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Computational Design and Synthesis of Novel Fluoro-Analogs of Combretastatins A-4 and A-1

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Abstract

Combretastatin A-1 (CA-1) and combretastatin A-4 (CA-4) isolated from the African bush willow *Combretum caffrum* are highly potent tubulin polymerization inhibitors, possessing strong antitumor activities because of their vascular disrupting properties. Extensive SAR studies have been done for CA-4 analogs. Because of poor solubility, water-soluble prodrugs of CA-4 and CA-1 have been developed, which are currently in human clinical trials. Fluorine plays an important role in the current drug discovery and development due to its unique properties. Thus, several fluorine-containing analogs of CA-4/CA-1 have been studied. However, no analogs, which have a CF₃O-, CF₂HO- or CF₃- group instead of the 4'-methoxy group in the B ring, have been investigated. Therefore, we set out to design and synthesize those novel fluoro-analogs of CA-4/CA-1. For the design of the new analogs, we took a structure-based design approach based on the X-ray crystal structure of colchicine-tubulin complex (PDB: 4O2B) and computational docking analysis using the AutoDock Vina program. A library of novel fluoro-analogs of CA-4/CA-1 was generated and their docking energy scores obtained. It was found that those novel fluoro-analogs exhibited better docking energy scores than CA-4/CA-1. Also, docking poses of all of these fluoro-analogs were virtually superimposable and very good fit to the colchine binding site. Among 15 compounds designed and analyzed, we have synthesized 5 compounds and evaluated their cytotoxicity against drug-sensitive and multidrug-resistant cancer cell lines. All fluoro-analogs exhibited strong cytotoxicity even against multidrug-resistant cell line. However, the critical activity of this class of compounds is its vascular disrupting activity. Thus, further biological evaluations are warranted for those novel fluoro-analogs of CA-4/CA-1.

Graphical Abstract

Novel fluoro-analogs of potent vascular disrupting agents (VDAs), combretastatins A-4 and A-1, were designed, synthesized and preliminary biological activity of select compounds examined.

First, a library of novel fluoro-analogs was created by a structure-based drug design based on the

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co-crystal structure of colchicine-tubulin complex and their docking energy scores were evaluated by AutoDock Vina. Then, select compounds were synthesized for biological evaluations.

Novel fluoro-analogs of combretastatin A-4/A-1 as vascular disrupting anticancer agents

Keywords

Combretastatin A-4 and A-1; Anticancer agent; Vascular disrupting agent; Fluoro-analog; Structure-based drug design; In silico screening; Structure-activity relationship

1. Introduction

The combretastatins are “stilbenoid” natural products isolated from *Combretum*, a genus including numbers of shrubs and trees. Although their chemical structures are simple, combretastatins are highly potent inhibitors of cell division. Among all the natural compounds extracted and characterized, combretastatin A-1 (CA-1) [1] and combretastatin A-4 (CA-4) [2] (Fig. 1) isolated from the African bush willow *Combretum caffrum*, were found to be the strongest tubulin polymerization inhibitors.

CA-4 binds to the colchicine site in the β -tubulin [3]. CA-4 is a microtubule-destabilizing agent that inhibits the polymerization of tubulin/microtubules [4]. Treating cells with CA-4 causes cell mytosis arrest at the G2/M phase, leading to apoptosis, and thus CA-4 is cytotoxic [5]. Although it is a cytotoxic agent, its main anticancer activity is attributed to its vascular disrupting property instead of cytotoxicity in clinically relevant dosing regimens [3]. As a vascular disrupting agent (VDA), CA-4 can cause complete tumor vasculature shutdown within 20 minutes [3]. Its vascular disrupting activity is believed to be associated with CA-4's binding to the microtubules of the endothelial cells in the blood vessels linked to tumors. Studies have shown that CA-4 mainly affects newly formed endothelial cells and does not affect quiescent endothelial cells [6].

Although CA-4 exhibits potent anticancer activities, it has limited water solubility [2]. Thus, a number of prodrugs were developed. In particular, CA-4 phosphate (CA-4P) [2] (also known as fosbretabulin) and OXI4503 (Fig. 2) are two combretastatin prodrugs that are currently in clinical trials. On the other hand, OXI4503 is the diphosphate prodrug of CA-1 and it is more effective than CA-4P in terms of antitumor activity [7]. Also, Ombrabulin, a 3'-amino-CA-4 prodrug, exhibited highly promising activities and advanced to phase III clinical trials, but later withdrawn [8] (Fig. 2).

Since the discovery of CA-4 and CA-1, a variety of CA-4/CA-1 derivatives and analogs have been synthesized for structure-activity-relationship (SAR) studies. It has been shown that (i) the trimethoxy groups in the A ring are critical for binding, but among the three methoxy

moieties, there is some flexibility in one of the *m*-methoxy groups, e.g., the chloro- and bromo-analogs exhibited a higher potency against certain cancer cell lines than CA-4, and (ii) the *para*-methoxy group in the ring B is essential for potency, but the hydroxyl group does not play an important role in terms of cytotoxicity and anti-tubulin activity [9].

The introduction of fluorine atoms into molecules of biological interest, such as steroids, carbohydrates, and others, proved beneficial. This growing interest is due to the unusual and unique chemical properties that fluorine can exert on the parent compound without altering its steric bulk [10, 11]. Electron withdrawing property of fluorine results in a strong polarization of the C-F bond, which exerts pronounced electronic effects on the reactions in adjacent positions, as well as on the drug-target interactions [12]. In addition, the incorporation of fluorine into a drug increases its lipophilicity, thus enhancing absorption into biological membranes, while the small covalent radius of fluorine can facilitate the docking of drugs with their receptor(s). The hydrophobic character of this covalent fluorine-carbon assembly is associated with a high dipole moment. This effect was recently described as polar hydrophobicity [13]. Accordingly, fluorine plays a significant role in medicinal chemistry, chemical biology and drug discovery, which is evident by the fact that a large number of fluorine-containing compounds have been approved by the FDA for medical and agricultural use and more than 20% of drugs currently available on the market contain at least one fluorine substituent [12, 14]. Thus, in the current drug discovery and development, it has become a common practice to explore fluorine-containing analogs of lead compounds for optimization.

Prompted by interesting results obtained with fluorinated colchicinoids against MDR cell lines [15], the first series of the fluoro-analogs of CA-4/CA-1 (FCAs) were synthesized by replacement of the hydroxyl [16], and/or methoxyl [17, 18] on the B-ring, as well as by replacement of the methoxy group(s) on the A-ring [19] and by introducing fluorine into the bridging olefin moiety [16] (Fig. 3). However, none of those fluoro-analogs exhibited substantial enhancement in their potencies in vitro. There are also examples where fluorine was introduced into both rings to examine the importance of the methoxy groups, especially in the A ring [20]. Wherein the replacement of three methoxy groups with three fluorine substituents was detrimental to both tubulin and cytotoxic activities [20].

Although several fluorine-containing CA-4 and CA-1 analogs have been synthesized and their tubulin polymerization inhibitory activity and cytotoxicity examined, no analogs incorporating a trifluoromethoxy, difluoromethoxy or trifluoromethyl group in the literature. Accordingly, we set out to design and synthesize those novel fluoro-analogs of CA-4/CA-1 for evaluation of their biological activities.

For designing the novel fluoro-analogs of CA-4/CA-1 and selection for chemical synthesis, we employed a structure-based design using the X-ray crystal structure of a tubulin-colchicine complex (PDB: 4O2B) [21], followed by docking to score their *in silico* binding ability.

2. Results and Discussion

2.1. Design and docking scores of novel fluoro-analogs of CA-4/CA-1

Prior to the docking study of novel fluoro-analogs of CA-4/CA-1 with the colchicine-binding site of tubulin, we examined the relevance and accuracy of the AutoDock Vina program by extracting the colchicine molecule from the crystal structure (PDB 4O2B, 2.3 Å resolution) [21], and then re-docked to the tubulin. Then, it was found that the docking pose with the lowest Autodock binding score (−7.11 kcal/mol) was nearly identical to that in the original X-ray crystal structure with an acceptable RMSD (< 2.0 Å). Next, CA-4 was docked to the colchicine binding site in the same manner. The docked pose of CA-4 overlapped very well with the colchicine molecule in the crystal structure with the docking score of −6.98 kcal/mole. Consequently, the AutoDock Vina program gave us confidence to use for the docking analysis of novel fluoro-analogs of CA-4/CA-1. Those operations are illustrated in Fig. 4.

The structure of tubulin-colchicine complex (PDB code: 4O2B [21]) is shown on the left, wherein the colchicine molecules (Cyan) bind to the interface of α,β -heterodimer in co-crystal structure. Red circle indicates the colchicine binding sites. In the top right circle are shown the binding poses of the docked CA-4 (light grey) and the colchicine (blue) in the crystal structure at the colchicine binding site. The docked CA-4 (score: −6.98 Kcal/mole) exhibits excellent overlap with colchicine (score of the docked colchicine: −7.11 Kcal/mol). In the bottom right circle are shown the binding poses of the docked CA-4 (light grey) and a fluoro-analog **Z-13** (binding score: −8.6 Kcal/mol) (pink) at the colchicine binding site. The fluoro-analog **Z-13** takes a very similar binding pose to that of CA-4.

For drug design, we introduced a trifluoromethoxy, difluoromethoxy or trifluoromethyl group at the 4-position of the B ring of CA-4/CA-1, replacing a methoxy group, to see how these fluorine-containing groups score in the docking. Next, we replaced the 3-hydroxyl group of CA-4 in the B ring with a fluorine or the 2',3'-dihydroxyl groups of CA-1 in the B ring with 2,3-difluoro substituents. We also introduced 2'-fluoro substituent without 3'-hydroxyl group. A 15 compounds library of novel fluoro-analogs of CA-4/CA-1, thus created, is shown in Fig. 5.

Each of all 15 compounds was docked and energy minimized. The docking scores are shown in Fig. 5, together with the Clog P values as estimated partition coefficient calculated by the ChemDraw program. As Fig. 5 indicates, the introduction of fluorine-containing substituents, i.e., CF₃O-, CF₂HO-, and CF₃-, to the 4' position of the B ring provides enhanced docking energy scores across the board. For CA-4, the replacement of the 3'-hydroxyl group in the B ring with fluorine increased the docking energy score by 1.0 Kcal/mol (see 3-F-DOH-CA-4). However, this fluorine-substitution effect is not obvious for 4'-CF₃O-, 4'-CF₂HO-, and 4'-CF₃-analogs. The introduction of 2'-fluorine substituent (without 3'-hydroxyl group) in place of 3'-fluorine substituent displayed very similar results. Interestingly, the substitution of 2',3'-dihydroxyl groups of CA-1 with 2',3'-difluoro substituents showed clear enhancement in the docking energy scores.

In order to examine the docking poses of select fluoro-analogs of CA-4/CA-1 with high docking scores, as well as to see how good the fitting to the binding pocket is, we created the close view of the tubulin surface with each fluoro-analog. Fig. 6 shows the docking poses of CA-4, **Z-2**, **Z-7** and **Z-13**. The Columbic surface of β -tubulin is colored in red and blue, showing positively and negatively charged surfaces. The van der Waals sizes of CA-4 and fluoro-analogs are indicated by mesh. The A ring with three methoxy group are deeply embedded in the hydrophobic area, providing an anchor for binding. The B ring extends to the shallower surface. The CF₃O-, CF₂HO- and CF₃-moieties of the fluoro-analogs can rotate relatively freely in the binding pocket. No clash is observed in all cases and docked molecules appear to fit to the binding pocket very well.

A zoom-up of the binding site of **Z-7** in tubulin is illustrated in Fig. 7. Other fluoro-analogs and CA-4 take virtually superimposable binding poses except for the differences at the 4'-position substituents. Close amino acid residues are mostly aliphatic ones such as Leu246, Leu253, Ile358, and Ile306. Only cys239 may have a polar interaction. Thus, the vast majority of interactions should be based on the van der Waals interactions.

2.2. Synthesis of novel fluoro-analogs of CA-4/CA-1

Among the 15 novel fluoro-analogs of CA-4/CA-1 shown in Fig. 5, we selected five analogs for chemical synthesis, which are **Z-2**, **Z-3**, **Z-7**, **Z-13** and **Z-14** (highlighted in blue in Fig. 5), based on the docking scores and commercial availability of fluorine-containing starting materials. The synthesis of these novel fluoro-analogs was carried out using basically the same method as that for CA-4 reported by Petit et al. [2]. Thus, the Wittig reactions of (3,4,5-trimethoxybenzyl)triphenylphosphonium bromide (**17**) with fluorine-containing benzaldehydes (**16a-e**) in the presence of *n*-butyllithium in THF at -78 °C gave the corresponding stilbenes as a mixture of *Z* (major) and *E* (minor) isomers in good to high overall yields (Fig. 8). These geometrical isomers were separated and isolated by column chromatography on silica gel to afford the bioactive *Z* isomers in moderate to fairly good yields. Although *E*-isomers were not the desirable products, we fully characterize the compounds since those are new chemical entities. Reaction conditions were not optimized. The very low yield of **Z-14** was due to non-optimal recrystallization conditions.

2.3. Preliminary biological evaluation of novel fluoro-analogs of CA-4/CA-1

The cytotoxicities of five novel fluoro-analogs, **Z-2**, **Z-3**, **Z-7**, **Z-13** and **Z-14**, were evaluated against three cancer cell lines, i.e., MCF-7 (human breast), NCI/ADR (human ovarian), ID8 (murine ovarian) and LCC6/MDR (human breast), using the standard MTT assay. Results are summarized in Table 1. MCF-7 and ID8 are drug-sensitive cancer cell lines, wherein P-glycoprotein (Pgp) is not overexpressed (Pgp-), while NCI/ADR and LCC6/MDR are multidrug-resistant cancer cell lines in which Pgp is overexpressed (Pgp+). As Table 1 shows, these five novel fluoro-analogs exhibited 53–118 nM IC₅₀ values against three cancer cell lines employed.

Notably, these fluoro-analogs showed no difference between drug-sensitive (Pgp-) and drug-resistant (Pgp+) cell lines. This means that these fluoro-analogs are not the substrates for Pgp. However, the docking energy scores and cytotoxicities are obviously not correlated.

Thus, CA-4 exhibited extremely strong cytotoxicities (ca. 3 nM IC₅₀ values) against these three cancer cell lines. These results are not surprising or disappointing since it has been shown that there is even larger difference in cytotoxicity between CA-4 and CA-1, as exemplified for the comparison of these two drugs against K562 (human myelogenous leukemia) and P388 (murine leukemia) cell lines. However, the inhibition of tubulin polymerization (ITP) activities of these two compounds are essentially the same, i.e., ca. 2 uM ID₅₀ [1, 9]. As mentioned in the Introduction, the key antitumor activity of CA-1 and CA-4 is not cytotoxicity, but vascular disruption. In fact, the water-soluble prodrug of CA-1 (Oxi4503) [7] is even more efficacious than the prodrug of CA-4 (CA-4P) [2]. Thus, the inhibitory activity of tubulin polymerization in endothelial cells is very important for this class of vascular disrupting agents (VDAs). Accordingly, we should look at the tubulin-polymerization inhibitory activity as well as vascular disruption activity of these novel fluoro-analogs. Those studies will be performed in due course and will be published elsewhere.

3. Experimental

3.1. General Method

¹H and ¹³C NMR spectra were measured on a Bruker 400, 500 or 700 MHz spectrometer in CDCl₃ using the residual proton of the solvent as the internal standard. ¹⁹F NMR were recorded on a Bruker 400 MHz spectrometer using CF₃C₆H₅ as the external standard. Melting points were measured on a Thomas–Hoover capillary melting point apparatus and are uncorrected. TLC was performed on Sorbent Technologies aluminum-backed Silica G TLC plates (Sorbent Technologies, 200 μm, 20 cm × 20 cm), and column chromatography was carried out on silica gel 60 (Merck, 230–400 mesh ASTM). Low-resolution mass spectra were obtained using flow injection analysis on an Agilent LC-MSD mass spectrometer, and high-resolution mass spectrometry (HRMS) analysis was carried out on an Agilent LC-UV-TOF mass spectrometer at the Institute of Chemical Biology and Drug Discovery, Stony Brook, New York.

3.2. Materials

The chemicals were purchased from Sigma-Aldrich, Fisher Scientific, and VWR International, and used as received or purified before use by standard methods. Tetrahydrofuran was freshly distilled from sodium and benzophenone. Dichloromethane was also distilled immediately prior to use under nitrogen from calcium hydride. 3,4,5-Trimethoxybenzyl bromide was prepared from 3,4,5-trimethoxybenzyl alcohol by the literature method [2]. 4-Trifluoromethoxybenzaldehyde (Oakwood Chemical), 3-fluoro-4-trifluoromethoxybenzaldehyde (Alfa Aesar), 4-difluoromethoxybenzaldehyde (Ox-Chem), 3-fluoro-4-trifluoromethyl-benzaldehyde (Alfa Aesar) and 2-fluoro-4-trifluoromethylbenzaldehyde (Alfa Aesar) were commercially available and used as received.

3.3. Docking evaluation of fluoro-analogs of CA-4/CA-1 to the colchicine site in microtubules

Fluoro-analogs of CA-4/CA-1 were first constructed using the ChemDraw (PerkinElmer) program, energy minimized, and converted to mol2 files. The structures in the MOL format

was translated into the Protein Databank (PDB) format via UCSF Chimera (UCSF, San Francisco, California). Once in PDB format, the molecules were individually loaded into the AutoDock Tools program (The Scripps Research Institute, La Jolla, California). They were assigned as ligands, assigned rotatable bonds, and a root, then saved as a pdbqt file. The co-crystal structure of the tubulin-colchicine was downloaded from rcsb.org (PDB code 4O2B). Chains C and D (α,β -tubulin) of the crystal structure were isolated for docking analysis and the colchicine at the interface of α,β -tubulin dimer was deleted during docking. The α,β -tubulin dimer, thus produced, was subjected to a brief energy minimization using the AMBER force field. The docked structures were energy minimized using the Merck Molecular Force Field 94s (MMFF94s). Docking was performed using the AutoDock Vina with the following parameters (untransformed coordinates of the protein).

Box center (x axis) = -13.871

Box center (y axis) = -9.733

Box center (z axis) = -20.602

Box size (x axis) = 50

Box size (y axis) = 50

Box size (z axis) = 50

Exhaustiveness = 64

The docking results were analyzed and visualized in both AutoDock Tools and UCSF Chimera.

3.4. Synthesis of (Z) and (E)-fluoro-analogs of CA-4/CA-1

A typical procedure is described for the synthesis of (Z) and (E)-4'-trifluoromethoxy-3,4,5-trimethoxystilbene (**Z-2** and **E-2**). All other fluoro-analogs of CA-4/CA-1 were synthesized in the same manner.

3,4,5-Trimethoxybenzylphosphonium bromide (**17**) (500 mg, 0.96 mmol) was dissolved in THF (15 mL, 0.067M) and cooled to -78 °C for 2 h. Then, *n*-BuLi (2.5 M in hexane, 0.40 mL, 0.96 mmol, 1.0 eq) was added to this solution. The reaction mixture turned red. After 1 h at -78 °C, 4-trifluoromethoxybenzaldehyde (**16**) (240 mg, 1.26 mmol, 1.3 eq) was added dropwise. The reaction mixture turned from red into orange to yellow. After stirring overnight, the reaction was quenched by addition of water, and the mixture was extracted with ethyl acetate. The organic layer was dried over anhydrous magnesium sulfate, filtered and concentrated on a rotary evaporator. The residue was subjected to column chromatography on silica gel (hexanes:ethyl acetate 9:1) to afford **Z-2** as a clear oil (177 mg, 52% yield), which became a white solid afterwards, and **E-2** as a white solid (78 mg, 23% yield).

3.4.1. (Z)-4'-trifluoromethoxy-3,4,5-trimethoxystilbene (Z-2)—Mp 70–71 °C; ^1H NMR (700 MHz, CDCl_3) δ 3.67 (s, 6 H), 3.86 (s, 3 H), 6.43 (s, 2 H), 6.58 (ABq, $J = 12.4$ Hz, 2H), 7.14 (d, $J = 8.2$ Hz, 2 H), 7.33 (dq, $J = 8.2, 1.4$ Hz, 2 H); ^{13}C NMR (175 MHz, CDCl_3) δ 55.76, 60.93, 106.01, 120.46 (q, $J = 254.6$ Hz), 120.89, 128.36, 130.38, 131.15,

131.93, 136.33, 137.39, 148.06, 152.96; ^{19}F NMR (376 MHz, CDCl_3) δ -57.82, -57.97; HRMS (TOF, m/z): Calcd. for $\text{C}_{18}\text{H}_{17}\text{F}_3\text{O}_4\cdot\text{H}^+$: 355.1152; Found: 355.1156 (= -1.18 ppm).

3.4.2. (E)-4'-trifluoromethoxy-3,4,5-trimethoxystilbene (E-2)—Mp. 92–93 °C; ^1H NMR (400 MHz, CDCl_3) δ 3.90 (s, 6 H), 3.94 (s, 3 H), 6.76 (s, 2 H), 7.02 (ABq, J = 16.34 Hz, 2H), 7.22 (d, J = 8.36 Hz, 1H), 7.53 (d, J = 8.36 Hz, 1 H); ^{13}C NMR (125 MHz, CDCl_3) δ 56.17, 61.00, 103.69, 120.50 (q, J = 255.6 Hz), 121.23, 126.58, 127.58, 129.67, 132.65, 136.90, 138.25, 148.05, 153.47; ^{19}F NMR (376 MHz, CDCl_3) δ -57.83; HRMS (TOF, m/z): Calcd. for $\text{C}_{18}\text{H}_{17}\text{F}_3\text{O}_4\cdot\text{H}^+$: 355.1152; Found: 355.1157 (= -1.54 ppm).

3.4.3. (Z)-3'-fluoro-4'-trifluoromethoxy-3,4,5-trimethoxystilbene (Z-3)—Colorless oil; 57% yield; ^1H NMR (400 MHz, CDCl_3) δ 3.70 (s, 6 H), 3.87 (s, 3 H), 6.43 (s, 2 H), 6.51 (d, J = 12.14 Hz, 1 H), 6.62 (d, J = 12.14 Hz, 1 H), 7.04 (d, 1H, J = 8.68 Hz), 7.15 (dd, J = 11.08, 1.84 Hz, 1 H), 7.23 (t, J = 8.68 Hz, 1 H); ^{13}C NMR (100 MHz, CDCl_3) δ 55.86, 60.97, 106.04, 117.47, 117.62, 120.47 (q, J = 257.6 Hz), 123.48, 125.27 (d, J = 1.8 Hz), 127.27 (d, J = 3.7 Hz), 130.25, 132.24, 137.98 (d, J = 60.43 Hz), 153.00, 154.30 (d, J = 241.4 Hz), 155.51; ^{19}F NMR (376 MHz, CDCl_3) δ -58.97 (d, J = 4.3 Hz, 3 F), -129.96 (q, J = 4.3 Hz, 1 F); HRMS (TOF, m/z): Calcd. for $\text{C}_{18}\text{H}_{16}\text{F}_4\text{O}_4\cdot\text{H}^+$: 373.1057; Found: 373.1065 (= -2.12 ppm).

3.4.4. (E)-3'-fluoro-4'-trifluoromethoxy-3,4,5-trimethoxystilbene (E-3)—White solid; mp 109–110 °C; 21% yield; ^1H NMR (400 MHz, CDCl_3) δ 3.90 (s, 3 H), 3.94 (s, 6 H), 6.75 (s, 2 H), 6.94 (d, J = 16.34 Hz, 1 H), 7.03 (d, J = 16.34 Hz, 1 H), 7.25–7.32 (m, 2 H), 7.36 (dd, J = 11.08, 1.84 Hz, 1 H); ^{13}C NMR (125 MHz, CDCl_3) δ 56.18, 61.00, 103.87, 114.35 (d, J = 19.0 Hz), 120.45 (d, J = 257.5 Hz), 122.57, 123.84, 125.61 (d, J = 2.6 Hz), 131.05, 132.65, 138.33 (d, J = 58.6 Hz), 144.45, 154.72 (d, J = 241.2 Hz); ^{19}F NMR (376 MHz, CDCl_3) δ -58.80, (d, J = 4.4 Hz, 3 F), -128.82 (q, J = 4.4 Hz, 1 F). HRMS (TOF, m/z): Calcd. for $\text{C}_{18}\text{H}_{16}\text{F}_4\text{O}_4\cdot\text{H}^+$: 373.1057; Found: 373.1062 (= -1.12 ppm).

3.4.5. (Z)-4'-Difluoromethoxy-3,4,5-trimethoxystilbene (Z-7)—Colorless oil; 61% yield; ^1H NMR (400 MHz, CDCl_3) δ 3.69 (s, 6 H); 3.89 (s, 3 H); 6.46 (s, 2 H), 6.51 (t, J = 73.6 Hz, 1 H), 6.55 (ABq, J = 12.30 Hz, 2 H), 7.04 (d, J = 8.68 Hz, 2 H), 7.30 (d, J = 8.68 Hz, 2 H); ^{13}C NMR (125 MHz, CDCl_3) δ 55.87, 60.95, 106.01, 115.71 (t, J = 259.4 Hz), 119.52, 128.64, 130.42, 130.61, 132.50, 134.76, 137.36, 149.98, 152.98; ^{19}F NMR (376 MHz, CDCl_3) δ -81.05 (d, J = 73.6 Hz); HRMS (TOF, m/z): Calcd. for $\text{C}_{18}\text{H}_{18}\text{F}_2\text{O}_4\cdot\text{H}^+$: 337.1246; Found: 337.1249 (= -0.78 ppm).

3.4.6. (E)-4'-Difluoromethoxy-3,4,5-trimethoxystilbene (E-7)—Colorless oil; 30% yield; ^1H NMR (500 MHz, CDCl_3) δ 3.90 (s, 3 H), 3.95 (s, 6 H), 6.55 (t, 1 H, J = 73.9 Hz), 7.00 (s, 2 H) 7.14 (d, 2 H, J = 8.60 Hz), 7.52 (d, 2 H, J = 8.60 Hz); ^{13}C NMR (125 MHz, CDCl_3) δ 56.16, 61.00, 103.61, 115.89 (t, J = 258.38 Hz), 119.81, 126.84, 127.70, 128.97, 132.81, 134.66, 138.12, 150.50 (t, J = 2.84 Hz), 153.46; ^{19}F NMR (376 MHz, CDCl_3) -80.69 (d, J = 23.1 Hz); HRMS (TOF, m/z): Calcd. for $\text{C}_{18}\text{H}_{18}\text{F}_2\text{O}_4\cdot\text{H}^+$: 337.1246; Found: 337.1250 (= -0.96 ppm).

3.3.7. (Z)-3'-Fluoro-4'-trifluoromethyl-3,4,5-trimethoxystilbene (Z-13)—Colorless oil; 63% yield: ^1H NMR (400 MHz, CDCl_3) δ 3.71 (s, 6 H), 3.87 (s, 3 H), 6.43 (s, 2 H), 6.54 (d, $J = 12.0$ Hz, 1 H), 6.69 (d, $J = 12.0$ Hz, 1 H), 7.14 (d, $J = 11.24$ Hz, 1 H), 7.17 (d, $J = 8.36$ Hz, 1 H), 7.50 (t, $J = 8.36$ Hz, 1 H); ^{13}C NMR (125 MHz, CDCl_3) δ 55.94, 60.98, 106.04, 117.11 (m), 122.58 (q, $J = 269.8$ Hz), 124.66, 124.69, 126.99 (d, $J = 31.6$ Hz), 127.32, 131.25, 133.32, 137.94, 143.89, 153.16, 159.60 (d, $J = 256.5$ Hz); ^{19}F NMR (376 MHz, CDCl_3) δ -61.22 (d, $J = 12.3$ Hz, 3 F), -114.82 (t, $J = 12.3$ Hz, 1 F); HRMS (TOF, m/z): Calcd. for $\text{C}_{18}\text{H}_{16}\text{F}_4\text{O}_3 \cdot \text{H}^+$: 357.1108; Found: 357.1120 ($\delta = -3.19$ ppm).

3.4.8. (E)-3'-Fluoro-4'-trifluoromethyl-3,4,5-trimethoxystilbene (E-13)—White solid; mp 158–159 °C; 25% yield; ^1H NMR (400 MHz, CDCl_3) δ 3.91 (s, 3 H), 3.95 (s, 6 H), 6.78 (s, 2 H), 6.98 (d, $J = 16.24$ Hz, 1 H), 7.13 (d, $J = 16.24$ Hz, 1 H), 7.34 (d, 1 H, $J = 12.8$ Hz), 7.35 (d, $J = 7.68$ Hz, 1 H), 7.59 (t, $J = 7.68$ Hz, 1 H); ^{13}C NMR (125 MHz, CDCl_3) δ 56.20, 61.02, 104.08, 113.83 (m), 116.73 (m), 122.0, 122.70 (q, $J = 270.1$ Hz), 125.40, 127.42 (m), 131.80, 132.51, 138.85, 143.50, 153.53, 160.10 (d, $J = 253.8$ Hz); ^{19}F NMR (376 MHz, CDCl_3) δ -61.07 (d, $J = 12.3$ Hz, 3 H), -114.76 (t, $J = 12.3$ Hz, 1 F); HRMS (TOF, m/z): Calcd. for $\text{C}_{18}\text{H}_{16}\text{F}_4\text{O}_3 \cdot \text{H}^+$: 357.1108; Found: 357.1122 ($\delta = -3.84$ ppm).

3.4.9. (Z)-2'-Fluoro-4'-trifluoromethyl-3,4,5-trimethoxystilbene (Z-14)—White solid; mp. 77–78 °C; 18% yield after recrystallization for hexanes/EtOAc; ^1H NMR (700 MHz, CDCl_3) δ 3.66 (s, 6H), 3.85 (s, 3H), 6.40 (s, 2H), 6.57 (d, $J = 12.2$ Hz, 1H), 6.75 (d, $J = 12.2$ Hz, 1H), 7.27 (d, $J = 7.58$ Hz, 1H), 7.34 (d, $J = 9.59$ Hz, 1H), 7.41 (t, $J = 7.58$ Hz, 1H); ^{13}C NMR (175 MHz, CDCl_3) δ 55.83, 60.94, 105.86, 113.12 (m), 120.43 (d, $J = 3.7$ Hz), 120.60 (d, $J = 47.1$ Hz), 123.24 (q, $J = 270.6$ Hz), 129.10 (d, $J = 14.6$ Hz), 131.44 (d, $J = 5.23$ Hz), 131.47, 134.05, 137.822, 153.06, 159.87 (d, $J = 248.9$ Hz); ^{19}F NMR (376 MHz, CDCl_3) δ -62.80 (3F), -112.4 (1F); HRMS (TOF, m/z): Calcd. for $\text{C}_{18}\text{H}_{15}\text{F}_4\text{O}_3 \cdot \text{H}^+$, 357.1108; found 357.1118 ($\delta = 1.58$ ppm)

3.5. In vitro cell growth inhibition assay for fluoro-analogs of CA-4/CA-1

Cancer cell growth inhibition was determined according to the method established by Skehan et al. [23]. Cancer cell lines, MCF7 (human breast), NCI/ADR (human ovary), and ID8 (murine ovary) were cultured as specified by ATCC (Manassas, Virginia). Cancer cells were cultured at 37°C in a humidified incubator with an atmosphere of 95% air and 5% CO_2 . For cytotoxicity assays the cells were plated at a density of $6\text{--}8 \times 10^3$ cells/well in 24-well plates and allowed to adhere overnight. The media was changed the following morning and replaced with media containing fluoro-analogs of CA-4/CA-1 or vehicle control. Fluoro-analogs of CA-4/CA-1 were dissolved in sterile DMSO to 10 mM concentration as a stock solution and were further diluted in appropriate media prior to addition to cells. The highest concentration of DMSO in drug solution was 0.5 %. Each dose of drug or vehicle was tested in triplicate, and the experiment is representative of at least 3 independent trials. After 72 hours of treatment the media was aspirated and the cells were washed in warm PBS. MTT reagent (Sigma-Aldrich) was diluted in RPMI-1640 media without phenol red (Invitrogen), and added to the cells at a concentration of 0.5 mg/mL. After 3 hours of incubation, the reagent was aspirated, the plate was washed with PBS and MTT formazan

crystals were dissolved in DMSO (50 μ L/well). Absorbance at 570 nm was measured on a thermomax plate reader (Molecular Devices). The IC₅₀ values were then calculated by fitting the concentration-effect curve data with the sigmoid- $E_{\max}$ model using nonlinear regression, weighted by the reciprocal of the square of the predicted effect [24].

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Highlights

- Structure-based drug design of novel fluoro-analogs of combretastatin A-4/A-1.
- Docking study suggested enhanced binding of fluoro-analogs to tubulin.
- Select fluoro-analogs with high docking scores were synthesized and tested.
- Select novel fluoro-analogs of CA-4/CA-1 exhibited strong cytotoxicity.
- Novel fluoro-analogs may serve as new vascular disrupting anticancer agents.

Fig. 1.
Structure of CA-4 and CA-1

Fig. 2.
Prodrugs of CA-4/CA-1 and 3'-amino-CA-4

Fig. 3.
Known fluoro-analogs of CA-4/CA-1

Fig. 4. Binding structures of docked colchicine and CA-4 (top right circle), as well as those of the docked CA-4 and a fluoro-analog **Z-13** (bottom right circle) at the colchicine binding sites (PDB: 4O2B) [21].

Fig. 5. Design of novel fluoro-analogs of CA-4/CA-1 (only Z isomers are shown) and their docking scores at the colchicine-binding site of tubulin, using AutoDock Vina

Fig. 6.
Close view of the tubulin surface at the colchicine binding site with CA-4 (light grey in **A**) and fluoro-analogs **Z-2** (dark grey in **B**), **Z-7** (pink, in **C**), **Z-13** (green in **D**).

Fig. 7.
Closeup of the docked **Z-7** (−8.3 Kcal/mol) in the binding site in tubulin and key amino acid residues in vicinity (<5Å).

Fig. 8.
Synthesis of select novel fluoro-analogs of CA-4/CA-1

Cytotoxicities (IC₅₀, μM) of select fluoro-analogs of CA-4/CA-1 against three cancer cell lines^a

Table 1

Compound	Binding affinity Kcal/mol ^b	MCF-7 ^c	NCI/AD R-RES ^d	ID8 ^e	LCC6/MDR ^f	K562 ^g	P388 ^h	ITP ⁱ
Z-2	-7.7	0.076	0.057	0.062	--	--	--	--
Z-3	-8.1	0.100	0.080	0.059	--	--	--	--
Z-7	-8.3	0.069	0.054	0.053	--	--	--	--
Z-13	-8.6	0.118	0.113	0.118	--	--	--	--
Z-14	-8.1	0.095	0.105	--	0.088	--	--	--
CA-4	-7.0	0.003	0.003	0.003	--	0.003 ^j	0.0003 ^k	1.8 ^k
CA-1	-6.9	--	--	--	--	0.332 ^j	0.251 ^k	2 ^l

^aIC₅₀ values are average of three experiments with less than 10% SD;

^bDetermined by AutoDock Vina with PDB 4O2B [21];

^chuman breast cancer cell line (Pgp-);

^dhuman ovarian cancer cell line (Pgp+);

^emurine ovarian cancer cell line;

^fhuman breast cancer cell line (Pgp+);

^ghuman myelogenous leukemia cell line;

^hmouse leukemia cell line;

ⁱInhibition of tubulin/microtubule assembly (ID₅₀, μM);

^jvalues from Ref. [22];

^kvalues from Ref. [9];

^lvalue from Ref. [1].