination studies of vinflunine with anti-Her2 antibody trastuzumab produced relatively high 62.5% and 73.7% response rates, at 280 or 320 mg/m^2 vinflunine respectively.

A phase III trial was conducted in transitional cell carcinoma of the urinary tract (TCCU) to follow-up on the preliminary phase I and II activity in this subtype of bladder cancer (Bellmunt et al. 2009). A 2 month survival advantage was observed in patients treated with vinflunine plus best supportive care (6.9 months) vs. BSC alone (4.6 month) and a significant 23% improvement in overall survival. These data suggest that vinflunine can benefit patients for second-line treatment of TCCU.

Specific vascular endpoints do not appear to have been evaluated or reported systematically across the majority of clinical trials, so it is difficult to determine if anti-vascular activity is a major contributor of the clinical efficacy. Also, it remains unknown whether the reduced affinity of vinflunine for tubulin ultimately imparts an improved clinical profile compared with first generation *Vincas*. Interestingly, the tubulin binding properties of the *Vincas*, more so than the cytotoxicity potencies, corresponded to the doses utilized in the clinic. As noted above, tubulin binding affinities were vincristine > vinblastine > vinorelbine > vinflunine. In contrast, the cellular potency for vincristine, vinblastine, vinorelbine in a panel of tumor cell lines were within fivefold of each other, while vinflunine was typically >10-fold higher. Weekly dosing regimens for these drugs were approximately 0.4–1.4 mg/m^2 for vincristine, 4–20 for vinblastine, 25–35 for vinorelbine, and 280–320 mg/m^2 for vinflunine. These activity differences among the highly related *Vinca* alkaloids further emphasizes that minimal but selective structural changes in analogs can significantly alter binding to the therapeutic target and translate to relevant pharmacological differences. Chemotherapeutic agents are typically administered to patients at maximally tolerated doses. Hence, it may be difficult to tease out the subtle biological differences in tubulin binding and anti-vascular effects observed at lower concentrations for a drug like vinflunine. At high doses, cytotoxicity in normal proliferating cells, such as hematopoietic and gastrointestinal compartments, can reduce the therapeutic index. Rational and well-titrated combinations of novel anti-vascular agents plus debulking cytotoxics remain an important focus in oncology.
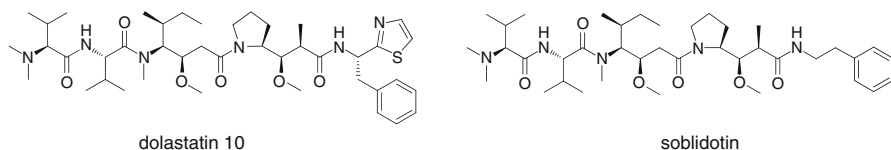
Fig. 2.4 Peptide tubulin inhibitors

2.4.2 Soblidotin

Soblidotin, also known as auristatin PE or TZT-1027, is an analog of dolastatin-10 (Kobayashi et al. 1997) (Fig. 2.4). Dolastatin-10 was originally extracted in 1987 from the Indian ocean mollusk *Dolabella auricularia.* Soblidotin demonstrated anti-vascular activity that was more pronounced than dolastatin and related anti-microtubule agents. Soblidotin inhibits purified tubulin in a cell-free system with an IC_{50} of approximately 2.2 µM, within range of other tubulin destabilizing agents. Soblidotin was found to bind to tubulin at both high ($K_d = 0.2$ nM) and low ($K_d = 10$ µM) affinity sites, and to non-competitively inhibit the binding of vinblastine from tubulin (Natsume et al. 2000). The anti-vascular activity of soblidotin was first observed as altered cell contraction and membrane blebbing in HUVEC upon acute exposure that was more pronounced than vincristine (Otani et al. 2000). Soblidotin induces mitotic arrest and apoptosis in cultured tumor and endothelial cells and is cytotoxic at subnanomolar concentrations. The effect of drug exposure time and cytotoxicity was reported for soblidotin and other anticancer agents in murine colon carcinoma C26 cells and HUVEC. Soblidotin was highly potent against both cell types, with an IC_{50} of 0.014 ng/mL against C26 after 3 day treatment; soblidotin was threefold more potent against HUVEC at approximately 0.0046 ng/mL (Watanabe et al. 2007). Interestingly, soblidotin retained ultra-potency after only 24 h treatment in HUVEC (0.098 ng/mL) while other chemotherapeutics were 10–100,000-fold less potent. Short-term exposure of all drugs to C26 cancer cells was ineffective ($IC_{50} > 1,000$ ng/mL). These findings were extended to the assessment of vascular permeability with HUVEC cells, where the ability of inhibitors to damage the junctions between ECs was assessed by using FITC-dextran (Watanabe et al. 2006, 2007). Soblidotin increased permeability by approximately 12-fold, compared with 9- and 4-fold changes for vinblastine and vincristine, respectively, and no effect for docetaxel, 5-fluorouracil, and cisplatin (Watanabe et al. 2007). These data suggest differential effects of soblidotin on ECs compared with other chemotherapeutics, including inhibitors of tubulin and DNA function.

The effect of soblidotin on HUVEC vascular tube formation was explored using cells plated on extracellular basement membrane (Watanabe et al. 2007). Drug added at the time of HUVEC plating impairs the development of newly formed tubules and when soblidotin was added 20 h after cell plating, it disrupted

existing tubules with an IC_{50} range of approximately 0.1–1 ng/mL. The effect of soblidotin on angiogenesis was assessed using a chick embryo CAM assay (Watanabe et al. 2007). After 2 days of exposure to soblidotin, >80% inhibition of neovascularization was observed. Transcriptional profiling was also conducted in a lung carcinoma cell line treated with various anti-tubulin agents, and the observed soblidotin gene expression pattern was distinct from that of vinblastine, vincristine, paclitaxel, and docetaxel (Shimoyama et al. 2006). Interestingly, soblidotin increased the expression of the MMP inhibitor, TIMP3, which is known to interfere with VEGF binding to its receptor. Proteomic or RNA profiling of soblidotin-treated ECs may provide additional support for the role of the peptide on anti-vascular activity.

The in vivo efficacy of soblidotin was evaluated in a VEGF-dependent model where human small cell carcinoma cell line SBC-3 was transfected with VEGF cDNA and implanted into nude mice (Natsume et al. 2003). SBC-3/VEGF tumors showed increased neovascularization and faster growth rate compared with control cells. Nearly complete regressions were observed against both control tumors and SBC-3/VEGF tumors treated with soblidotin. This activity was more pronounced than combretastatin-A4P, vincristine, docetaxel, or cisplatin at or near their maximally tolerated doses. Histological analyses showed enhanced erythrocyte accumulation, leakage, and scattering from tumor vasculature of SBC-3/VEGF cells compared with SBC-3/neo cells 3–48 h post-administration of soblidotin. The authors suggest that soblidotin preferentially induced thrombi, extravasation and necrosis in a VEGF-dependent manner. Combretastatin-A4P was ineffective in these models, suggesting that a combination of both cytotoxic and anti-vascular activity is required for maximal effect. Soblidotin also reduced tumor perfusion in C26 tumor-bearing mice, caused erythrocyte leakage, and potentiated vascular permeability induced by VEGF (Watanabe et al. 2006).

Multiple clinical trials have been conducted with soblidotin. Separate Phase I studies were performed in patients with various advanced solid tumors, as well as in a cohort of non-small cell lung cancer (NSCLC) (Schoffski et al. 2004; Greystoke et al. 2006; Tamura et al. 2007). In the NSCLC trial (Horti et al. 2008), 49 patients treated by dose-escalation had dose-limiting toxicities of neutropenia, fever, myalgia, and neuropathic pain. One complete response, 3 partial responses, and 20 stable disease were reported.

A Phase II trial was conducted in 32 NSCLC patients previously treated with platinum-based therapy, and the majority had also been treated with microtubule inhibitor taxanes or vinblastine (Riely et al. 2007). Predominate adverse events included leukemia and neutropenia, which are commonly seen with tubulin inhibitors at MTD. However, no objective responses were observed and median disease progression was 1.5 months. No data was reported specifically on anti-vascular markers. A Phase II study was also conducted in patients with metastatic soft-tissue sarcomas; 21% of patients showed stable disease but no partial responses were observed (Patel et al. 2006). No further Phase II development work has been conducted on soblidotin as a free drug beyond these trials.

2.5   Conclusions and Future Directions

A variety of natural products exert pronounced anti-angiogenic effects in preclinical models, however, their clinical development was frequently limited by the onset of side effects, including damage in normal tissues. A key question to overcome the current limitations of VDAs is how to select for natural products with optimal selectivity for tumor vasculature, sparing normal, resting vasculature.

The balance between efficacy and safety is evident in clinical studies with ultra-potent anti-microtubule agents such as soblidotin, dolastatin, and related molecules. Clearly it is necessary to minimize toxicities associated with tubulin inhibitors against rapidly proliferating cell compartments, such as hematological and gastro-intestinal tissues.

A class of therapeutics targeting tumor vasculature, also referred to as VTAs, that interfere selectively with key regulators of tumor angiogenesis, including agents blocking VEGF-A and VEGFR-2 signaling, showed promising antitumor activities in preclinical studies and in clinical trials (Ferrara et al. 2007). However, the increase in overall survival by targeted therapeutics is frequently limited, and these agents are most commonly administered in combination with standard of care cytotoxic regimens. For example, the use of bevacizumab is approved only when combined with cytotoxic or cytokine therapy (with the exception of patients with GBM) and patients with metastatic disease are refractory or acquire resistance to VEGF inhibitors (Jain et al. 2006; Bergers and Hanahan 2008). Therefore, novel compounds or therapeutic modalities targeting tumor vasculature with improved efficacy and safety characteristics are needed to increase the response to anti-angiogenic treatment.

One potential strategy to achieve this goal is to combine the unique potency and selectivity of VDAs including tubulin inhibitors and flavonoids with the selectivity of large molecules binding to antigens expressed on tumor vasculature. This approach is showing success, in particular with auristatin-based tubulin inhibitors, as evidenced by the clinical approval of brentuximab vedotin (SGN-35) for Hodgkin lymphoma. The delivery of soblidotin and other peptide inhibitors to anti-vascular antigen targets via tumor endothelial-specific antibodies may enhance the therapeutic index and clinical activity of such agents.

Additional therapeutic modalities are currently developed in oncology which may achieve this goal, including nanoparticles targeted to the tumor vasculature. It will be interesting to test the potential of VDAs in the context of ADCs or nanoparticles and to study their ability to interfere with tumor angiogenesis and to block tumor growth.

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