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Short communication

# A novel class of *trans*-methylpyrazoline analogs of combretastatins: Synthesis and *in-vitro* biological testing

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ABSTRACT

Thirteen methylpyrazoline analogs (**1a**-**m**) of combretastatin A-4 (CA-4, **2**) were synthesized. The *trans*-geometry of the two substituted phenyl moieties was ascertained by a single crystal X-ray diffraction study of compound **1d**. The cytotoxicities of the analogs against the growth of murine B16 melanoma and L1210 lymphoma cells in culture were measured using the MTT assay. One of the derivatives, **1j**, which has the same substituents as CA-4 was the most active in the series with $IC_{50}$ values of 3.3 μM and 6.8 μM against the growth of L1210 and B16 cells, respectively. The activity of this analog against human cancer cell lines was confirmed in the NCI 60 panel. The other active analogs against L1210 were **1b** and **1f**, which gave $IC_{50}$ values in the 6–8 μM range. Compound **1j** caused microtubule depolymerization with an $EC_{50}$ value of 4.1 μM. This compound has good water solubility of 372 μM. Molecular modeling studies using DFT showed that compound **1j** adopts a "twisted" conformation mimicking CA-4 that is optimal for binding to the colchicine site of tubulin.



## 1. Introduction

There is an intense effort in cancer research to develop novel compounds that are capable of rapid destruction of tumor vasculature leading to tumor necrosis and antitumor efficacy. Such vasculature disrupting agents (VTAs) are promising anticancer drugs. One of the most prominent lead compounds is combretastatin A-4 (CA-4, **2**, see Fig. 1). CA-4 was originally isolated from the african willow tree, *combretum caffrum*, by G.R. Pettit *et al.* in 1989 [1]. Combretastatin A-4 is an effective antitumor agent. This compound is capable of rapid and selective disruption of the abnormal tumor vasculature [2]. CA-4 binds to tubulin within the colchicine binding site, thereby disrupting microtubules and altering endothelial cell shape and disrupting the vascular barrier causing rapid vasculature shutdown and tumor necrosis [3]. It had been suggested that these anti-vasculature actions might be mediated through the vascular endothelial-cadherin signaling pathway [4]. Yet, CA-4 has a major drawback for development as a potential antitumor drug, that is, its poor bioavailability and low solubility in biological media [5]. This hindrance has led to the synthesis of many structural analogs of CA-4, including CA-4P (**3**), a phosphate containing pro-drug, which is currently undergoing clinical trials [6]. Another analog that has received significant attention is A105972, **4**, which has shown potent and promising antitumor activity in mice [7].

A wide range of structural analogs of CA-4 have been synthesized, evaluated, and reported from the author's laboratory. Examples include pyrazole [8], pyrazoline [9], cyclohexenone [10], and oxadiazoline analogs [11]. Interestingly, the pyrazole-containing compounds were not active at inhibiting the growth of L1210 cancer cells in culture [8], but the pyrazoline analogs were active [9]. This result was explained by the analysis of an X-ray structure of a pyrazole compound [8] and molecular models of both classes of compounds. The results revealed that the pyrazoles essentially adopted a planar conformation but the pyrazolines adopted a "twisted" geometry that was similar to the conformation of CA-4 as determined by X-ray crystallography [12]. Several review articles that catalog all combretastatin analogs have recently been reported [6,13].

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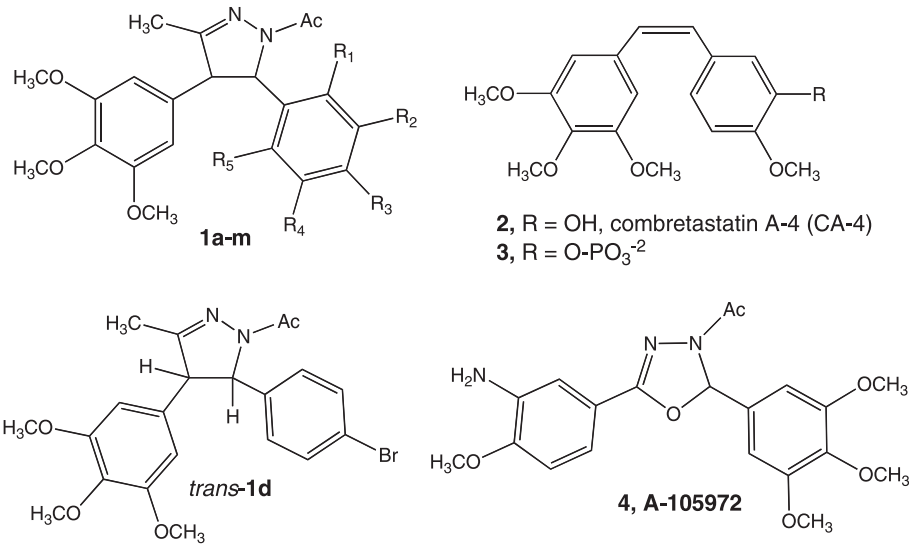



**Fig. 1.** Structures of the methylpyrazolines 1a-m (the definition of $R_1$ to $R_5$ is given in Scheme 1 and drawn in Table 1), combretastatin A-4 (CA-4, 2), CA-4P (3), A-105972 (4), and the 4-bromomethylpyrazoline analog 1d.

The authors' laboratory has also developed a series of acetylated analogs of combretastatins [14]. Specifically, the acetylated analogs of combretastatin were found to exhibit good cytotoxicity and microtubule depolymerization potency, making them worthy of further investigation [14]. One potential challenge for the acetylcombretastatins is their susceptibility to detoxification by biological nucleophiles such as glutathione [15]. To overcome this challenge while concomitantly enhancing the cytotoxic potency, the acetylcombretastatins were modified by converting the α,β-unsaturated enone functionality into a methylpyrazoline unit. The methylpyrazoline analogs were also designed because it was proposed that they would adopt the necessary "twisted geometry" similar to CA-4, and the substituted phenyl moieties would be fixed in a *trans* configuration (see analog 1d in Fig. 1).

## 2. Results and discussion

### 2.1. Molecular modeling

Thirteen methylpyrazoline analogs were designed for this study. The analogs were divided into two groups as shown in Table 1. The first group I contains a single substituent on the B ring (**1a-g**). The second group II contains two- or three substituents on the B ring (**1h-m**). The conformation of analog **1d** was assessed by molecular modeling studies using MacSpartan to determine if it adopted the "twisted geometry". The molecular structures were optimized by molecular mechanics (MMFF) using a molecular equilibrium conformer procedure. The equilibrium geometry was subsequently optimized using Hartree-Fock (3-21 G) calculations, followed by another geometry optimization using a Density Functional calculation (B3LYP and 6-31G*). The results given in Fig. 2A provided evidence that compound **1d** does adopt a "twisted" conformation similar to that of CA-4 [12].

### 2.2. Chemistry

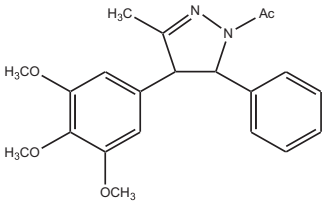
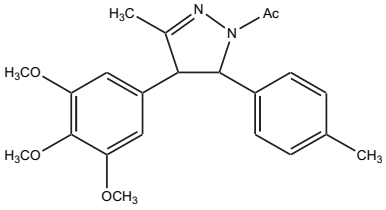
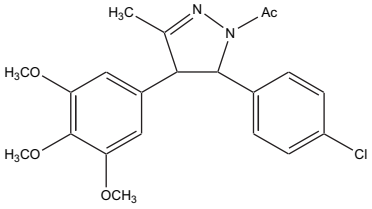
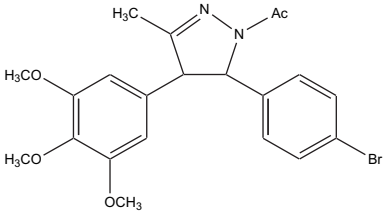
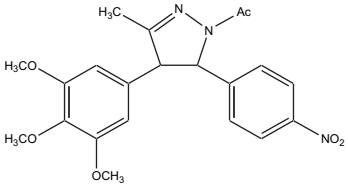
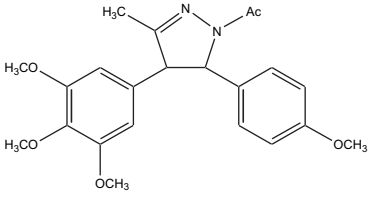
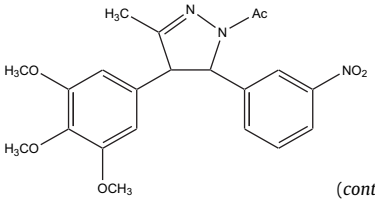
As described in Scheme 1, all thirteen derivatives were synthesized in a two-step process. The first step included the reaction of the appropriately substituted benzaldehyde with 3,4,5-trimethoxyphenylacetone [16] in the presence of benzoic acid and piperidine in refluxing toluene to yield acetylcombretastatins **5a-m** in 15–95% yield. The acetylcombretastatins were subsequently reacted with hydrazine hydrate and acetic acid for 4 h in refluxing methanol. The resulting methylpyrazoline analogs were collected and purified by silica gel column chromatography using an ethyl acetate/hexane solvent system. The chemical yields varied between 25 and 60%. The structures of the methylpyrazoline analogs were confirmed using mass spectrometry, infrared, and 400 MHz $^1$H NMR measurements. The *trans* configuration of the two substituted phenyl moieties on the pyrazoline unit was initially inferred from the coupling constants of about 4 Hz between the two protons on the ring, which is consistent with reported values [17]. A single crystal X-ray crystallography study was performed on one of the methylpyrazoline analogs, **1d**, which contained a bromine atom in the *para*-position. The X-ray results as shown in Fig. 2B provided unambiguous evidence of the *trans* configuration for the phenyl groups. The results also confirmed the "twisted" shape of **1d** determined from molecular modeling studies, which is similar to the shape of combretastatin A-4 [12].

### 2.3. Cytotoxicity

Methylpyrazolines **1a-m** were tested to determine their cytotoxicity against the growth of cancer cells in culture using an *in-vitro* 72 h continuous exposure-MTT assay [8–11,14]. Two murine cancer cell lines L1210 (lymphoma) and B16 (melanoma) were used in this study. The $IC_{50}$ values (μM) of each methylpyrazoline analog and CA-4 were determined and are provided in Table 1. It is immediately apparent from the results that three compounds (**1b**, **1f**, and **1j**) display significant cytotoxicity. These compounds have $IC_{50}$ values of less than 10 μM against L1210 cells, and for compound **1j** its $IC_{50}$ value against B16 was also less than 10 μM. With $IC_{50}$ values of 3.3 μM and 6.8 μM for L1210 and B16 cells, respectively, compound **1j** is the most potent. Compounds **1f** and **1b** have $IC_{50}$ values of 8.4 μM and 6 μM for the L1210 cells, and 95 μM and 45.5 μM for the B16 cells, respectively. For comparison, CA-4 is significantly more cytotoxic over the methylpyrazoline compounds described in this paper. The reported $IC_{50}$ values for CA-4 against the growth L1210 and B16 cells in culture were 0.003 and 0.002 μM, respectively [1b,7b].

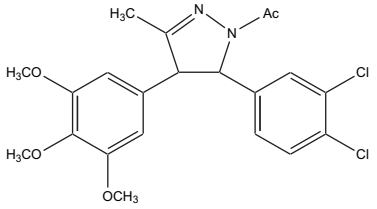
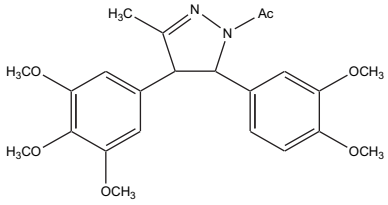
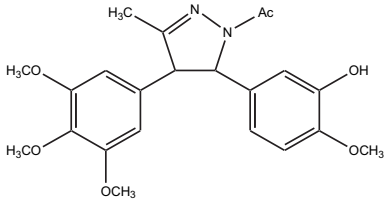
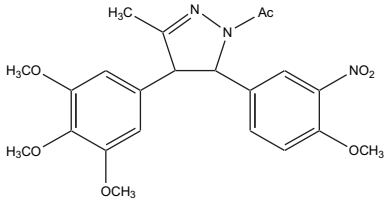
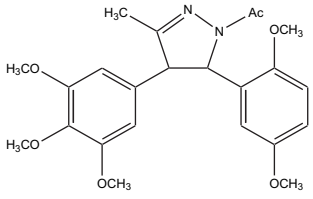
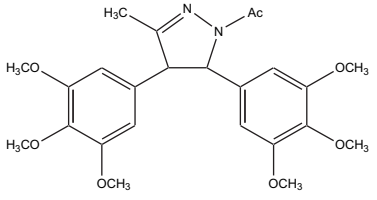
With $IC_{50}$ values of less than 50 μM, compounds **1a**, **1d**, **1h**, **1i**, and **1k** showed modest activity against L1210 cells. The other

**Table 1**
$IC_{50}$ values against the growth of L1210 and B16 cells. $IC_{50}$ values are expressed in μM.

| Compounds | | L1210 $IC_{50}$ (μM) | B16 $IC_{50}$ (μM) |
|---|---|---|---|
| Group I | | | |
| **1a** |  | 47 | >100 |
| **1b** |  | 6 | 45.5 |
| **1c** |  | >100 | >100 |
| **1d** |  | 33 | >100 |
| **1e** |  | >100 | >100 |
| **1f** |  | 8.4 | 95 |
| **1g** |  | >100 | >100 |
| Group II | | | |

(*continued on next page*)

**Table 1** (*continued*)

| Compounds | | L1210 $IC_{50}$ (μM) | B16 $IC_{50}$ (μM) |
|---|---|---|---|
| Group I | | | |
| **1h** |  | 36 | >100 |
| **1i** |  | 34 | >100 |
| **1j** |  | 3.3 | 6.8 |
| **1k** |  | 32.5 | >100 |
| **1l** |  | >100 | >100 |
| **1m** |  | >100 | >100 |
| CA-4 (**2**) [1b,7b] | | 0.003 | 0.002 |

compounds described in this study did not show any cytotoxicity against either cell line; their $IC_{50}$ values were greater than 100 μM. It is interesting to note that the results are consistent with earlier reports from our laboratory [8–11,14], the L1210 cells were more sensitive to the methylpyrazoline analogs and other potential vascular targeting agents than the B16 cells. This is presumably because the L1210 cells are more aggressive than B16 cells, making them more sensitive to tubulin inhibitors.

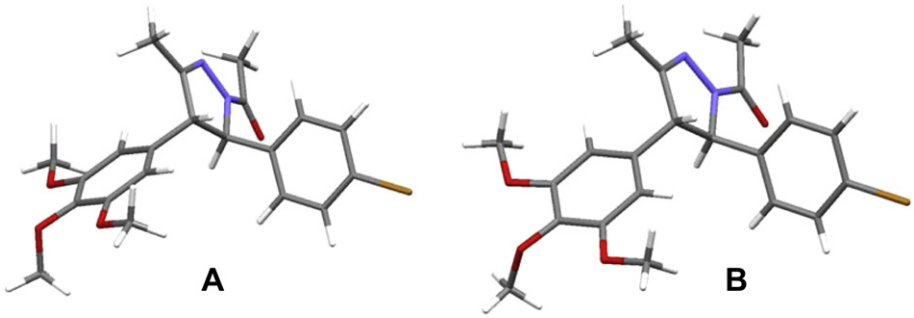
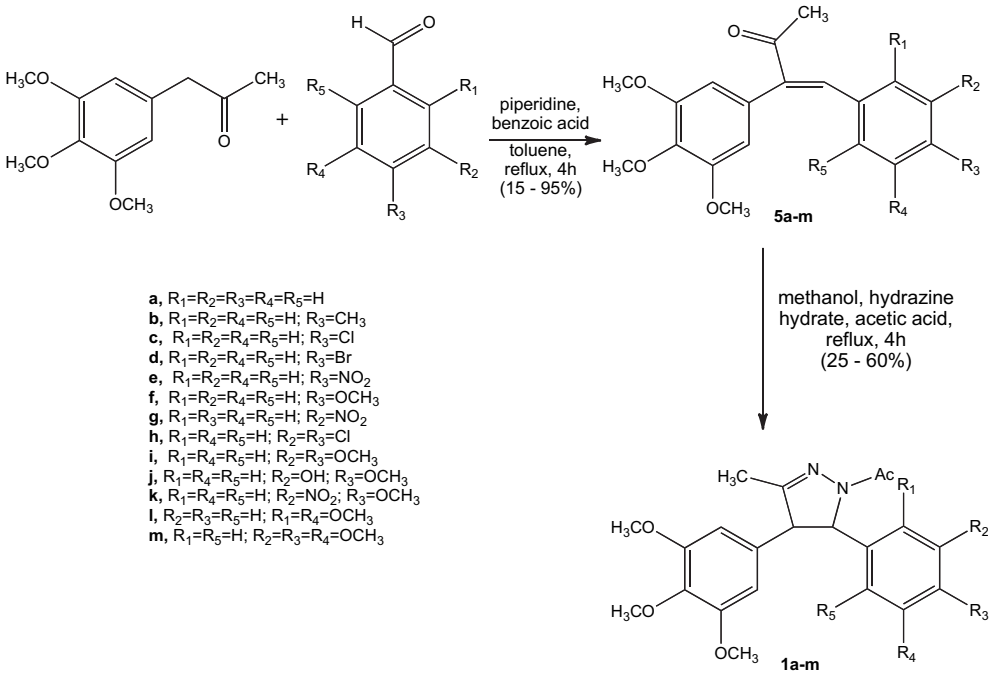



**Fig. 2.** Structure and conformation of the 4-bromomethylpyrazoline analog 1d as determined by (A) molecular modeling and (B) single crystal X-ray diffraction. Complete X-ray structural data for 1d were deposited with the Cambridge Structural Database.




**Scheme 1.** Synthesis of the target methylpyrazoline analogs of CA-4 (**1a-m**).

From the cytotoxicity data obtained four other trends were also noted. First, for the methylpyrazoline derivatives tested, there is no correlation between the substitution pattern of group I or II with cytotoxicity. Second, the data suggests that substituents in the 4-position (*para*) contribute to optimal cytotoxicity. It can be seen from compounds **1f** and **1j** that having a methoxy substitution at the *para*-position makes the compounds more potent. Third, the hydroxyl substitution in the 3-position of compound **1j** seems to have a significant effect on improving cytotoxicity against both the L1210 and B16 cells. The fourth trend evident from the data is that halides and nitro groups do not increase cytotoxicity. The most cytotoxic compound **1j** was selected for further evaluations to

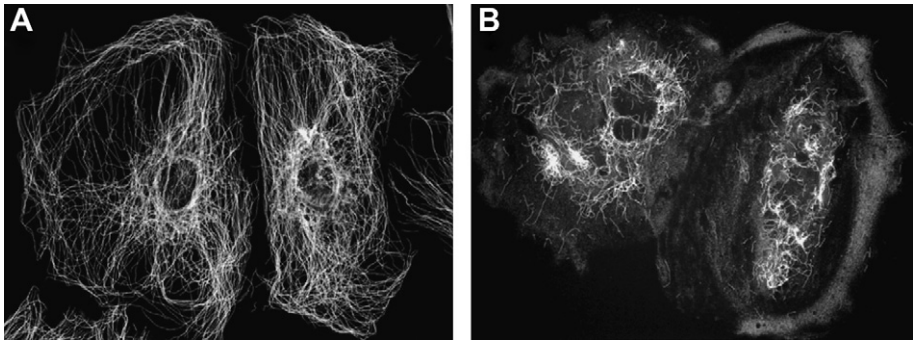



**Fig. 3.** Effects of compound 1j (6.25 μM) on interphase microtubule depolymerization in A-10 aortic smooth muscle cells.

determine its mechanism of action, cytotoxicity to other cancer cell lines, and aqueous solubility.

Analog **1j** was sent to the NCI (National Cancer Institute) for further testing against a panel of 60 different human cancer cell lines [18]. At a fixed concentration of 10 μM, compound **1j** was found to be cytotoxic and selective against certain human cancer cell lines. It was most cytotoxic against SF-539 CNS cancer, MDA-MB-435 melanoma, OVCAR-3 ovarian cancer, and HS-578T breast cancer cells. The results are summarized in a figure given in the supplementary materials section. In a separate study conducted in our laboratory, the cytotoxicity of compound **1j** against human melanoma MDA-MB-435 was found using an SRB assay (48 h exposure) to have an $IC_{50}$ value of 0.90 μM [11], which is consistent with NCI's $GI_{50}$ value for the same cells of 0.2 μM.

### 2.4. Microtubule depolymerization

Further studies were conducted to determine the mechanism of action for compound **1j** and its methylpyrazoline derivatives. The effect of compound **1j** on interphase microtubules was examined on A-10 aortic cells (See Fig. 3). The $EC_{50}$ value, the concentration that is required to cause 50% loss of cellular microtubules in A-10 cells, was found to be 4.1 μM. The results were further analyzed by calculating the ratio of $EC_{50}/IC_{50}$ (MDA-MB-435) for analog **1j**. Compounds that display a low ratio of $EC_{50}/IC_{50}$, such as compound 4.5 for **1j**, are expected to derive their cytotoxic activity from causing microtubule depolymerization. For comparison, the ratio of $EC_{50}/IC_{50}$ (MDA-MB-435) for CA-4 is 2.3 [11]. This suggests that microtubule depolymerization is most likely the mechanism of action for the methylpyrazoline analogs.

### 2.5. Water solubility

One important goal of this project was to design CA-4 analogs that possess an increased solubility in biological media. Thus, the lead compound **1j** was subjected to a test in which the aqueous solubility was measured. This assay utilized a modified version of the Multi Screen Solubility Filter Plate protocol developed by Millipore [11]. The maximum aqueous solubility for the assay is 500 μM, and the results showed that compound **1j** had solubility of 372 μM, which is slightly higher than that for CA-4, which was recorded as 350 μM [11].

## 3. Conclusion

In conclusion, methylpyrazoline compounds **1a-m** are effective analogs of combretastatin A-4. Several derivatives are cytotoxic against a wide variety of cancer cell lines grown in culture. The mechanism of action of the most cytotoxic analog **1j** was found to be microtubule depolymerization, consistent with the actions of CA-4. In addition, methylpyrazoline compounds were found to have noteworthy advantages in comparison to CA-4, which include increased water solubility and an easier synthetic scheme.

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Research Excellence award (SLM) is gratefully acknowledged. The X-ray diffractometer at Youngstown State University was funded by NSF Grant 0087210, Ohio Board of Regents Grant CAP-491, and by Youngstown State University.

## Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.ejmech.2011.03.064.

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