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Brief Article

# Vascular Disrupting Activity of Tubulin-Binding 1,5-Diaryl-1*H*-imidazoles†

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Highly cytotoxic 1,5-diaryl-1*H*-imidazoles were studied to clarify the relationship between cytotoxicity and activity as vascular disrupting agents (VDA). All the compounds disorganized the tubulin cytoskeleton, affected endothelial cell morphology and capillary formation in vitro, and caused vessel shutdown and tumor necrosis in vivo, thus confirming their vascular disrupting properties. Nonetheless, the substitution patterns on the imidazole ring, responsible for greater interaction energy with tubulin and higher cytotoxicity, were not associated to greater vascular disrupting activity.

## Introduction

Microtubule-binding drugs possess a well documented antitumor activity as well as vascular targeting properties.[1−3] The recognized ability of the tubulin-binding agents, in particular the microtubule destabilizing compounds, to selectively damage the tumor vasculature has been exploited in the development of vascular disrupting agents (VDA[a]). In contrast to drugs that target tumor angiogenesis, VDA selectively act on the newly formed tumor vasculature at doses that are substantially lower than those required to cause cytotoxicity.[4] By affecting the microtubule cytoskeleton, they cause morphologic and functional changes in endothelial cells, triggering a cascade of events that lead to rapid vasculature collapse, reduction of blood flow, and ultimately to central tumor necrosis.[4] One outstanding class of compounds for such an approach are the combretastatins, which have received a great deal of attention due to their relatively simple structures, high potency as cytotoxic agents, and antivascular activity. Combretastatin A-4 (CA-4, **1**) is the most active member of the combretastatins A family, isolated from the African tree *Combretum caffrum*. CA-4 exhibits strong antitubulin activity by binding to the colchicine binding site on tubulin.[5] A limitation to the clinical development of **1** (CA-4) has been its poor water solubility. Hence, more soluble structural analogues have been developed, and, at present, CA-4-like VDA, which are currently subjected to clinical testing include compounds **2** (CA-4P, a water-soluble prodrug of **1**), **3** (AVE8062, an analogue of **1**), and **4** (Oxi4503, a prodrug of combretastain A-1) (Figure 1).[6−9]

A reported critical structural requirement for the activity of these compounds is the cis configuration of the double bond. In fact, the trans isomer of **1** and its analogues have an activity lower than that of the corresponding cis isomers.[10] However, the cis double bond in **1** and its cis analogues easily undergoes isomerization during storage and administration[10] and in the course of metabolism in liver microsomes.[11]

As a consequence, considerable efforts have been devoted at modifying **1**, and bioavailable cis-restricted analogues based on the bioisosteric replacement of the olefinic double bond of the natural derivative with vicinal diaryl-substituted five-membered heteroaromatics, which include oxazole, isoxazole, thiazole, pyrazole, imidazole, triazole, and tetrazole, have been developed.[10,12,13]

As far as the imidazole derivatives are concerned, it should be noted that in 2002 Wang and co-workers evaluated the cytotoxicity against NCI-H460 and HCT-15 cancer cell lines and the antitubulin activity of a series of 1,2-, 1,5-, and 4(5),5(4)-diaryl-substituted imidazole derivatives.[14] From this study emerged the existence of a lack of correlation between tubulin depolymerizing activity, cytotoxicity, and in vivo antineoplastic activity. This finding implicated that the only cytotoxicity, which may be related to biological targets different to that postulated, cannot be taken alone as a predictor of vascular disrupting activity for **1**.

Recently, in continuation of our studies on the synthesis and evaluation of the antitumor activity of vicinal diaryl-substituted five-membered heterocycles, which can be considered cis-restricted CA-4 analogues, we developed efficient and selective procedures for the synthesis of 1,5-[15] and 1,2-diaryl-1*H*-imidazoles[16,17] of general formulas **5** and **6**, respectively (Figure 2).

We reported the cytotoxic activity in vitro of these heterocycles against the NCI-60 human cancer cell lines screening panel, and from these data it emerged that 1,5-diaryl-1*H*-imidazoles **5** are generally more active than their corresponding 1,2-diaryl substituted isomers **6** (Supporting Information, Table 1).[18] We also described the results of molecular modeling studies concerning the interactions of these CA-4 analogues at the colchicine binding site of $\alpha\beta$-tubulin. These studies showed that there was a good linear correlation between experimental cytotoxicity data and calculated interaction energies of compounds **5** and **6** with the colchicine binding

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[a] Abbreviations; CA-4, combretastatin A-4; FGF-2, fibroplast growth factor 2; FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cells; MTD, maximum tolerated dose; VDA, vascular disrupting agents.

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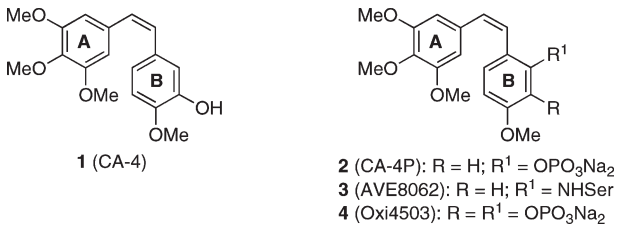



**Figure 1.** Combretastatin A-4 (CA-4) and its soluble analogues CA4-P, AVE8062, and Oxi4503.

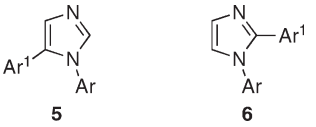



**Figure 2.** Chemical structures of 1,5-diaryl-1*H*-imidazoles **5** and 1,2-diaryl-1*H*-imidazoles **6**.

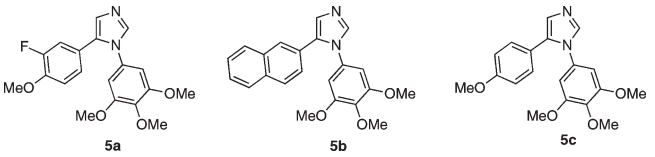



**Figure 3.** Chemical structures of 1,5-diaryl-1*H*-imidazoles **5a**−**c**.

site on tubulin.[18] Moreover, the higher activity of the 1,5-diaryl-1*H*-imidazoles **5** in comparison with that of the corresponding 1,2-diarylated isomers **6** was attributed to the higher total interaction energies of compounds **5** with the colchicine site on tubulin.[18]

Taking into account the results reported by Wang and co-workers,[14] in order to assess whether the substitution patterns associated with increased tubulin-binding properties and cytotoxicity are also responsible for greater vascular disrupting properties, in the present study we have evaluated the activity of the three most cytotoxic 1,5-diaryl-1*H*-imidazoles, compounds **5a**, **5b**, and **5c** (Figure 3), and of the corresponding hydrochlorides on the endothelial cells and the tumor vasculature and we have compared this activity with that of **1**.

## Chemistry

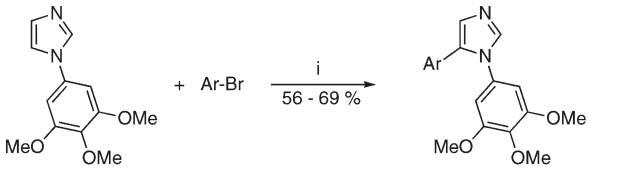
1,5-Diaryl-1*H*-imidazoles **5a**−**c** were regioselectively prepared by palladium-catalyzed direct arylation of 1-(3,4,5-trimethoxyphenyl)-1*H*-imidazole (**7**) with the required aryl bromides in DMF at 140 °C in the presence of CsF as the base and a catalyst precursor consisting of a mixture of $Pd(OAc)_2$ and P(2-furyl)$_3$ as the ancillary ligand (Scheme 1).[15]

Imidazoles **5a**−**c** were then converted in high yields into the corresponding water-soluble hydrochloride salts **8a**−**c** by reaction of their benzene solutions with aqueous hydrochloric acid at room temperature. Using SPARC, a computer program that evaluates physical properties of organic compounds from molecular structure,[19] we estimated the p$K_a$ values of hydrochlorides **8a**, **8b**, and **8c** to be 6.63, 5.58, and 6.82, respectively.

## Biological Results and Discussion

Alteration of endothelial cell morphology is the distinctive trait of the activity of tubulin-targeting VDA, in vitro. We therefore investigated the effect of **5a**−**c** on human umbilical vein endothelial cells (HUVEC) morphology. After 1 h of treatment, all the compounds caused profound changes in the morphology of endothelial cells, which retracted by assuming a rounded shape and tended to detach from the substrate (Figure 4). We tested also the hydrochlorides **8a**−**c** and, interestingly, we observed that in all cases they resulted more potent than the corresponding free bases, although only **8b** displayed an activity comparable to that of **1** (Figure 4).

**Scheme 1.** Preparation of 5-Aryl-1-(3,4,5-trimethoxyphenyl)-1*H*-imidazoles **5a**−**c**[a]




[a] Reagents and conditions: (i) ArBr (2.0 equiv), $Pd(OAc)_2$ (5 mol %), P(2-furyl)$_3$ (10 mol %), CsF (2.0 equiv), DMF, 140 °C, 48 h.

Importantly, the concentrations of the compounds active in affecting cell morphology were not cytotoxic in comparable experimental conditions (1 h exposure, Supporting Information (SI), Table 2). This finding might have relevant implications for the future development of these compounds because it suggests that vascular disrupting activity occurs at doses lower than the cytotoxic ones and hence possibly lower than the maximum tolerated dose (MTD).

In line with the hypothesis that cytotoxicity is not predictive of VDA activity, we found no correlation between vascular disrupting activity and potency in inhibiting the proliferation of endothelial cells (SI, Table 2) or tumor cells (SI, Table 1). For example, the potency of **5a** in affecting endothelial cell morphology was lower than that of **1** or **5c**, despite its cytotoxicity, is superior to that of the other imidazoles and similar to that of **1** (SI, Table 1).[18]

Compound **5b** and its salt **8b** were selected for further in vitro experiments and for in vivo testing. Alteration of morphology induced by **8b** was accompanied by alteration in the organization of the tubulin cytoskeleton (Figure 5). Concentrations of **8b** active in affecting cell shape (0.1−1 $\mu$M) caused a rapid and concentration-dependent disorganization of microtubules, associated with a blebbing morphology of the rounded cells, as typically reported for VDA.[20,21] Although less potent than **1**, **8b** was more potent than the free base **5b**, confirming that a correct formulation is essential not only for in vivo testing but also for preliminary in vitro assays.

The imidazole derivatives were able to disrupt a network of capillary-like structures formed by endothelial cells on a 3D layer of Matrigel. On this permissive matrix, HUVEC spontaneously align forming a network of interconnecting cords reminiscent of immature vessels, complete 24 h after seeding. Addition of the compounds **5b** and **8b** to the formed cords caused, in 1 h, their disruption, with HUVEC maintaining less contact with neighboring cells and forming only short, distorted cords. Also in this assay, **8b** was more active than **5b** (Figure 5).

We next evaluated the effects of imidazole derivatives in an in vivo model of newly formed vessels induced by FGF-2 in a Matrigel plug. Vessels formed in 7 days, as shown by confocal microscopy images of vehicle-treated animals showing functional perfused vessels stained by FITC-conjugated lectin.

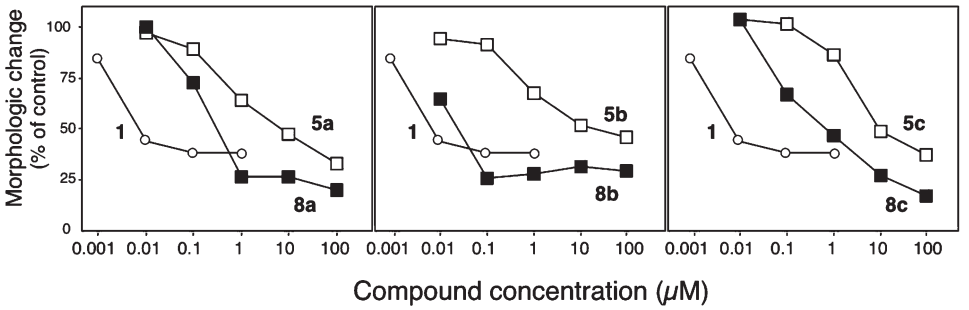



**Figure 4.** Effect of imidazole derivatives **5a**−**c** (open squares) and their corresponding hydrochlorides **8a**−**c** (black squares) on endothelial cell morphology. **1** (CA-4) was tested as the reference compound (circles).

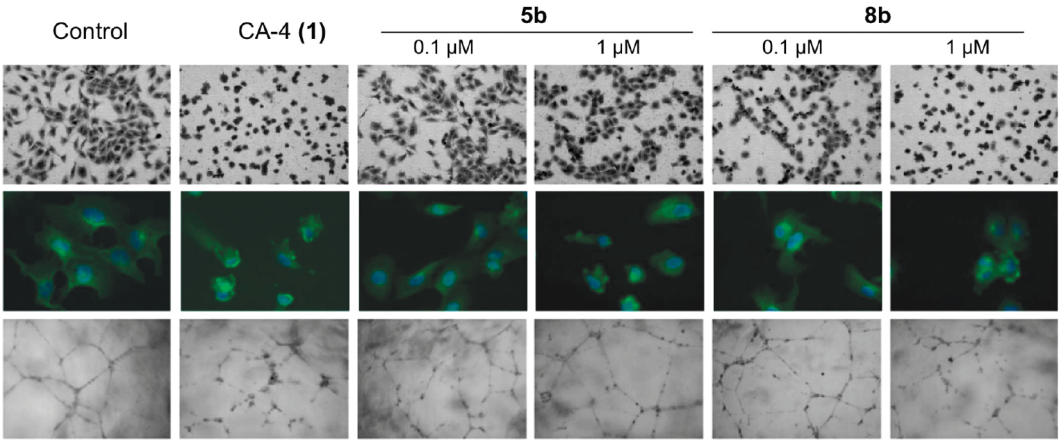



**Figure 5.** Effect of **1** (CA-4) (0.1 $\mu$M), **5b**, and **8b** on endothelial cell morphology, cytoskeleton organization, and cord formation. Top panel: representative images of HUVEC exposed to the indicated concentration of compounds for 1 h (×100). Middle panel: immunofluorescence analysis of the tubulin cytoskeleton in HUVEC exposed to vehicle or the indicated concentration of compounds for 1 h (×200). Bottom panel: the compounds were added to cords formed by HUVEC on Matrigel, 24 h after seeding. Images (×40) were taken 1 h after addition of the compounds.

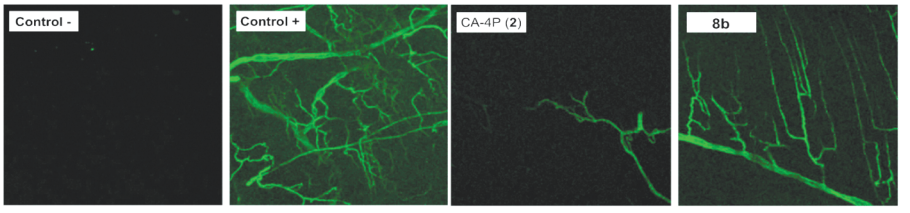



**Figure 6.** Effect of **8b** and **2** (CA-4P) on newly formed vessels in vivo. Confocal microscopy images (×200) showing functional, FITC-isolectin B4 staining of the vasculature within the Matrigel plug lacking FGF-2 (control −) or containing FGF-2 in mice treated with vehicle (control +), **2** (100 mg/kg, ip), or **8b** (20 mg/kg, ip). At least three images were analyzed for each sample ($n$ = 5).

At that time, a single treatment with **8b** (20 mg/kg ip) caused a rapid shutdown of the vessels (Figure 6), as shown by the reduced number of vessels perfused by the fluorescent tracer 1 h after treatment with the VDA.

Although on the whole **8b** appeared less potent than CA-4P, treatment with **8b** apparently affected the most tortuous vessels, while sparing the straight ones, suggesting a role for hemodynamics forces in vessels occlusion in response to this compound.

The hallmark of vascular disrupting activity in tumors is the induction of massive central tumor necrosis.[4] Mice bearing subcutaneous MDA-MB-435 tumors received a single treatment with **8b**. Tumors were removed and the amount of necrotic tissue evaluated 24 h later. A single administration of **8b** induced extensive central tumor necrosis, significantly increased compared to vehicle-treated tumors (Figure 7).

A similar effect was observed when mice were treated with 20 or 80 mg/kg. Necrosis was evident in the central region of the tumor, whereas a rim of vital cells remained at the periphery of the tumor, hence with the typical pattern described for VDA.[21]

## Conclusions

We have demonstrated that imidazoles **5a**−**c**, particularly when formulated as their water-soluble salts **8a**−**c**, have vascular disrupting activity because they are able to disorganize the tubulin cytoskeleton, to affect endothelial cell morphology and capillary formation in vitro, and to cause vessels shutdown in vivo in experimental models. More importantly, the compounds cause a rapid central necrosis of experimental tumors in vivo, namely they possess the main feature of VDA.

This study also indicates that the compounds with greater interaction energy with the colchicine binding site on tubulin and higher cytotoxicity do not always possess a greater

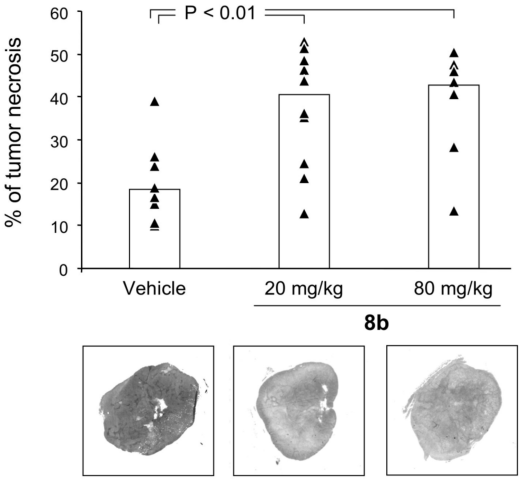



**Figure 7.** Induction of tumor necrosis by **8b**. MDA-MB-435 tumors growing sc were treated with vehicle or **8b** (20 or 80 mg/kg ip). After 24 h of treatment, tumors were excised, sections were stained with H&E, and the percentage of necrotic area evaluated as described in the Supporting Information. Columns indicate the median value. $P < 0.01$ compared to vehicle. Representative sections showing the typical pattern of central necrosis and the viable rim at the periphery of the tumor, characteristic of VDA activity are shown below.

vascular disrupting activity, and this fact implies that substitution patterns associated to an increased cytotoxicity are not necessarily associated to a greater activity on the tumor vasculature. Therefore our results raise cautions in using interaction energy and cytotoxicity values as the only parameters indicative of vascular damaging activity in SAR studies and in the development of VDA. Morphologic changes in endothelial cells and vessel damaging activity in vitro and in vivo appear more representative of the VDA activity.

Our study also confirms the feasibility of developing imidazole derivatives containing the 3-fluoro-4-methoxyphenyl moiety as cis-locked analogues of the potent vascular disrupting agent **1** and establishes that the 2-naphthyl moiety is a good candidate for surrogating the CA-4 B ring. It is also worth noting that the results of this study discloses the possibility of designing new, more potent derivatives with improved water solubility and vascular disrupting activity.

## Experimental Section

**General Procedure for the Synthesis of 1,5-Diaryl-1*H*-imidazoles 5a−c.** To a flame-dried reaction vessel were added compound **7** (0.70 g, 3.0 mmol), $Pd(OAc)_2$ (33.6 mg, 0.15 mmol), $P(2\text{-furyl})_3$ (70 mg, 0.3 mmol), an aryl bromide (6.0 mmol), if a solid, and CsF (0.91 g, 6.0 mmol). Deareated DMF (15 mL) and an aryl bromide, if a liquid, were then added successively under a stream of argon by syringe at room temperature. The resulting mixture was stirred under argon at 140 °C for 48 h. After being cooled to room temperature, the reaction mixture was diluted with AcOEt (50 mL), poured into a sat. aq NaCl solution (150 mL), and extracted with AcOEt (4 × 30 mL). The organic extract was washed with brine (2 × 30 mL), dried over $Na_2SO_4$, and concentrated under reduced pressure. The residue was purified by MPLC on silica gel. The chromatographic fractions containing the required compound were collected and concentrated. A $CH_2Cl_2$ solution of the residue was then stirred for 2 h at room temperature with 3-(mercapto)propyl-functionalized silica gel (0.50 g, loading 1.2 mmol/g), which was used as a metal scavenger. The resulting heterogeneous mixture was filtered on celite and concentrated. Compounds **5a−c** possess a purity of at least 98%, determined by GLC.

**General Procedure for the Synthesis of 1,5-Diaryl-1*H*-imidazole Hydrochlorides 8a−c.** To a solution of compound **5** (0.61 mmol) in benzene (7.7 mL) was added an aq 0.5 M HCl solution (2.2 mL, 1.1 mmol) and the resulting mixture was stirred at room temperature for 6 h. After this period of time, the volatile solvents were removed under reduced pressure and the residue was recrystallized from $Et_2O$ and EtOH. Compounds **8a−c** possess a purity of at least 98%, determined by elemental analysis.

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**Supporting Information Available:** Analytical and spectroscopic data for compounds **5a−c** and **8a−c**, experimental details on biological assays, effect on endothelial cell morphology, and comparative cytotoxicity data. This material is available free of charge via the Internet at http://pubs.acs.org.

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