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Minireviews

Combretastatins: More Than Just Vascular Targeting Agents?

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ABSTRACT

Several prodrugs of the naturally occurring combretastatins have undergone extensive clinical evaluation as vascular targeting agents (VTAs). Their increased selectivity toward endothelial cells together with their innate ability to rapidly induce vascular shutdown and inhibit tumor growth at doses up to 10-fold less than the maximum tolerated dose led to the clinical evaluation of combretastatins as VTAs. Tubulin is well established as the molecular target of the combretastatins and the vast majority of its synthetic derivatives. Furthermore, tubulin is a highly validated molecular target of many direct anticancer agents routinely used as front-line chemotherapeutics. The unique vascular targeting properties of the combretastatins have somewhat overshadowed their development as direct anticancer agents and the delineation of the various cell death pathways and anticancer properties associated with such chemotherapeutics. Moreover, the ongoing clinical trial of OXi4503 (combretastatin-A1 diphosphate) together with preliminary preclinical evaluation for the treatment of refractory acute myelogenous leukemia has successfully highlighted both the indirect and direct anticancer properties of combretastatins. In this review, we discuss the development of the combretastatins from nature to the clinic. The various mechanisms underlying combretastatin-induced cell cycle arrest, mitotic catastrophe, cell death, and survival are also reviewed in an attempt to further enhance the clinical prospects of this unique class of VTAs.

Introduction

Natural Combretastatins. The combretastatins were originally isolated from the Bushwillow tree *Combretum caffrum* by Pettit et al. (1987, 1995a). The Bushwillow tree is indigenous to riverbanks in the Eastern Cape Province of South Africa. Pettit is a world-renowned organic chemist whose work mainly focuses on the isolation and development of antineoplastic compounds of natural origin. More than 60% of currently used anticancer agents were originally derived from natural sources (Dall'Acqua, 2014). Structurally combretastatins consist of two substituted aromatic (aryl) rings (ring A and B) linked by a two-carbon alkene bridge (Lin et al., 1988). In total, 17 natural combretastatin analogs were originally isolated and characterized from *C. caffrum* (Lin et al., 1988). However, it is just two *cis* stilbene compounds, namely combretastatin A-1 (CA-1) and combretastatin A-4 (CA-4) (Fig. 1), the most active compounds of the series that attracted profound interest from chemists, biologists, and clinicians.

Synthetic Combretastatins. Rational development of combretastatin analogs has been pretty much ongoing since the original characterization of CA-4 in 1989. The ease of synthesis led to an unprecedented production of natural combretastatin compounds and synthetic analogs, which contrasts significantly to the two decades devoted to devise a strategy to synthesize the natural antimitotic agent Taxol (Nicolaou et al., 1994). The structural simplicity of the compounds rendered them readily amenable to chemical manipulation, thus modifying therapeutic efficacy, solubility, and stability of the lead compounds (Fig. 1). The combretastatins are naturally very potent anticancer agents with activity in the low nanomolar range. However, some structural modifications yielded novel synthetic derivatives with activity in the subnanomolar range (O'Boyle et al., 2010; Schobert et al., 2011). B ring substitutions were found to be more favorable in terms of improving drug potency. A ring substitutions were deemed less favorable; however, some benefit was observed with halogen atom substitutions (Beale et al., 2012), pointing to a preference to smaller substitutions/deletions as opposed to larger bulkier ones. Generally, alkene modifications do not drastically alter activity; however, some improvements can be obtained alone and in conjunction with

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ABBREVIATIONS: ABC, ATP-binding cassette; AML, acute myelogenous leukemia; BCRP, breast cancer resistance protein; CA-1, combretastatin A-1; CA-1P, combretastatin A-1 diphosphate; CA-4, combretastatin A-4; CA-4P, combretastatin A-4 phosphate; HIF-1, hypoxia-inducible factor 1; JNK, c-jun terminal kinase; MAPK, mitogen-activated protein kinase; MTA, microtubule targeting agent; MTD, maximum tolerated dose; NSCLC, nonsmall cell lung carcinoma; P-gp, P-glycoprotein; SAC, spindle assembly checkpoint; VTA, vascular targeting agent.

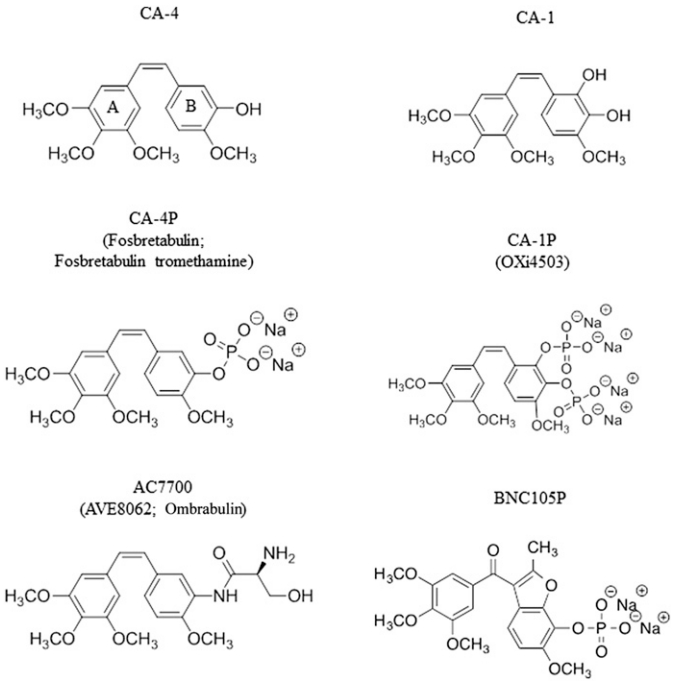

Fig. 1. Chemical structures of selected combretastatins.

B ring substitutions (O'Boyle et al., 2010; Schobert et al., 2011). Increasing the potency of the combretastatins alone was not enough to progress the compounds into clinical trials. CA-1 and CA-4 are *cis* stilbenes and can readily isomerize into inactive *trans*-isomers in response to heat, light, or acidic conditions. Stability issues were addressed by extensive alkene substitutions to a conformationally restricted structure: many in the form of heterocyclic groups (rings containing elements other than carbon) (Rajak et al., 2013; Lipeeva et al., 2014; Galli et al., 2015). Examples of alkene modifications include the following: imidazoles, furanones, thiazoles, pyrazoles, indoles, isoxazoles, and β-lactams (Medarde et al., 1999; Kaffy et al., 2006; O'Boyle et al., 2010; Banimustafa et al., 2013; Lin et al., 2013; Tsyganov et al., 2013; Mahal et al., 2015). To date, no *cis*-restricted synthetic analogs have progressed to clinical trials. It was the inherent insolubility of the compounds that ultimately prevented clinical progression.

Solubility limitations were solved following the development of a series of CA-4 prodrugs, including ammonium, potassium, and sodium phosphate salt derivatives (Pettit et al., 1995a). Prodrugs are defined as compounds that require a chemical conversion by a metabolic process into an active pharmacological agent. The soluble phosphate prodrugs are cleaved into the natural lead compounds by endogenous phosphatases. Synthetic conversion of CA-1 into a prodrug by means of diphosphorylation soon followed (Pettit and Lippert, 2000). Substitution of the B ring OH group with serinamide offered an alternative approach to solving the solubility issues and led to the development of the CA-4 analog AVE8062 (Ohsumi et al., 1998). In this study, the serine is cleaved by aminopeptidase to the CA-4 active compound. The combretastatin A-1 diphosphate (CA-1P) and combretastatin A-4 phosphate (CA-4P) prodrugs were converted into their active counterparts over 7 times faster in tumor and liver preparations than in blood (Kirwan et al., 2004). The prodrugs soon became the preferred structures to progress to clinical trials (Young and Chaplin, 2004; Chaplin et al., 2006; Patterson et al., 2012).

Vascular Targeting Agents

The combretastatins are primarily undergoing clinic evaluation as vascular targeting/disrupting agents. The clinical attributes of a vascular targeting agent (VTA) have been described in depth by Thorpe (2004). Basically, a VTA targets existing tumor blood vessels, which ultimately leads to tumor cell death from ischemia and tumor hemorrhagic necrosis. VTAs act rapidly (within minutes) and independently of tumor type, making them an attractive anticancer agent. Microtubule targeting agents (MTAs) are ideal candidates for VTAs as they target tubulin, which provides essential structural support to the immature tumor vasculature (Siemann et al., 2004). The additional structural support in mature vessels provided by the actin cytoskeleton together with a mature basement membrane and vessel-associated pericytes protects them from disruption by tubulin-targeting VTAs. The ability of other MTAs, namely vinblastine and colchicine, to inhibit tumor growth by a vascular mechanism is well established (Baguley et al., 1991). However, neither the vinca alkaloids nor colchine progressed to clinical evaluation as a VTA due to toxicity and a low therapeutic window (Levy et al., 1991; Hill et al., 1993). Watts et al., (1997) first noted that CA-1 caused a rapid increase in endothelial permeability, suggesting that this class of compounds could ultimately target the tumor vasculature, leading to tumor necrosis. It was the subsequent discovery that CA-4P could inhibit tumor blood flow at concentrations 10-fold less than the maximum tolerated dose (MTD) (Dark et al., 1997) that prompted the first clinical trials of CA-4 as a VTA. Further preclinical data confirmed a vascular mechanism for CA-4– and CA-4P–mediated inhibition of tumor growth (Grosios et al., 1999). The vascular targeting properties of CA-4 were soon linked to the tubulin-binding properties of the compound (Kanthou and Tozer, 2002). Original observations of the tumor vasculature were initially made following a single injection and tumor analysis carried out after short time frames (1–24 hours). Subsequent studies demonstrated that tumor perfusion and bioenergetic and oxygenation status returned to normal levels by 24 hours following a single injection of CA-4P (Horsman et al., 2000). Improved therapeutic benefits were later observed using multiple dose scheduling as opposed to a single dose at the MTD (Nabha et al., 2001).

Resistance to CA-4P monotherapy was repeatedly observed in the tumor rim (Tozer et al., 2008). Given that these cells are more susceptible to conventional chemotherapy, the combretastatin prodrugs soon presented as ideal candidates to complement traditional anticancer approaches (Chaplin and Hill, 2002). As anticipated, preclinical studies demonstrated improved activity when combretastatins were combined with nitric oxide synthetase inhibitors (Tozer et al., 2009), established chemotherapeutics (Grosios et al., 2000; Siemann et al., 2002; Morinaga et al., 2003; Yeung et al., 2007), hyperthermia (Horsman et al., 2000; Eikesdal et al., 2001; Horsman and Murata, 2002), and ionizing radiation (Landuyt et al., 2001). In some reports, the sequence of administration of the aforementioned treatment schedules determined the outcome, thus identifying a schedule dependence of combretastatin-mediated

TABLE 1
Combretastatin-based phase I–III clinical trials

Compound/(s)	Patients Enrolled	Tumor Type/ Pathology	Regimen	Main Outcomes	Reference
			Phase I		
CA-4P	n = 25	Refractory solid tumors	Single-dose CA-4P 18–90 mg/m(2) repeated 3-week interval	cr = 1, 60 mg/m(2) upper boundary MTD, 6/7 decline in gradient peak tumor blood flow.	Dowlati et al., 2002
CA-4P	n = 34	Refractory solid tumors	CA-4P 5–114 mg/m(2)	Well tolerated 14/16 patients at 52 or 68 mg/m(2). Improvement liver metastases n = 1.	Rustin et al., 2003
CA-4P	n = 13	Refractory solid tumors	CA-4P 5–114 mg/m(2)	Significant dose-dependent reductions in tumor perfusion 30 min post–CA-4P	Anderson et al., 2003
CA-4P	n = 37	Refractory solid tumors	CA-4P 6–75 mg/m(2) daily for 5 days repeated on 3-week intervals	pr = 1 (metastatic soft tissue carcinoma), sd = 14 52–65 mg/m(2), well-tolerated antitumor efficacy was observed	Stevenson et al., 2003
CA-4P	n = 25	Refractory solid tumors	Single-dose CA-4P 18–90 mg/m(2) repeated every 3 weeks	CA4P prolongs QTc interval. Advisable to limit patients with known coronary heart disease or risk factors from future trials.	Cooney et al., 2004
CA-4P + carboplatin	n = 16	Refractory solid tumors	40 cycles of carboplatin (AUC 4) and 5 mg min/mL; 60 min later CA-4P 27–36 mg/m(2) repeated on 3-week cycle	sd = 6, dose-limiting thrombocytopenia	Bilenker et al., 2005
CA4P + bevacizumab	n = 16	Refractory solid tumors	Bevacizumab (10 mg/kg) given 4 h after 2nd dose CA-4P 45–63 mg/m(2)	Apparent diffusion coefficient measurements were reproducible in a two-center clinical trial setting	Koh et al., 2009
CA-4P + (131)I-A5B7 anti-CEA antibody	n = 12	Refractory colon/rectum or pancreatic tumors	1800 MBq/m(2) (113)I-A5B7 on day 1, and 2–3 days later 45 mg/m(2) CA-4P repeated weekly	mr = 1, sd = 3, pd = 7	Meyer et al., 2009
CA-4P	n = 25	Refractory solid tumors	CA-4P single-dose 5–85 mg/m(2)	Well tolerated in Chinese patients at $\leq$65 mg/m(2), n = 7 dose-limiting toxicities at doses $\geq$65 mg/m(2), n = 1 obvious clinical response	He et al., 2011
CA4P + bevacizumab	n = 15	Refractory solid tumors	CA-4P 45–63 mg/m(2) on days 1, 8 repeated every 2 weeks. Bevacizumab 10 mg/kg day 8 of subsequent cycles 4 h after CA-4P.	CA-4P and bevacizumab combinations are well tolerated. sd = 3.	Nathan et al., 2012
CA-1P	n = 43	Refractory solid tumors	CA-1P 0.06–15.4 mg/m(2)	MTD 8.5 mg/m(2). MTD can be raised to 14 mg/m(2) by excluding hypertension patients. Tumor response at 14 mg/m (2). Antivascular effects at $\geq$11 mg/m (2).	Patterson et al., 2012
CA-4P	n = 8	Age-related macular degeneration	CA-4P 27 or 36 mg/m(2) weekly for 4 cycles	Results suggest potential efficacy. Transient hypertension.	Ibrahim et al., 2013

(*continued*)

TABLE 1—*Continued*

Compound/(s)	Patients Enrolled	Tumor Type/ Pathology	Regimen	Main Outcomes	Reference
AVE8062 + docetaxel	*n* = 58	Advanced solid tumors	AVE8062 11.5–42 mg/m(2) followed by docetaxel 75–100 mg/m(2) in 3-week cycles until disease progression or unacceptable toxicity	pr = 10	Eskens et al., 2014
CA-4P	*n* = 25	Refractory solid tumors	CA-4P 20–85 mg/m(2)	MTD = 65 mg/m(2). CA-4P is well tolerated with mild adverse events.	Liu et al., 2014
CA-1P	*n* = 20	Hepatic tumor burden	CA-1P on days 1, 8, and 15 of 28-day cycle	Completed. No results posted.	https://clinicaltrials.gov
CA-1P	*n* = 16	Hepatic tumor burden	CA-1P on days 1, 8, and 15 of 28-day cycle.	Completed. No results posted.	https://clinicaltrials.gov
AVE8062	*n* = 15	Advanced solid tumors	AVE8062 15–50 mg/m(2) once every 3 weeks	Recommended dose is 50 mg/m(2).	Murakami et al., 2014
AVE8062 + platinum salts + taxanes	*n* = 71	Advanced solid tumors	AVE8062 + platinum salts + taxanes every 3 weeks	Completed. No results posted.	https://clinicaltrials.gov
AVE8062 + bevacizumab	*n* = 39	Advanced solid tumors	AVE8062 on day 1 Bevacizumab on day 2 every 3-week cycle	Completed. No results posted.	https://clinicaltrials.gov
AVE8062 + cisplatin	*n* = 12	Advanced solid tumors	AVE8062 combined with 75 mg/m(2) cisplatin once every 3 weeks	Completed. No results posted.	https://clinicaltrials.gov
AVE8062 + docetaxel + cisplatin	*n* = 11	Advanced solid tumors	AVE8062 + docetaxel + cisplatin once every 3 weeks	Completed. No results posted.	https://clinicaltrials.gov
AVE8062 + paclitaxel + carboplatin	*n* = 18	Advanced solid tumors	AVE8062 + paclitaxel + carboplatin once every 3 weeks	Completed. No results posted.	https://clinicaltrials.gov
CA-1P	Recruiting	Relapsed and refractory AML and MDS	CA-1P [5 mg/m(2) ± 25% until MTD reached] given days 1, 8, 15, and 22 of a 28-day cycle	To determine safety and MTD.	https://clinicaltrials.gov
BNC105P	*n* = 21	Advanced solid tumors	BNC105P 2.1–18.9 mg/m(2)	Recommended dose 16 mg/m(2).	Rischin et al., 2011
Everolimus + BNC105P	*n* = 15	Advanced RCC	Everolimus 10 mg + BNC105P 4.2–16 mg/m(2); 21-day cycle Phase Ib	Everolimus 10 mg + BNC105P 16 mg/m(2) daily identified as recommended dose to enter Phase II	Pal et al., 2015
CA4P + carboplatin or paclitaxel	*n* = 21	Refractory solid tumors	CA-4P 27–60 mg/m(2), 18–22 h later carboplatin (AUC 4–5) or paclitaxel 135–175 mg/m(2) every 3 weeks	pr = 6/9 ovarian cancer patients. Combinations are well tolerated with antitumor activity observed.	Rustin et al., 2005
CA4P + carboplatin + paclitaxel	*n* = 48	Refractory solid tumors	CA-4P 27–72 mg/m2 (10-min infusion), 20 h later carboplatin AUC 4–5 paclitaxel 135–175 mg/m(2)	Combinations were well tolerated. pr = 10 (22%).	Rustin et al., 2010
CA-4P + radiotherapy	*n* = 39	NSCLC prostate adenocarcinoma SCCHN	27–66 Gy in 6–33 fractions over 3–6 weeks, CA-4P 50–63 mg/m(2) 1, 3, or 6 doses. Patients with SCCHN also received cetuximab.	Combinations were well tolerated. Responses were seen in 7/18 NSCLC patients.	Ng et al., 2012
Pazopanib ± CA-4P	Recruiting	Advanced recurrent ovarian cancer	Pazopanib 600 or 800 mg each day of 28-day cycle until disease progression. ±CA-4P 45–60 mg/m(2) every week for 3 weeks of a 4-week cycle. Max 6 cycles.	Ongoing. Results will determine doses for Phase II.	https://clinicaltrials.gov

(*continued*)

TABLE 1—*Continued*

Compound/(s)	Patients Enrolled	Tumor Type/ Pathology	Regimen	Main Outcomes	Reference
			Phase II		
CA-4P	*n* = 26	Anaplastic thyroid cancer	CA-4P 45 mg/m(2) on days 1, 8, and 15 over 2 cycles and end of therapy	sd = 7; 1/3 survived more than 6 months. Low baseline sICAM predictive of event-free survival.	Mooney et al., 2009
Carboplatin + paclitaxel ± CA-4P	*n* = 18	Relapsed ovarian cancer	CA-4P 63 mg/m(2), 18 h later paclitaxel 175 mg/m(2) and carbopatin AUC 5 repeated every 3 weeks	Combination is well tolerated. Addition of CA-4P to carboplatin and paclitaxel gave a higher response rate.	Zweifel et al., 2011
CA-4P ± carboplatin/paclitaxel	*n* = 80	Anaplastic thyroid cancer	CA-4P 60 mg/m(2) on days 1, 8, and 15. Carboplatin AUC 6 and paclitaxel 200 mg/m(2) day 2 every 3 weeks for 6 cycles. Then CA-4P days 1, 8 every 3 weeks or similar doses carboplatin and paclitaxel every 3 weeks for 6 cycles.	Thyroidectomy followed by CA-4P showed a nonsignificant improvement in patient survival.	Sosa et al., 2012
BNC105P	*n* = 30	Advanced MPM	BNC105P 16 mg/m(2) on days 1, 8 of a 21-day cycle. Repeated until progression or toxicity.	BNC105P was safe and tolerable, but results were insufficient to warrant use as a single agent. pr = 1; sd = 13.	Nowak et al., 2013
Carboplatin + paclitaxel + bevacizumab ± CA-4P	*n* = 60	Chemotherapy naive NSCLC	Carboplatin (AUC 6), paclitaxel [200 mg/m(2)], bevacizumab (15 mg/kg) day 1 of 21-day cycle. CA-4P [60 mg/ m(2)] or placebo on days 1, 7, and 14 every 3 weeks until progression or 12 months from randomization	Regimen including CA-4P cr = 0/32, pr = 18/32, sd = 8/32, pd = 1/32.	https://clinicaltrials.gov
CA-4P	*n* = 23	Choroidal neovascular-iz-ation secondary to pathologic myopia	CA-4P 27–45 mg/m(2) on days 0 and 7 (±2 days) up to 3 additional treatments	Completed. No results posted.	https://clinicaltrials.gov
CA-4P	*n* = 20	Asian patients with PCV	CA-4P single-dose 15–45 mg/m(2)	Completed. No results posted.	https://clinicaltrials.gov
Bevacizumab ± CA-4P	*n* = 107	Recurrent fallopian tube, ovarian or peritoneal carcinoma	Bevacizumab ± CA-4P repeated every 21 days in absence of disease progression or unacceptable toxicity	Ongoing. Not recruiting. No results posted.	https://clinicaltrials.gov
CA-4P	Recruiting	GI-NET with elevated biomarkers	CA-4P 60 mg/m(2) on days 1, 8, and 15 of a 3-week cycle until progression or unacceptable toxicity	Ongoing. No results posted.	https://clinicaltrials.gov
Taxane + platinum ± AVE8062	*n* = 176	Metastatic NSCLC with no prior treatment	AVE8062 35 mg/m(2) or placebo day 1. Followed by taxane-platinum. Repeated every 3 weeks. Max 6 cycles.	Addition of AVE8062 to taxane-platinum did not improve progression- free survival.	Von Pawel et al., 2014
Everolimus + BNC105P	*n* = 139	Advanced RCC	Everolimus 10 mg ± BNC105P 16 mg/m(2) daily	No difference in mean PFS observed. Several biomarkers associated with BNC105P outcome.	Pal et al., 2015
AVE8062 + paclitaxel + carboplatin	*n* = 157	Platinum-sensitive recurrent ovarian cancer	AVE8062 or placebo and paclitaxel for min 6 cycles until disease progression or unacceptable toxicity	Completed. No results posted.	https://clinicaltrials.gov

(*continued*)

TABLE 1—*Continued*

Compound/(s)	Patients Enrolled	Tumor Type/ Pathology	Regimen	Main Outcomes	Reference
Carboplatin + gemcitabine ± BNC105P	*n* = 134	Partially sensitive ovarian cancer	Carboplatin AUC 4 on day 1 Gemcitabine 800–1000 mg/m(2) (determined in phase 1) on days 1 and 8, BNC105P (determined in phase 1) on days 2 and 9, 21-day cycle for 6 cycles. Single-agent maintenance BNCP105P 16 mg/m(2) for 6 additional cycles. Phase III	Completed. No results posted.	https://clinicaltrials.gov
Cisplatin ± AVE8062	*n* = 355	Refractory soft tissue sarcoma	AVE8062 25 mg/m(2) or placebo, followed by cisplatin 75 mg every 3 weeks	Completed. No results posted.	https://clinicaltrials.gov

AML, acute myeloid leukemia; auc, area under the concentration-time curve; AVE8062, AC7700 or ombrabulin; BNC105P, 7-hydroxy-6-methoxy-2-methyl-3-(3,4,5-trimethoxybenzoyl)benzo[b]furan phosphate; CA-1P, combretastatin A-1 phosphate or OXi4503; CA-4P, combretastatin A-4 phosphate or fosbretabulin or fosbretabulin tromethamine or zybrestat; cr, complete response; GI-NET, gastrointestinal neuroendocrine tumors; MDS, myelodysplastic syndromes; MPM, malignant pleural mesothelioma; mr, minor response; MTD, maximum tolerated dose; NSCLC, nonsmall cell lung cancer; PCV, polypoidal choroidal vasculopathy; pd, progressive disease; PFS, progression-free survival; pr, partial response; RCC, renal cell carcinoma; SCCHN, squamous cell carcinoma of the head and neck; sd, stable disease; sICAM, soluble intracellular adhesion molecule-1.

inhibition of tumor growth when combined with other anticancer therapies (for review, see Horsman and Siemann, 2006). For instance, recent preclinical data demonstrated that prevention of cancer recurrence in the tumor rim could be achieved by administrating AVE8062 72 hours following irradiation, which prevented microcirculation in the tumor and tumor–host interface (Hori et al., 2014). Furthermore, identifying optimum scheduling can also avoid unnecessary toxicity. For example, administrating carboplatin and CA-4P on adjacent days as opposed to simultaneous administration avoided bone marrow toxicity (Bilenker et al., 2005). The extensive clinical evaluation of the combretastatins as single agents and in combination with various anticancer agents is detailed in Table 1. Initial phase I clinical trials identified a MTD of 50–65 mg/m(2) for CA-4 derivatives and 8.5-14 mg/m(2) for CA-1P (Table 1). Hypertension was identified as the major dose limiting side effect, but can be managed with appropriate medication. Upper limits of established MTDs can be administered by excluding patients with known heart disease or associated risk factors. Partial tumor responses appear to be the predominant positive clinical outcome of both combretastatin monotherapy and combination regimens. Higher response rates were recorded in relapsed ovarian cancer patients treated with standard chemotherapy and combretastatin as compared with patients treated with chemotherapy alone (Zweifel et al., 2011). Recruitment of patients for combretastatin-based clinical trials has progressed from patients with advanced chemotherapy-resistant carcinomas to chemotherapy-naive patients and also to phase III clinical trials, thus displaying clinical confidence in combretastatin-based regimens. Although clinical evaluation of the combretastatins is primarily focused on their pronounced vascular targeting properties, the combretastatins are naturally tubulin-targeting agents and can directly target cancer cells. To date, reviews on this interesting class of compounds have focused on their primary role as VTAs (Tozer et al., 2002, 2008; Siemann et al., 2004, 2009; Porcù et al., 2014), strategic chemical modifications (Hsieh et al., 2005; Rajak et al., 2013), their natural origin (Kingston, 2009; Pereira et al., 2012), or their stilbene moiety (Mikstacka et al., 2013). In this work, although acknowledging the former, we also extend our review to include other less conversed, but equally compelling direct anticancer properties endowed by the combretastatins.

Inhibitors of Neovascularization/Angiogenesis

Angiogenesis is defined as the growth of new capillaries from existing blood vessels. It plays a critical role in human physiology and facilitates various pathologies such as tumor growth, inflammation, disorders of the immune system, and age-related macular degeneration (Carmeliet, 2003). Antiangiogenic agents prevent the formation of new blood vessels from existing blood vessels by primarily targeting the recruitment of endothelial cells to existing blood vessels. It is worth noting that VTAs have indirect antiangiogenic effects; by disrupting pre-existing vasculature, they destroy the scaffold that supports the sprouting of new capillaries. In addition, accumulating research suggests that the combretastatins also display direct antiangiogenic properties. CA-4P can potently and rapidly (within 2 hours) inhibit the migration of endothelial cells, thus inhibiting the formation of new tumor blood vessels (Ahmed et al., 2003). Furthermore, in this study, CA-4P inhibited sprout formation at concentrations that did not inhibit the proliferation of endothelial cells, confirming direct antiangiogenic properties of CA-4P. Similarly, modulation of endothelial cells by combretastatin analogs has been reported in vitro (Dupeyre et al., 2006; Kong et al., 2010; Romagnoli et al., 2010; Sanna et al., 2010; Arthuis et al., 2011; Porcù et al., 2013) and in vivo (Fortin et al., 2011; Porcù et al., 2013; Zheng et al., 2014). The molecular mechanism of the antiangiogenic properties of TR-644 CA-4 analog was linked to the inhibition of vascular endothelial growth factor-induced phosphorylation of VE-cadherin (Porcù et al., 2013). It has also been suggested that CA-4 may exert its antiangiogenic properties by downregulation of connective tissue growth factor, a negative regulator of angiogenesis (Samarin et al., 2009). Preclinical studies combining the combretastatins with other antiangiogenic agents gave conflicting results. In more detail, therapeutic benefits were observed when combretastatins

were combined with the antiangiogenic agent Endostar (Fu et al., 2011), but not with the antiangiogenic agent TNP-470 (Landuyt et al., 2001; Lambin and Landuyt, 2003). Phase I clinical trials demonstrated that CA-4P–induced vascular changes were maintained by addition of the antiangiogenic drug bevacizumab (Nathan et al., 2012). Apart from tumor angiogenesis, CA-4 can also impair physiologic (Hussain et al., 2012; Feng et al., 2013) and pathologic angiogenesis (Griggs et al., 2002; Jockovich et al., 2007). Clinical trials of CA-4P in patients with neovascular age-related macular degeneration demonstrated potential clinical efficacy; however, future clinical use is unlikely due to associated adverse side effects (Ibrahim et al., 2013).

Antimetastasis/Migration Agents

Combretastatins can not only inhibit the migration of endothelial cells, but can also inhibit the migration of cancer cells and prevent tumor metastasis and progression. Specifically, CA-4 can inhibit the attachment, migration, and invasion in vitro as well as metastasis in vivo of implanted human gastric cancer-derived cells (Lin et al., 2007). Similarly, CA-4 inhibited the migration of human bladder cancer cells in vitro (Shen et al., 2010). The CA-4 analog AVE8062 inhibited lymph node metastasis in a murine model of LY80 Yoshida sarcoma tumor (Hori et al., 2002). In this study, the tumor blood flow was completely shut off even in small metastases, highlighting the therapeutic potential of combretastatins to manage lymph node micrometastases. Combination of CA-4 with other chemotherapeutics in artificial delivery systems such as nanocapsules (Wang and Ho, 2010) or electrospun fibers (Luo et al., 2014) also demonstrated antimetastasis efficacy. Small interfering RNA technology demonstrated a role for the RhoA/RhoA-associated kinase signaling pathway in the CA-4–mediated inhibition of T cell migration (Pollock et al., 2014). Other synthetic combretastatatin analogs also demonstrated significant antimetastasis/migration properties (Nathwani et al., 2013; Porcù et al., 2013; Pollock et al., 2014). Importantly, CA-4 demonstrated therapeutic activity in patients with metastatic anaplastic thyroid cancer (Granata et al., 2013). CA-1P underwent phase I clinical trials in patients with hepatic tumor burden (Table 1). However, AVE8062 plus a taxane-platinum regimen failed to improve progression-free survival in patients with metastatic nonsmall cell lung carcinoma (NSCLC) (von Pawel et al., 2014). Taken together, these findings imply that the combretastatins may function to prevent tumor metastasis by a dual mechanism: first, reducing cell migration of cancer cells from the primary site; second, by preventing the tumor blood flow to arising metastases and ultimately inhibiting tumor progression. However, further clinical evaluation is required to identify efficacious combretastatin-based regimens for first-line treatment of metastasis.

Microtubule-Targeting Agents

Tubulin Binding. Early studies showed that combretastatin could reverse the differentiation of glioma cells into astrocytes, indicating that they may function as antimitotic agents (Hamel and Lin, 1983). The cellular target of most antimitotic agents is tubulin. Tubulin is a protein that forms the main constituent of the microtubule cytoskeleton and has many cellular functions, including maintenance of cell shape, transport of vesicles, and involvement in cell division. Tubulin turbidity assays demonstrated that combretastatin could inhibit the polymerization of tubulin (Hamel and Lin, 1983). CA-4 was later found to interact with tubulin at or near the colchicine binding site using a competitive binding assay (Pettit et al., 1995b). The binding affinity of combretastatins to the colchicine binding site is attributed to the presence of the trimethoxyaryl ring A, a common pharmacophore among colchicine-like agents (McGown and Fox, 1989). Interactions of combretastatins and tubulin were confirmed by cell-free turbidity assays, immunofluorescence, and sedimentation assays (Carr et al., 2010; Greene et al., 2010, 2011; Shen et al., 2010). Modern molecular modeling and docking studies using x-ray structures of tubulin complexed with colchicine have facilitated the generation of algorithms to predict the binding probability of the copious number of emerging synthetic combretastatin analogs (ter Haar et al., 1996; Bellina et al., 2006). Due to its potent binding affinity toward the colchicine binding site of tubulin, CA-4 and its numerous active derivatives fall into the classification of MTAs. MTAs are classified as inhibitors (vinca alkaloids, colchicine) or promoters (taxanes) of microtubule polymerization (Jordan and Wilson, 2004). Microtubules are protein biopolymers formed by the polymerization of heterodimers of α- and β-tubulins (Kaur et al., 2014). MTAs interact with tubulin and alter the natural equilibrium of free tubulin dimers and assembled polymers. Some MTAs can suppress microtubule dynamics without changing microtubule mass (Jordan et al., 1993). Two groups of naturally occurring MTAs, the taxanes and the vinca alkaloids, are front-line chemotherapeutic agents used for the direct treatment of many types of human cancers (Kumar et al., 2010; Ujjani and Cheson, 2013), thus verifying tubulin as a highly validated target in cancer therapy. The combretastatins are classified as microtubule inhibitors and are thus capable of directly targeting cancer cells.

G_2/M Cell Cycle Arrest. MTAs are principally antimitotic agents in that they disrupt the microtubule dynamics during mitosis and trigger cell cycle arrest in G_2/M. Combretastatin-induced G_2/M arrest was widely observed in vitro (Table 2); however, an increase in mitotic arrested tumor cells was not observed following exposure to combretastatin in vivo (Eikesdal et al., 2001; Nabha et al., 2001). However, it may be suggested that the dual-deprivation effects of both glucose and oxygen deficiency induced by targeting the tumor vasculature would in turn create an environment incompatible with cell division, making a notable increase in mitotic arrested cells unlikely. Furthermore, destroying the tumor vasculature would also impede the sustained accumulation of combretastatin within the tumor. The extent and length of combretastatin-induced G_2/M arrest in vitro were cell-type specific (Greene et al., 2010; Shen et al., 2010) and reversible (Cenciarelli et al., 2008). Combretastatin-induced mitotic arrest was confirmed using several markers of mitosis, including the following: mitotic protein monoclonal 2, nuclear translocation of cyclin B1, increased expression of cyclin B, and cyclin-dependent kinase 1 (Nabha et al., 2002; Kanthou et al., 2004; Fang et al., 2008; Shen et al., 2010; Zhu et al., 2010; Qiao et al., 2012). Approaches that enhance or abrogate G_2/M phase arrest can often improve the therapeutic potential of anticancer agents (DiPaola, 2002). Inhibition of

p38 mitogen-activated protein kinase enhanced both CA-4–induced G_2/M arrest and cytotoxicity in the BEL-7402 hepatocellular carcinoma cells (Quan et al., 2008). Further studies investigating potential synergies between agents that modulate G_2/M arrest with combretastatins are clearly warranted.

Spindle Assembly Checkpoint Activation. Disruption of microtubule dynamics or alteration of polymer mass can in turn alter the tension across sister chromatids, preventing chromosome attachment and correct chromosome bi-orientation, leading to activation of the spindle assembly checkpoint (SAC). Analysis of the SAC regulators is often carried out to confirm mitotic arrest. Activation of the SAC by combretastatins was confirmed by kinetochore localization of Bub1 and Mad1 (Vitale et al., 2007) and the phosphorylation and activation of BubR1 (Greene et al., 2010, 2011). A requirement for BubR1 in combretastatin-induced G_2/M cell cycle arrest was demonstrated in cervical carcinoma cells (Greene et al., 2011). In this model, caspase-dependent cleavage of BubR1 associated with mitotic release and onset of apoptosis (Greene et al., 2011). Likewise, a decline in the levels of BubR1 correlated with the onset of CA-4–induced apoptosis in bladder cancer-derived cells (Shen et al., 2010). Combretastatin-induced apoptosis was associated with reduced levels of the SAC protein Bub3 in bladder cancer cells (Shen et al., 2010); however, no change in Bub3 expression was observed in chronic myelogenous leukemia cells (Greene et al., 2010). The role of the SAC in combretastatin-induced mitotic arrest and cell death remains largely unknown. It is known that the combetastatins induce a G_2/M arrest in endothelial cells; however, whether activation of the SAC contributes to mitotic arrest has yet to be determined. The expression profiles of SAC regulators vary in cancer cells of different origin and determine cellular response to various MTAs. Further characterization of combretastatin-induced activation of SAC in endothelial cells and cells of varying neoplastic origin is required to further understand combretastatin-induced mitotic arrest.

Induction of Cell Death

Various outcomes following combretastatin-induced mitotic arrest have been reported, including classic apoptotic cell death, mitotic catastrophe, and polyploidy (>4N DNA content). In contrast, mitosis-independent apoptosis in response to CA-4 was reported in ex vivo chronic lymphocytic leukemia (Bates et al., 2013). Other forms of cell death, including autophagy and necrosis, have not yet been reported as a direct consequence of combretastatin exposure to cancer cells, although induction of autophagy-mediated prosurvival pathways has been observed and will be discussed in more detail below. It is also worth clarifying that combretastatins indirectly induce both autophagy and necrosis in tumors by targeting the tumor vasculature. Original reports indicate that apoptosis is executed by caspases; however, more recent studies have demonstrated that it can occur independently of caspases (Bröker et al., 2005). It is worth noting that caspase-independent cell death is more likely to be indicative of cell death mechanisms other than apoptosis. Caspase-dependent and -independent cell death have been reported for the combretastatins, although the former is more frequently documented (Table 2).

Apoptosis. Apoptosis is initiated by two main pathways: the intrinsic and extrinsic (for review, see Elmore, 2007). The intrinsic pathway is triggered inside the cell and mainly by stress-induced release of cytochrome c from the mitochondria, resulting in the formation of the apoptosome and activation of a series of cysteine proteases known as caspases. The extrinsic pathway is activated outside the cell via death receptors, leading to the recruitment and activation of initiator caspases such as caspase-8. A review of the current literature suggests that the intrinsic pathway is the most frequently reported form of apoptotic cell death in direct response to combretastatins and sometimes occurs secondary to mitotic catastrophe (Table 3). Several factors pointing to a combretastatin-induced mitochondria-mediated apoptotic pathway include loss of mitochondrial potential, increase in mitochondrial accumulation of Bim (BH3-only protein of the Bcl-2 family), cytochrome c release, and activation of caspase-9 and -3. Knockdown experiments demonstrated that Bim is an absolute requirement for CA-4–induced apoptosis in H460 lung cancer cells (Mendez et al., 2011). A more recent report showing caspase-8 activation together with an increase in Fas ligand in response to a novel CA-4 analog G-1103 suggested an additional involvement of the extrinsic pathway (Zuo et al., 2015). However, cross-talk between both the intrinsic and extrinsic apoptotic pathways is a common occurrence; hence, in the former study, additional experiments are required to ultimately confirm the extrinsic pathway as the dominant mode of G-1103–induced apoptosis in Hela cells. It is well documented that combretastatin-induced apoptosis is subsequent to mitotic arrest in proliferating cancer cells. A recent report identified a role for c-Jun terminal kinase (JNK) and NOXA (a BH3-only member of the Bcl-2 family) in combretastatin-induced apoptosis in quiescent ex vivo chronic lymphocytic leukemia cells pointing to a mitosis-independent apoptotic pathway (Bates et al., 2013). Chemotherapy-induced apoptosis frequently associated with the downregulation/loss of key mediators of cell survival. Likewise, combretastatin-induced apoptosis associated with a loss of cell survival factors, including x-linked inhibitor of apoptosis (Fang et al., 2008; Xu et al., 2008; Zhu et al., 2010; Demchuk et al., 2014) and AKT (Lin et al., 2007; Fang et al., 2008; Shen et al., 2010). Similarly, modulation in the form of downregulation, cleavage, or phosphorylation of the antiapoptotic protein Bcl-2 was associated with apoptosis induced by combretastatins (Hu et al., 2006; Wu et al., 2009; Greene et al., 2010; Liu et al., 2011; Qiao et al., 2013; Zuo et al., 2015). However, others report no effect on Bcl-2 expression, clearly indicating a cell-specific response (Billard et al., 2008). P53-dependent apoptosis was reported in NSCLC (Mendez-Callejas et al., 2014). Several studies have observed enhanced apoptosis in vitro by combining combretastatins with other anticancer agents, including dasatinib (Zhang et al., 2013), tumor necrosis factor–related apoptosis-inducing ligand/Apo-2L (Zhang et al., 2011), Bcl-2 inhibitors (ABT-263) (Bates et al., 2013) (ABT-737) (Li et al., 2014), or JNK inhibitor/small interfering RNA (Li et al., 2014). Bcl-2 inhibitors are undergoing phase I and II clinical evaluation for advanced and metastatic solid tumors (https://clinicaltrials.gov) and may also complement combretastatin-based regimens within the clinic. However, overall the signaling pathways leading to combretastatin-induced apoptotic cell death remain vague and poorly defined with scope for improved definition.

TABLE 2
Combretastatins induce G_2/M cell cycle arrest in a broad spectrum of normal and carcinoma-derived cell lines

Compound	Cell Type	Reference
CA-4P	Endothelial	Kanthou et al., 2004; Ding et al., 2011
CA-4	Malignant human B-lymphoid	Nabha et al., 2001
CA-4P	Leukemia	Petit et al., 2008
CA-4	Hepatocellular carcinoma	Quan et al., 2008
CA-4	Nonsmall cell lung cancer (NSCLC)	Cenciarelli et al., 2008
CA-4	Bladder cancer	Shen et al., 2010
CA-4	Leukemia, cervical	Greene et al., 2010, 2011
CA-4	NSCLC	Méndez-Callejas et al., 2014
CA-4–like ethers	Leukemia	Lawrence et al., 2001
Naphthalene CA-4 analogs	Cervical, colon, and lung carcinoma	Maya et al., 2005
β-Lactam CA-4 analogs	Endothelial, leukemia, cervical, breast, and colon carcinoma	Carr et al., 2010; Greene et al., 2010, 2011, 2012; Nathwani et al., 2013; O'Boyle et al., 2011, 2013
Benzo[b]thiophene and benzofuran combretastatin analogs	Lung cancer	Simoni et al., 2006;
Carbazole sulfonamide CA-4 analog	Breast cancer	Hu et al., 2006
N-phenyl-N′-(choroethyl)urea derivatives	Melanoma	Fortin et al., 2007
4/5-Hydroxy-2,3-diaryl (substituted)-cycopent-2-en-1-ones CA-4 analogs	Endothelial cancer	Gurjar et al., 2007
MZ3 CA-4 analog	Leukemia	Fang et al., 2008
Benzil derivatives of CA-4	Colon cancer and NSCL	Mousset et al., 2008
2,5-Diaryl-2,3-dihydro-1,3,4-oxadiazoline CA-4 analog	Cervical	Lee et al., 2010
XN0502 CA-4 analog	NSCLC	Zhu et al., 2010
DAT-230 CA-4 analog	Fibrosarcoma	Qiao et al., 2012, 2013
Combretastatin-amidobenzothiazole conjugate	Breast carcinoma	Kamal et al., 2012
3,5-Dihalogenation A-ring tetrazoles	Ovarian carcinoma	Beale et al., 2012
Cyclopropylamide CA-4 analog	Lung carcinoma	Chen et al., 2013
3,4-Diaryl squaric acid CA-4 analog	Breast carcinoma	Liu et al., 2013
2-Alkylthio-4-(2,3,4-trimethoxyphenyl)-5-aryl-thiazole CA-4 analogs	Mouse embryonic fibroblast	Salehi et al., 2013
CA-4 derivatives containing a 3′-O-substituted carbonic ether moiety	Breast cancer	Ma et al., 2013
TR-644 CA-4 analog	Endothelial	Porcù et al., 2013
1,2,3-Triazole CA-4 analog	Leukemia	Demchuk et al., 2014
3,4-Diaryl-1,2,5-selenadiazol analog	Colon cancer	Guan et al., 2014
Benzo[b]furan combretastatin analog	Lung cancer	Kamal et al., 2014b
1,2,3-Trizole–linked aminocombretastatin conjugates	Lung cancer	Kamal et al., 2014a
COH-203 CA-4 analog	Liver cancer	Qi et al., 2014
G-1103 CA-4 analog	Cervical and fibrosarcoma	Zuo et al., 2015

COH-203, 5-(3-hydroxy-4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)-3*H*-1,2-dithiol-3-one; DAT-230, 2-methoxy-5-(2-(3,4,5-trimethoxyphenyl) thiophen-3-yl)aniline; G-1103, 3-(3-hydroxy-4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)-1,2,5-selenadiazole; MZ3, (4-(4-bromophenyl)-2,3-dihydro-N,3-bis (3,4,5-trimethoxyphenyl)-2-oxoidmidazole-1-carboxamide); TR-644, 2-amino-4-(3′,4′,5′-trimethoxyphenyl)-5-aryl thiazole; XN0502, 4-(4-methoxphenyl)-5-(3,4,5-trimethoxyphenyl)-1H-pyrazol-3-amine.

Mitotic Catastrophe. Perturbations within the mitotic spindle induced by MTAs can trigger mitotic catastrophe. The morphologic features of mitotic catastrophe in response to combretastatin exposure were first noted by Nabha et al. (2000) in malignant human B-lymphoid cells. Mitotic catastrophe was later defined as the principal method of cell death induced by CA-4P in these cells, followed by apoptosis (Nabha et al., 2002). The morphologic hallmarks of mitotic catastrophe induced by combretastatins were also described in NSCLC cells (Vitale et al., 2007; Cenciarelli et al., 2008; Mendez et al., 2011), bladder cancer cells (Shen et al., 2010), cervical (Romagnoli et al., 2011), acute lymphoblastic leukemia (Romagnoli et al., 2011; Demchuk et al., 2014), and breast cancer-derived cells (O'Boyle et al., 2011, 2013). However, although originally believed to be a distinct form of cell death, mitotic catastrophe is now acknowledged as a process that precedes other forms of cell death, such as necrosis or apoptosis (Vakifahmetoglu et al., 2008). Mitotic catastrophe was recently defined as an oncosuppressive event that occurs failing an adequate mitotic arrest to prevent genomic instability (Vitale et al., 2011). Due to this recent advance, it is important to reassess the role of mitotic catastrophe induced by combretastatins.

Cell Survival/Resistance

Cancer cells can develop cell survival properties leading to resistance to various cancer therapeutics. Intrinsic and acquired cellular resistance to chemotherapy is often associated with tumor heterogeneity, drug efflux and metabolism, mutations in drug binding sites, survival signaling, autophagy, and tumor microenvironment, such as hypoxia. As previously discussed, the combretastatins are one of a few groups of anticancer agents that act independently of cancer type and tumor site by targeting the associated vasculature as opposed to the tumor itself and thus bypassing associated problems of resistance due to tumor heterogeneity. However, targeting the tumor vasculature can reduce tumor glucose and oxygen levels

TABLE 3
Combretastatins induce mitotic catastrophe and cell death in endothelial and cancer-derived cells

Compound	Cell Type	Type of Cell Death	Reference
CA-4	Endothelial	Apoptosis	Iyer et al., 1998
CA-4	NSCLC	Mitotic catastrophe and apoptosis	Vitale et al., 2007; Cenciarelli et al., 2008
CA-4	Bladder cancer	Apoptosis, caspase-dependent and -independent cell death	Shen et al., 2010
CA-4	NSCLC	Caspase-dependent mitotic catastrophe, apoptosis, intrinsic	Mendez et al., 2011; Méndez-Callejas et al., 2014
CA-4	Leukemia	Apoptosis	Greene et al., 2010
CA-4	Colon cancer	Caspase-dependent apoptosis	Zhang et al., 2011
CA-4P	Endothelial	Apoptosis	Ahmed et al., 2003
CA-4P	Endothelial	Caspase-independent, some hallmarks of apoptosis	Kanthou et al., 2004
CA-4P	Leukemia	Mitototic catastrophe and apoptosis	Nabha et al., 2002
CA-4P	Leukemia	Apoptosis, intrinsic, caspase-dependent and -independent cell death	Petit et al., 2008
4-Arylcoumarin CA-4 analogs	Leukemia	Apoptotic and nonapoptotic	Bailly et al., 2003
CA-4 Benso[b]thiophene and benzofuran analogs	Lung cancer	Apoptosis	Simoni et al., 2006
4-Arylcoumarin CA-4 analogs	Human breast	Apoptosis	Rappl et al., 2006
MZ3 CA-4 analog	Leukemia	Apoptosis, intrinsic	Fang et al., 2007, 2008; Xu et al., 2008
Boronic acid analog CA-4	Leukemia	Apoptosis	Nakamura et al., 2006
Carbazole sulfonamide CA-4 analog	Breast cancer	Apoptosis	Hu et al., 2006
ST2151 synthetic stilbenoid CA-4 analogs	NSCLC	Mitotic catastrophe and apoptosis, intrinsic	Vitale et al., 2007
2,3-Diaryl-4/5-hydroxy-cycopent-2-en-1-one CA-4 analogs	Endothelial	Apoptosis	Gurjar et al., 2007
Arylcoumarin combretastatin analogs	Leukemia	Apoptosis, intrinsic caspase-dependent	Billard et al., 2008
XN0502 CA-4 analog	NSCLC	Apoptosis, caspase-dependent	Zhu et al., 2010
1,5-Disubstituted 1,2,4-triazoles CA-4 analogs	Leukemia	Apoptosis, intrinsic	Romagnoli et al., 2010
β -Lactam CA-4 analogs	Endothelial, leukemia, ex vivo CML cells, cervical carcinoma, and breast cancer	Apoptosis, caspase-dependent Apoptosis, mitotic catastrophe	Greene et al., 2010, 2011; O'Boyle et al., 2011, 2013; Nathwani et al., 2013
Imidazole CA-4 analogs	Leukemia	Apoptosis	Schobert et al., 2010
FPTB CA-4 analog	Chondrosarcoma	Apoptosis, intrinsic	Liu et al., 2011
Thiazole CA-4 analogs	Cervical cancer	Mitotic catastrophe, apoptosis caspase-dependent and-independent	Romagnoli et al., 2011
1,5-Disubstituted tetrazoles CA-4 analogs	Cervical cancer	Apoptosis, intrinsic	Romagnoli et al., 2012
DAT-230 CA-4 analog	Fibrosarcoma; gastric adenocarcinoma	Apoptosis, intrinsic	Qiao et al., 2012, 2013
Cyclopropylamide analog CA-4	Lung cancer	Apoptosis	Chen et al., 2013
3-(Trimethoxyphenly)-2(3*H*)-2(3*H*)-thiazole thiones CA-4 analogs	Breast cancer	Apoptosis	Banimustafa et al., 2013
CA-4E containing 3′-O–substituted carbonic ether	Breast cancer	Apoptosis	Ma et al., 2013
1,2,3-Triazole CA-4 analog	Leukemia	Apoptosis, mitotic catastrophe	Demchuk et al., 2014
1,2,3-Triazole–linked aminocombretastatin conjugates	Lung cancer	Apoptosis	Kamal et al., 2014a
G-1103 CA-4 analog	Fibrosarcoma	Apoptosis, intrinsic and extrinsic	Zuo et al., 2015
Biaryl CA-4 analogs	Osteosarcoma, leukemia	Apoptosis	McNulty et al., 2015

CML, chronic myeloid leukemia; DAT-230, 2-methoxy-5-(2-(3,4,5-trimethoxyphenyl) thiophen-3-yl)aniline; FPTB, 2-(furanyl)-5-(pyrrolidinyl)-1-(3,4,5-trimethoxybenzyl) benzoimidazole; G-1103, 3-(3-hydroxy-4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)-1,2,5-selenadiazole; MZ3, (4-(4-bromophenyl)-2,3-dihydro-N,3-bis(3,4,5-trimethoxy-phenyl)-2-oxoidmi-dazole-1-carboxamide); XN0502, 4-(4-methoxphenyl)-5-(3,4,5-trimethoxyphenyl)-1H-pyrazol-3-amine.

and thus paradoxically create an environment that can trigger prosurvival pathways. In this review, we discuss drug resistance directly and indirectly associated with combretastatin exposure and how they can overcome drug resistance associated with other chemotherapeutics.

Lack of Interactions with Drug Efflux Pumps. Active drug efflux transporters of the ATP-binding cassette (ABC)–containing family of proteins can attribute tumor resistance to a wide spectrum of clinically relevant drugs, thus conferring a multidrug resistance cellular phenotype (for review, see Glavinas et al., 2004). Members of the ABC family include P-glycoprotein (P-gp; multidrug resistance protein 1; gene symbol ABCB1); multidrug resistance-associated protein (gene symbol ABCC1); and breast cancer resistance protein (BCRP; gene symbol ABCG2). The ABC transporters confer resistance by preventing ATP-dependent drug translocation across the plasma membrane and thereby preventing the accumulation of chemotherapeutics to levels required to induce cytotoxic effects. P-gp is the most characterized member of the ABC family. It is well established from numerous sources that the combretastatins

are not substrates for the P-gp and thus offer a therapeutic advantage over other clinically used MTAs such as the taxanes and the vinca alkaloids, which are known P-gp substrates (Shirai et al., 1998; Gwaltney et al., 2001; Xu et al., 2008; Greene et al., 2010; Lee et al., 2010; Romagnoli et al., 2012; Penthala et al., 2013). Similarly, the combretastatins demonstrated potent activity in cancer cells overexpressing BCRP (Greene et al., 2010). Interestingly, CA-4 analogs could restore mitoxantrone accumulation in BCRP-expressing cells, thus offering potential as a novel BCRP reversing agent (Combes et al., 2011). The multidrug resistance protein 1 inhibitor MK-571 failed to restore sensitivity of combretastatin-resistant HT-29 cells to CA-4 (Schobert et al., 2011). The molecular basis of the inherent resistance of HT-29 cells to CA-4 has yet to be identified. Overall, the expression of ABC transporters does not influence the therapeutic efficacy of lead combretastatins, thus offering a therapeutic advantage over many established chemotherapeutics against malignancies expressing drug efflux pumps.

Metabolism. The combretastatin metabolite profile has been described as complex. *O*-demethylation and aromatic hydroxylation were identified as the two principal phase I biotransformation pathways of CA-4 (Aprile et al., 2007). *Z-E* isomerization of the olefin bond was primarily observed during metabolic *O*-demethylation and aromatic hydroxylation of CA-4. Metabolites arising from aromatic hydroxylation of ring B were readily oxidized into *para*-quinone metabolites (Aprile et al., 2007). In this study, the resulting metabolites were not analyzed for activity or toxicity. The biologic attributes of such metabolites would be of clinical interest as quinones can be toxic (Bolton et al., 2000). In contrast, some metabolites may be active, as it has been postulated that the improved antitumor activity of CA-1 may be attributed to the formation of active metabolites (Kirwan et al., 2004). Folkes et al. (2007) demonstrated that CA-1 quinone intermediates could enhance oxidative stress by increasing free radicals. The active quinone metabolite was subsequently determined to display significant cytotoxicity independent of tubulin binding (Aprile et al., 2013). Phase II in vitro and in vivo metabolic studies identified CA-4 glucuronide and sulfate metabolites (Aprile et al., 2009). Importantly, glucuronidation of CA-4 was associated with inactivation of the compound in BEL-7402 hepatocellular carcinoma cells (Quan et al., 2009). CA-1 monoglucuronides were identified as the major CA-1–related compounds in human urine (Stratford and Folkes, 2012). In summary, the combretastatins may be metabolized into an array of active and nonactive metabolites.

Autophagy. Autophagy is an inherent cellular survival pathway triggered in response to starvation and various stress signals. During this highly regulated and conserved pathway, cellular components are sequestered in double-membraned autophagosomes. Fusion of the autophagosomes with lysosomes or vacuoles facilitate the breakdown of required cellular material providing metabolites and energy to fuel prosurvival effects. Activation of autophagy can stimulate death or prosurvival pathways in response to chemotherapy-induced stress. In recent times, the latter response appears to be reported more frequently. Likewise, a prosurvival response to direct combretastatin exposure has been reported in cancer cells (Greene et al., 2012; Li et al., 2014). Similarly, hypoxia and starvation-induced tumor cell autophagy as a consequence to antiangiogenic therapies can also mediate tumor resistance and survival (Hu et al., 2012; Guo et al., 2013). The JNK–Bcl-2 pathway has been implicated in CA-4–induced autophagy (Li et al., 2014). Inhibition of JNK in turn inhibited autophagy and promoted CA-4–induced cell death. Indirect inhibition of the autophagy pathway using Bcl-2 inhibitors also enhanced CA-4–induced cell death (Li et al., 2014). Several studies demonstrated preclinical therapeutic efficacy in combining autophagy inhibitors with conventional anticancer approaches. The antimalarial drugs chloroquine and hydroxychloroquine are currently undergoing open label clinical trials with established anticancer agents against various carcinomas (http://clinicaltrials.gov/). Preclinical data directly inhibiting autophagy by pharmacological or genetic means showed enhanced cell death induced by CA-4 (Li et al., 2014) and a synthetic *cis*-stable derivative (Greene et al., 2012). Furthermore, autophagy inhibitors can work in hypoxic areas and may complement combretastatin therapy in vivo. Autophagy was detected using electron microscopy in CA-4P–treated xenografts (Yeung et al., 2007). The significance of autophagy in these tumors was not determined. The antitumor effects of CA-4P were enhanced in autophagy-defective PC-3 xenografts as compared with autophagy-competent xenografts, suggesting autophagy can mediate resistance to combretastatins (Hoang et al., 2013). Given that the combretastatins are VTAs and consequently induce conditions that promote prosurvival autophagic pathways, further preclinical studies with clinically approved autophagic inhibitors are urgently required.

Mutations/Alterations in Drug Binding Site. The combretastatins interact at or near the colchicine binding site of tubulin and thereafter exert their potent antitumor effects. Mutations within the various tubulin binding sites and alterations of the tubulin isotype distribution can contribute to MTA drug resistance and efficacy. A reduction in the amount of class III β-tubulin isotype was observed in nonsmall cell lung carcinoma cells with acquired resistance to CA-4 (Wehbe et al., 2005). These findings contradict other studies demonstrating that the therapeutic efficacy of colchicine site-binding agents is not influenced by changes in class III β-tubulin expression (Stengel et al., 2010). However, both studies support the findings that the combretastatins are active in cells overexpressing class III β-tubulin displaying resistance to paclitaxel. Similarly, other studies have demonstrated activity of combretastatin analogs in cells overexpressing class III β-tubulin (Lee et al., 2010). In summary, the combretastatins would offer an effective alternative to carcinomas expressing alterations in tubulin isotype distribution and also to those expressing mutations within tubulin binding sites other than the colchicine binding site.

Survival Signaling. Prosurvival signaling in tumor cells and their microenvironment can contribute to chemotherapeutic resistance and tumor cell survival. Overexpression of the antiapoptotic protein survivin associated with resistance to the combretastatin analog BPR0L075 (Cheung et al., 2009). Ectopic expression of the antiapoptotic Mcl-1 reduced the extent of apoptosis induced by dasatinib and CA-4 combinations as compared with cells expressing basal levels of Mcl-1 (Zhang et al., 2013). Aberrant expression and activation of various kinases is a key factor mediating chemoresistance and tumor cell survival. In BEL-7402 cells, CA-4 stimulated the extracellular signal-regulated kinases 1/2 and p38 mitogen-activated protein kinase (MAPK) (Quan et al., 2008). P38 MAPK was associated with tubulin reassembly and resistance to CA-4 in these cells. Pharmacological inhibition of p38

MAPK enhanced CA-4–induced cell death in these cells. CA-4–induced phosphorylation and activation of JNK associated with autophagy-mediated cell survival (Li et al., 2014). Inhibition of JNK subsequently inhibited the CA-4–induced autophagy survival pathway and facilitated apoptosis. In contrast, exposure to combretastatins can also downregulate several mediators of prosurvival signaling associated with chemotherapeutic resistance, tumor cell survival, and poor patient outcome (see apoptosis section). Correlations between the combretastatins and various other cell survival proteins remain to be determined.

Hypoxia. Hypoxia in solid tumors correlates with an aggressive phenotype, increased metastases, and chemoresistance, which ultimately leads to poor patient outcomes. Early studies demonstrated that CA-4P rapidly induced tumor hypoxia (within 1 hour) as a consequence of tumor vasculature damage (Horsman et al., 1998; Sunar et al., 2007). Combretastatin-induced hypoxia is indeed a double-edged sword. On one hand, combretastatins target tumor vasculature and induce tumor death by ischemia. In contrast, hypoxic conditions can induce the upregulation of genes associated with drug resistance-associated cell survival. A reduction in CA-4P–induced hypoxia was noted in the resistant tumor rim consistent with a requirement of hypoxia for effective combretastatin-mediated tumor inhibition (Zhao et al., 2005). CA-4P induced the expression of glucose-regulated protein GRP78, a stress-inducible chaperone induced by glucose depletion and anoxia (a severe form of hypoxia) (Dong et al., 2005). In this report, CA-4P could not directly induce GRP78 expression in breast cancer cells in vitro under normal cell culture conditions, indicating that CA-4P–induced GRP78 is an indirect consequence of tumor vasculature disruption and associated with hypoxia and glucose deprivation. Furthermore, the authors state that increased endogenous expression of GRP78 is associated with resistance to etoposide and may have important clinical implications when combining combretastatins with etoposide. The transcription factor hypoxia-inducible factor 1 (HIF-1) is a key regulator of cellular response to hypoxia. Under hypoxic conditions, CA-4P reduced HIF-1 accumulation and increased HIF-1 expression under aerobic conditions (Dachs et al., 2006). Increased HIF-1 activation leads to activation of the transcription factor NFκB, which is associated with increased cell survival. Given that HIF-1 can regulate a range of angiogenic and metastatic activities, the clinical significance of combretastatin-induced modulation of HIF-1 needs to be determined. The development of hypoxia in the form of cardiac ischemia was observed as a dose-limiting adverse affect of CA-4P in clinical trials (Stevenson et al., 2003; He et al., 2011). Combretastatin-induced hypoxia, a friend or foe? Current data suggest that the benefits of combretastatin-induced hypoxia by far exceed the setbacks.

Future Directions

CA-4 Versus CA-1. The structural difference between CA-4 and CA-1 is attributed to a second hydroxyl group on ring B of CA-1. As such, the prodrug variant of CA-1 is diphosphorylated as opposed to CA-4, which is monophosphorylated. It has been postulated that the extra phosphate group of CA-1P may influence the pharmacokinetics, distribution, and release profile of active CA-1 (Kirwan et al., 2004). To date, the number of CA-4–based clinical trials by far exceeds those evaluating CA-1P. Current clinical data suggest that CA-1P is now the most potent clinically evaluated VTA (Patterson et al., 2012). Intriguingly, the vast majority of synthetic combretastatins are analogous to CA-4 despite emerging data demonstrating the superior therapeutic efficacy of CA-1 (Hua et al., 2003; Patterson et al., 2012). Similarly, combretastatin signaling pathways are primarily investigated using CA-4 and associated analogs. This may be somewhat attributed to the increased difficulty in synthesizing CA-1 analogs and lack of commercial availability of CA-1. CA-1P may well become the preferred combretastatin within the clinic and should hence be incorporated in further preclinical and clinical studies.

Are Combretastatins More Than Just VTAs? Collectively, the many forms of combretastatins functioning as VTAs have quickly evolved as a prevalent strategy to complement established chemotherapeutics for the treatment of solid tumors. Although the outcome of various clinical trials supports the continued use of combretastatin prodrugs as VTAs within the clinic, there is room for significant improvement. CA-4P was previously found to be 20–30 times more sensitive in tumor cells than human umbilical vein endothelial cells (Ahmed et al., 2003), demonstrating potential as a direct anticancer agent. An interesting study conducted by Petit et al. (2008) highlighted the antileukemic and antivascular properties of CA-4P in a preclinical acute myelogenous leukemia (AML) study. Similarly, a preclinical study of CA-1P revealed that blood vessel density alone could not account for observed tumor regression, pointing to direct cytotoxic effects on leukemia cells (Madlambayan et al., 2010). Ultimately, optimum use of combretastatins may be obtained by exploiting both direct cytotoxic properties and the vascular targeting properties of the compounds. CA-1P is currently undergoing phase I clinical evaluation for relapsed and refractory AML (http://clinicaltrials.gov). Preliminary results demonstrate that CA-1P is well tolerated with evidence of disease response (Turner et al., 2013). Final results from AML clinical trials are eagerly awaited and will ultimately determine whether combretastatins are more than just vascular targeting agents.

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This review was intended to be an overview of the multiple anticancer attributes of the combretastatins as opposed to a fully comprehensive report. We apologize to researchers who have contributed to combretastatin research and were not included in the manuscript.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Greene, Meegan, Zisterer.

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