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Design and synthesis of novel enhanced water soluble hydroxyethyl analogs of combretastatin A-4

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ABSTRACT

Thirteen hydroxyethyl- analogs of combretastatin A-4 (CA-4) that contain the 1-(1′-hydroxyethyl)-1-(3″,4″,5″-trimethoxyphenyl)-2-(substituted phenyl)ethene framework were synthesized. Molecular modeling studies at the DFT level showed that compound **3j** adopts a 'twisted' conformation mimicking CA-4. The cytotoxicity of the novel compounds against the growth of murine B16 melanoma and L1210 lymphoma cells in culture was measured using an MTT assay. Three analogs **3f**, **3h**, and **3j** were active. Of these, **3j**, which has the same substituents as CA-4 and IC_{50} values of 16.1 and 4.1 μM against B16 and L1210 cells, respectively, was selected for further biological evaluation. The activity of **3j** was verified by the NCI 60 cell line screen. Compound 3j causes microtubule depolymerization in A-10 cells with an EC_{50} of 21.2 μM. Analog **3j**, which has excellent water solubility of 479 μM, had antitumor activity in a syngeneic L1210 murine model.

Combretastatin A-4 (**1a**, CA-4), as shown in Figure 1 is a natural product and vascular disrupting agent (VDA), first isolated by G.R. Pettit et al. in 1989.[1] It was isolated from the bark of the African Willow Tree, *Combretum caffrum*. VDAs including CA-4 are effective antitumor agents that are capable of rapidly and selectively disrupting the abnormal tumor vasculature resulting in vascular collapse and subsequent tumor necrosis.[2] Combretastatin A-4 binds to tubulin within the colchicine binding site, altering endothelial cell structure and causing vascular permeability and rapid destruction of tumor vasculature.[3] However, CA-4's possibilities as a clinical antitumor agent is hindered by its low bioavailability and poor aqueous solubility.[4] These limitations have led to the development of many water-soluble derivatives and analogs, the most important of which include a phosphate containing pro-drug (**1b**, CA-4P) as shown in Figure 1.[5] Other promising analogs include AC-7739 (**1c**), the prodrug **1d** and A105972, **1e**.[6a] These derivatives of CA-4 have high potency and increased aqueous solubility as compared to CA-4. Several combretastatin analogs are advancing in clinical trials.[6]

Other examples of cytotoxic analogs of CA-4 that have been synthesized in the author's laboratory include pyrazole,[7] pyrazoline,[8] cyclohexenone,[9] oxadiazoline (**1f**)[10] 2-thioxo-pyrimidine,[11] and acetyl-combretastatin analogs.[12] The X-ray crystallography structure and molecular modeling studies revealed that

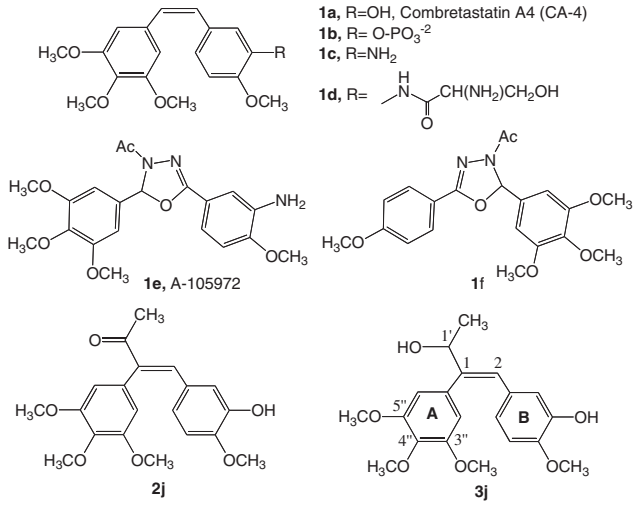

Figure 1. Structures of combretastatin A-4 derivatives **1a–d**, acetyl-analog **2j**, and the novel hyroxyethyl analog **3j**.

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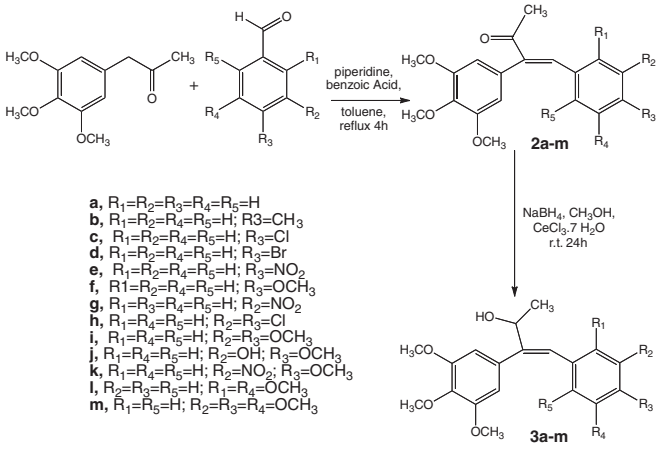
Table 1
Cytotoxicity of compounds **3a–m**.

Compounds	L1210 IC_{50} (μM)	B16 IC_{50} (μM)
GROUP A		
3a	>100	>100
3b	33.5	>100
3c	>100	>100
3d	>100	>100
3e	>100	>100
3f	3.9	17.5
3g	>100	>100
GROUP B		
3h	18.5	51.5
3i	69	>100
3j	4.1	16.1
3k	67.5	>100
3l	>100	>100
3m	51.5	84

IC_{50} values are given in μM. The cells were continuously treated with compounds for 72 h

compounds that lacked a 'twisted geometry' were inactive as microtubule depolymerizers and had lower cytotoxicity.[13]

Among these classes of analogs the acetyl-combretastatins (**2j**, Fig. 1), show great promise in both their potency and selectivity.



Scheme 1. Synthesis of the hydroxyethyl compounds **3a–m**.

But the task of creating analogs with the potent cytotoxicity of CA-4 and enhanced water solubility has proven much more challenging. For these reasons the related class of "hydroxyethyl" analogs were synthesized (**3j**, Fig. 1), with the goal of further increasing the aqueous solubility and potency of the acetyl analogs, while retaining the optimal twisted conformation. Thirteen specific analogs were synthesized and were divided into two groups shown in Table 1. The compounds in Group A contain a mono substitution on the B ring (**3a–g**), while compounds in Group B, contain a di- or tri-substitution on the B ring (**3h–m**). The conformation of analog **3j** was assessed by molecular modeling studies using MacSpartan. The molecular structures were optimized by molecular mechanics (MMFF) using a molecular equilibrium conformer procedure. The equilibrium geometry was subsequently optimized using the Hartree–Fock (3-21 G) calculations, followed by a density-functional calculation (B3LYP and 6-31 G). Results given in Figure 2, show that compound **3j** has a twisted conformation similar to CA-4 as well as the acetyl-CA-4, **2j**.

As described in Scheme 1, all 13 derivatives were synthesized in a two-step process by the reaction of the appropriately substituted benzaldehydes with 3,4,5-trimethoxy phenyl acetone[14] in the presence of benzoic acid and piperidine in refluxing toluene. This process yielded the acetyl-combretastatins **2a–m**,[12b] which were reduced using $NaBH_4$ and $CeCl_3{\cdot}7H_2O$ in methanol. The reaction was stirred for 24 h. The hydroxyethyl analogs **3a–m** were purified using silica gel column chromatography with ethyl acetate and hexane as eluents. The chemical yields were 25–80%. The structures of the compounds were confirmed by using IR, ^{1}H-NMR and mass spectral analysis. The *z*-configuration or *cis*-stilbene framework of analogs **2a–m** and **3a–m** was confirmed by a single crystal X-ray structure of **2e**.[12b] The chemical shift of the γ-vinylic proton of **2a–m** at the 7.50 ppm range[12a] also corroborated the *cis* geometry.

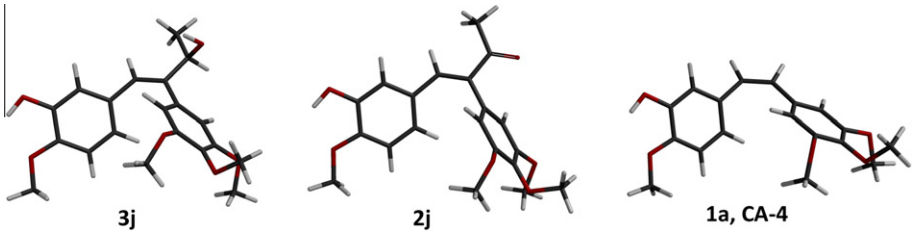


Figure 2. Molecular models of CA-4 (**1a**) and novel analogs **2j** and **3j** determined from Hartree–Fock and density functional calculations.

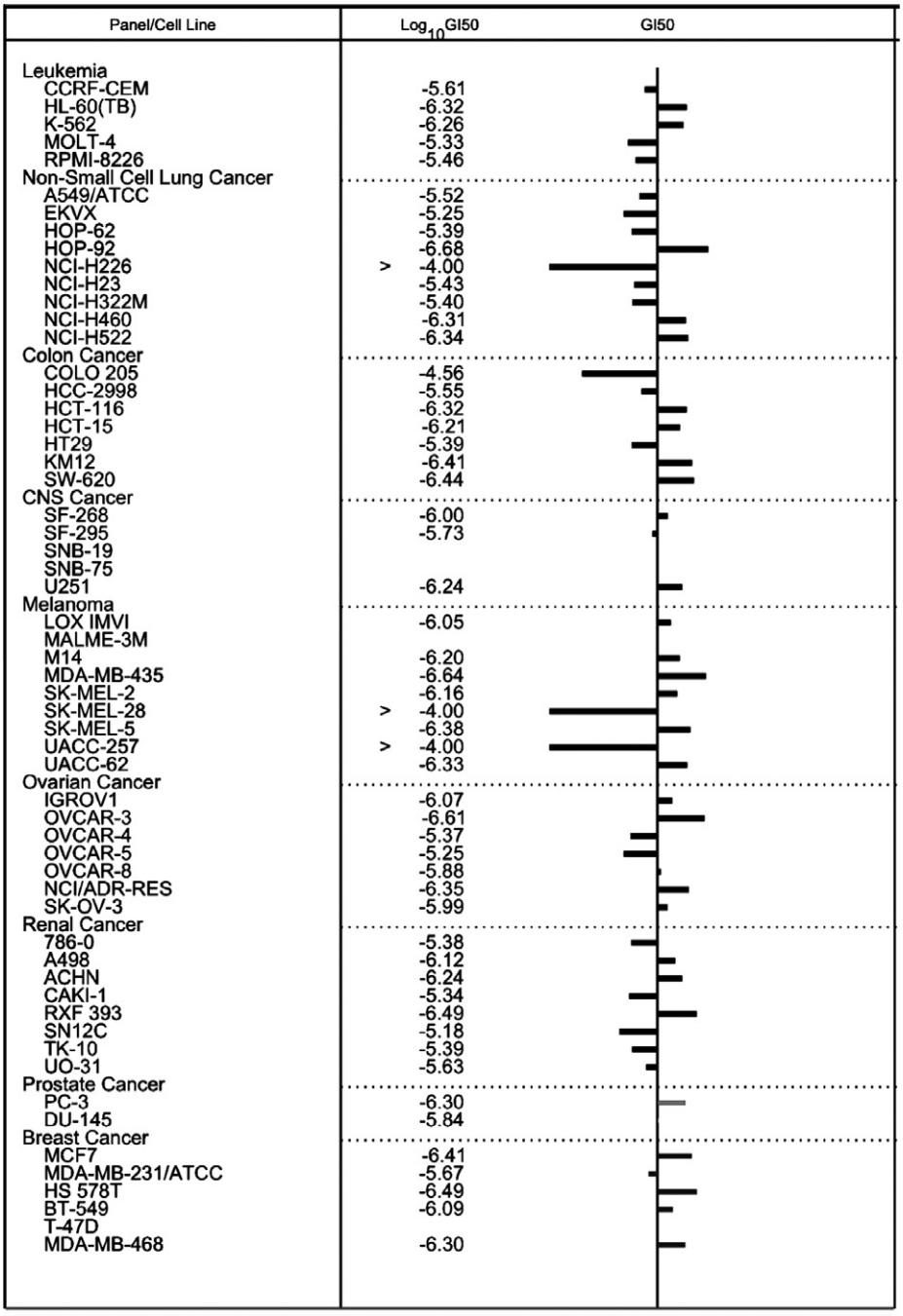


Figure 3. NCI screen data (GI_{50} values) for compounds **3j** against a panel of human cancer cell lines.

Table 2
Ratio of EC_{50} (microtubule depolymerization) to cytotoxicity (IC_{50}, MDA-MB-435)

Compound	EC_{50} (μM)	IC_{50} (μM)	EC_{50}/IC_{50}
1a[10]	0.003	0.007	2.3
1f[10]	0.5	0.14	3.6
2j[12b]	1.8	0.18	10
3j	21.2	0.88	24

Each of the hydroxyethyl compounds **3a–m** was evaluated for activity against L1210 and B16 cells (murine lymphoma and melanoma respectively) using a 72-h continuous exposure MTT assay.[15] The assays yielded IC_{50} values, the concentration required to inhibit cell growth by 50%. The IC_{50} values for each compound in B16 and L1210 cells are shown in Table 1.

In this class of 13 derivatives, two compounds (**3f** and **3j**) showed modest cytotoxicity with IC_{50} values of 3.9 and 4.1 μM against the L1210 cell line, respectively, and 17.5 and 16.1 μM for the B16 cell line, respectively. Compounds **3b**, **3i**, **3k**, and **3m** showed low activity with IC_{50} values in the range of 33.5–67.5 μM. The remaining analogs were inactive (IC_{50} >100 mM). It is worth noting that the L1210 cells are more sensitive to these compounds than B16 cells.[7–12] The nature of the differences in sensitivity might be related to the higher proliferation rate of L1210. Overall, the cytotoxicity results showed two clear trends. First, the results showed that substitution in the 4-position (*para*) significantly enhanced

cytotoxicity, especially for a methoxy group (**3f**) over its alkyl, halide, or nitro counterpart. We hypothesize that this is related to the size and electronic characteristics of the substituent. Second, the results showed that substitution on the 3-position further affected the potency of the compounds. Compound **3j**, which contains a hydroxy group on the 3-position and a methoxy group on the 4-position, is as equally active as compound **3f**. However, when the 3-position contains a bulkier substituent, such as a methoxy (**3i** and **3m**) or nitro (**3k**) group, the activity dropped significantly. Based on these results, analog **3j**, which has a substitution pattern closest to CA-4, was selected for further evaluation to investigate its mechanism of action, aqueous solubility, and activity in a murine tumor model.

The cytotoxicity of compound **3j**, was further evaluated by the NCI (National Cancer Institute). The NCI data provided the GI_{50} values of the compound against 60 human cancer cell lines.[16] The data show that this compound caused growth inhibition, with some selectivity. The results are summarized in Figure 3. In a separate study using a previously reported method,[10] the cytotoxicity of analog **3j** was determined to have an IC_{50} value of 0.88 μM against MDA-MB-435 human melanoma cells (Table 2), which was consistent with the GI_{50} value of 0.2 mM.

To gain insight into the mechanism of action of the hydroxyethyl CA-4 analogs, the effect of compound **3j** on interphase microtubules was examined using A-10 cells. The EC_{50} values, concentration required to cause 50% loss of interphase microtubules, for compounds **1a**, **1f**, **2j**, and **3j** are shown in Table 2. The results were further analyzed by calculating the ratio of EC_{50}/IC_{50} (MDA-MB-435) for analogs **1f**, 2j, and **3j**, and the results are given in Table 2. Compounds that give a ratio of 2:3, including **1a** and **1f**, show tight linkage of the microtubule depolymerizing and cytotoxic effects consistent with microtubule mediated primary mechanism of action. Oxadiazoline analog **1f** was previously confirmed to cause microtubule depolymerization.[10] With a ratio of EC_{50}/IC_{50} of 10 for analog **2j**, the result suggests that microtubule disruption contributes to the mechanism of action, but the linkage is not as tight as with **1a**, suggesting the possibility of a secondary mechanism of cytotoxicity.[12b] However, the hydroxyethyl analog, **3j**, which has an EC_{50}/IC_{50} ratio of 24, strongly suggests a secondary mechanism of action. This finding is not totally surprising, since small changes in the structure of CA-4 analogs have been shown to produce large changes in biological effects.[12] In fact, some CA-4 analogs are known to effect other biological targets, such as the kinesin spindle protein (KSP)[17] and DNA.[18]

With the significant in vitro activity of compound **3j** against cancer cells in culture, the effects of compound **3j** were preliminarily tested in vivo using DBA2 female mice that were inoculated with L1210 mouse lymphocytic leukemia cells on the right flank.[19] The mice were treated via an intraperitoneal (ip) route once a day with just the vehicle (control) or once a day with compound **3j** at a dose of 75 mg/kg starting on days 1, 3, 5, 7, 9, 11, 13, 15, and 17 after tumor inoculation. The volume of each injection was 100 μL.[20] On day 19, the tumor volume of **3j**-treated mice was reduced by 50% compared to the vehicle-treated control. It is worth noting that in a separate in vivo experiment, administration of compound **3j** at a dose of 75 mg/kg on days 1, 5, 9, 13, and 17 to healthy DBA2 female mice resulted in no visible toxicity as indicated by changes in body weight, blood cell counts, and the color and texture of the kidneys, liver, and spleen at the end of the experiment.

Since one of the goals of this work was to design water soluble CA-4 analogs, the aqueous solubility of **3j** was measured using a modified version of the Multi Screen Solubility Filter Plate protocol developed by Millipore.[10] The maximum aqueous solubility for the assay is 500 μM. A standard curve is developed for each compound by diluting into an 80:20 aqueous buffer:acetonitrile mixture with a final concentration of 5% DMSO (v/v). The concentration of the compound soluble in aqueous buffer without acetonitrile was determined from the standard curve. The aqueous sample was mixed for 6 h, centrifuged to pellet soluble material, and the supernatant removed. The absorbance of the supernatant was measured and the concentration of **3j** calculated from the standard curve. Our results show that compound **3j** has a solubility of 479 ± 20.0 μM. This is substantially higher than the solubility of CA-4, which was measured to be 350 μM in this assay.[6g,10]

In conclusion, hydroxyethyl CA-4 compounds **3a–m** are novel analogs of combretastatin A-4. Several of the compounds were cytotoxic against the growth of a number of cancer cells in vitro. The mechanism of action of compound **3j** is likely to be multifactorial, as indicated by the lack of close linkage of the concentration required to disrupt cellular microtubules and the IC_{50} concentration. The closest relative to CA-4, hydroxyethyl CA-4 **3j** demonstrated antitumor activity in mice with no toxicity. In addition, hydroxyethyl compounds have significant advantages, which include the ease of synthesis and enhanced aqueous solubility.

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20. The stock drug solutions were prepared by first dissolving 45 mg of each compound in a total of 450 μL of absolute ethanol, 900 μL of polyethylene glycol 400 and 150 μL Tween 80. Each solution was then diluted to a final volume of 3000 μL with a solution consisting of 1200 μL 5% glucose in sterile water and 300 μL of DMSO to give stock solutions for each compound comprising of a 75 mg/kg dosage. Both solutions were prepared in glass vials and stored at room temperature. Each 100 μL injection delivers 1.5 mg of the drug into the mice via an ip route.