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Original article

Design, synthesis and anti-proliferative effects in tumor cells of new combretastatin A-4 analogs

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ABSTRACT

A total of 11 novel combretastatin A-4 (CA-4) analogs were designed, synthesized, and evaluated for the anti-proliferative effects in tumor cells. The compounds represent four structural classes: (i) hydrogenated derivatives, (ii) ethoxyl derivatives, (iii) amino derivatives and (iv) pro-drugs. Biological evaluations demonstrate that multiple structural features control the biological potency. Three of the compounds, **sit-1**, **sit-2** and **sit-3**, have potent anti-proliferative activity against multiple cancer cell lines. Their pro-drugs were synthesized to increase water solubility. Structure–activity relationship study and Surflex-Docking were studied in this paper. These results will be useful for the design of new CA-4 analogs that are structurally related to the SAR study.

1. Introduction

Combretastatins were first extracted from the bark of the South African bush willow tree combretum caffrum in 1982 by Pettit et al. [1]. Those compounds may serve as a new anticancer drug that utilized starvation tactics to attack solid tumors. Among the combretastatin family, combretastatin A-4 (CA-4) (Fig. 1; (*Z*)-5-(3,4,5-trimethoxystyryl)-2-methoxyphenol) exerts a potent inhibition of tubulin polymerization by binding to the colchicine site and, as a consequence, demonstrates strong activity in suppressing tumor blood flow (TBF). Several studies described its ability to induce widespread necrosis of solid tumors, including multidrug-resistant ones, which suggested CA-4 is an attractive lead compound for the development of novel antitubulin anticancer agents [2,3].

However, CA-4 does not show *in vivo* efficacy due to its poor pharmacokinetics resulting from its high lipophilicity, low aqueous solubility and also isomerization of *cis*-double bond to the more thermally stable *trans*-isomer [4–6]. So, considerable efforts have gone into modifying CA-4 to improve its water solubility and *in vivo* efficacy. To date, various CA-4 analogs have been synthesized and reported to possess cytotoxic activity against sever cancer cell lines. For example, a pro-drug of CA-4, the water-soluble phosphate derivative CA-4P (fosbretabulin) is now in phase II clinical trials. Another combretastatin A-4 analog, the serine amide AVE8062 (ombrabulin) is not only in a phase II clinical trial in combination with taxanes and platinum salts in advanced solid tumors, but also in a phase II/III clinical trial in patients with advanced soft tissue sarcoma [7–9].

To study the relationship between the structure of CA-4 and the anti-proliferative effect in human cancer cells (SAR study), we synthesized 11 CA-4 analogs containing (i) hydrogenated derivatives, (ii) ethoxyl derivatives, (iii) amino derivatives and (iv) pro-drugs. The anti-proliferative effect of each class was tested using the MTT assay in human cancer cells *in vitro*. To understand the SAR of the CA-4 analogs, Surflex-Docking was applied to study the interactions between these analogs and tubulin. Furthermore, we have designed and synthesized a number of pro-drugs of potent CA-4 analogs to increase the water solubility.

2. Experimental

2.1. Synthesis

2.1.1. General methods

All the chemicals and reagents were commercially available and required no further purifications. Solvents (THF, DMF, CH_2Cl_2, benzene) were dried and freshly distilled before use according to

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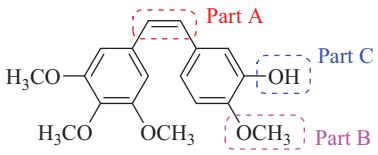


Fig. 1. Structure of combretastatin A-4.

literature procedures. Chromatographic separations were performed on silica gel flash columns. TLC analyses were performed on precoated silica gel polyester plates with a fluorescent indicator UV 254. Melting points were determined using a melting point apparatus (WRS-2A) and uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE III at 500 MHz or on a Bruker spectrometer at 101 Hz in chloroform-*d* using TMS (δ = 0.0 ppm) as an internal standard. IR was recorded on a NICOLET 6700 FT-IR. HRMS were recorded on a solanX 70 FT-MS spectrometer using methanol and water (v/v = 1:1) as solvent. LC–Mass spectra were recorded on a LCMS-2020 spectrometer from Shimadzu Corporation with acetonitrile and water as the mobile phase and the gradient was from 5% of acetonitrile at 0 min to 100% of acetonitrile at 10 min.

2.1.2. (Z)-2-Methoxy-5-(3,4,5-trimethoxystyryl)phenol (CA-4)

Following the synthetic method from Shen et al. [10], compound CA-4 was obtained in 35% yield. mp 116.8–117.6 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 3.69 (s, 6H), 3.84 (s, 6H), 3.85 (s, 3H), 5.58 (s, 1H), 6.41 (d, 1H, *J* = 12.0 Hz), 6.46 (d, 1H, *J* = 12.0 Hz), 6.53 (s, 2H), 6.73 (d, 1H, *J* = 8.5 Hz), 6.79 (dd, 1H, *J* = 8.5 Hz, *J* = 2.0 Hz), 6.92(d, 1H, *J* = 2.0 Hz).

*2.1.3. 2-Methoxy-5-(3,4,5-trimethoxyphenethyl)phenol (**sit-1**)*

A solution of compound CA-4 (1.0 g, 3.16 mmol) and palladium-carbon (10%, 0.1 g) in MeOH (20 mL) was stirred at room temperature for 2 h under hydrogen atmosphere. Palladium-carbon was filtered off, the filtrate was dried over Na_2SO_4 and concentrated. Compound **sit-1** was obtained in 91% yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.81 (d, 1H, *J* = 3.0 Hz), 6.77 (d, 1H, *J* = 8.0 Hz), 6.64–6.66 (m, 1H), 6.38 (s, 2H), 5.61(s, 1H), 3.87 (s, 3H), 3.83 (s, 9H), 2.82 (s, 4H).

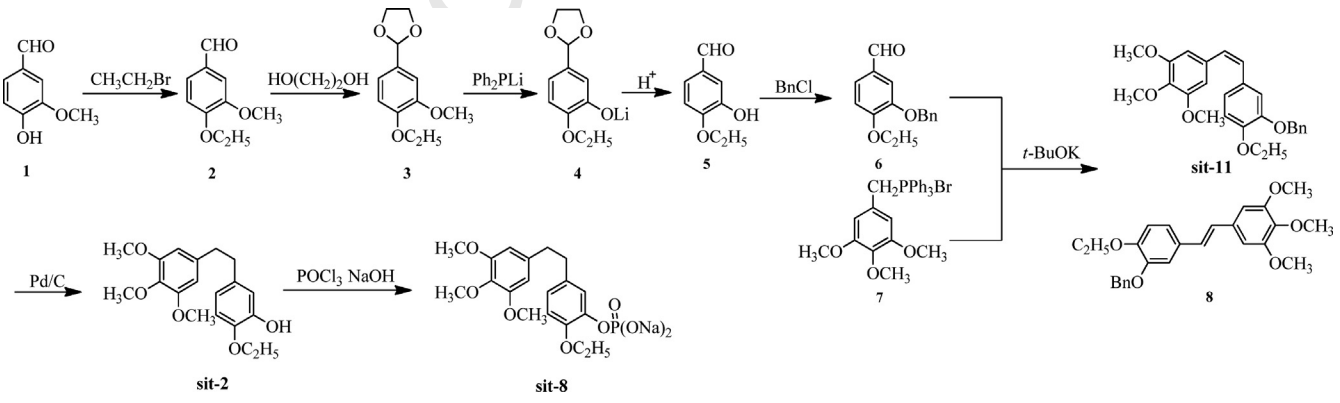
*2.1.4. 5-(3-(Benzyloxy)-4-ethoxystyryl)-1,2,3-trimethoxybenzene (**sit-11**) (Scheme 1)*

A mixture of 4-hydroxy-3-methoxybenzaldehyde **1** (1 g, 6.57 mmol) and potassium carbonate (1.36 g, 9.85 mmol) in DMF (20 mL) was stirred at 40 °C for 10 min. Ethyl bromide (0.98 mL, 13.1 mmol) was added through the septum after the mixture was raised to 70 °C. The reaction mixture was then stirred at this temperature until TLC analysis indicated the completion of the reaction, then quenched by water, extracted with EtOAc, dried over Na_2SO_4 and concentrated. Pure white 4-ethoxy-3-methoxybenzaldehyde **2** was obtained by crystallization (petroleum ether (60–90 °C): EtOAc = 9:1, v/v). mp 62.1–62.2 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 9.84 (s, 1H), 7.41–7.45 (m, 2H), 6.96 (d, 1H, *J* = 5.0 Hz), 5.80 (s, 1H), 4.19 (q, 2H, *J* = 5.0 Hz), 3.93 (s, 3H), 1.50 (t, 3H, *J* = 5.0 Hz).

A solution of 4-ethoxy-3-methoxybenzaldehyde **2** (1 g, 5.6 mmol), *p*-toluenesulfonic acid (60 mg, 0.31 mmol) and ethylene glycol (6 mL, 0.1 mol) in benzene (30 mL) was refluxed for 12 h. After cooling to room temperature, aqueous potassium carbonate (15%, 25 mL) was added. Organic layer was washed with aqueous potassium carbonate (15%, 50 mL), dried over Na_2SO_4 and concentrated, white 2-(4-ethoxy-3-methoxyphenyl)-1,3-dioxolane **3** was obtained. mp 75–77 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.21 (s, 1H), 6.83 (d, 2H, *J* = 5.0 Hz), 5.80 (s, 1H), 4.00 (t, 4H, *J* = 5.0 Hz), 4.04 (q, 2H, *J* = 5.0 Hz), 3.83 (s, 3H), 1.43 (t, 3H, *J* = 5.0 Hz).

A solution of diphenylphosphine (1 mL, 5.8 mmol) and *n*-butyl lithium (2.5 mol/L in hexanes, 3 mL) in anhydrous THF (10 mL) was stirred in an ice bath under nitrogen atmosphere. 2-(4-Ethoxy-3-methoxyphenyl)-1,3-dioxolan **3** (1 g, 4.4 mmol) was dissolved in anhydrous THF (5 mL) and added. Then the solution was stirred at room temperature until TLC analysis indicated the completion of the reaction. The mixture was quenched with water. Aqueous phase was acidified with HCl when the yellow mixture became tea green and the product was extracted with EtOAc, dried over Na_2SO_4 and evaporated in vacuum. Pure white 4-ethoxy-3-hydroxybenzaldehyde **5** was obtained after column chromatography purification (petroleum ether (60–90 °C): EtOAc = 5:1, v/v). mp 125–127 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 9.84 (s, 1H), 7.41–7.44 (m, 2H), 6.94 (d, 1H, *J* = 10.0 Hz), 5.80 (s, 1H), 4.22 (q, 2H, *J* = 5.0 Hz), 1.50 (t, 3H, *J* = 5.0 Hz).

A mixture of 4-ethoxy-3-hydroxybenzaldehyde **5** (1 g, 6.02 mmol) and potassium carbonate (1.24 g, 9 mmol) in EtOH (20 mL) was stirred at 40 °C for 10 min. Benzyl chloride (0.7 mL, 6.1 mmol) was added through the septum. Then the reaction mixture was refluxed for 3 h. After cooling to 50 °C, the solution was filtered. Pure white 3-(benzyloxy)-4-ethoxybenzaldehyde **6** was obtained after the filtrate was cooled in a fridge. mp 69.8–70.8 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 9.81 (s, 1H), 6.98–7.47 (m, 8H), 5.20 (s, 2H), 4.19 (q, 2H, *J* = 5.0 Hz), 1.50 (t, 3H, *J* = 5.0 Hz). ^{13}C NMR ($CDCl_3$, 101 MHz): δ 190.8, 154.7, 148.8, 136.6, 129.8, 128.5, 127.9, 127.2, 126.8, 112.3, 111.9, 71.0, 64.6, 14.6. MS (*m*/*z*): 257 (M^+). ESI-HRMS (*m*/*z*): calcd. for $C_{16}H_{17}O_3$ $(M+H)^+$, 257.1178. Found: 257.1196. IR (KBr) (υ_{max}, cm^{-1}): 2985, 2935, 2820, 1686, 1595, 1580, 1436, 1278, 1266, 1131, 1001, 874, 802, 750.



Scheme 1. Synthesis of **sit-2**, **sit-8**, **sit-11** by our group before.

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NaH (60%, 0.64 g, 26.6 mmol) was added to anhydrous DMSO (10 mL) and the mixture was stirred at room temperature (Scheme 2). A solution of 3,4-dihydroxybenzaldehyde **9** (1 g, 7.24 mmol) in anhydrous DMSO (5 mL) was added dropwise through a syringe. The reaction mixture was stirred for about half an hour. Benzyl bromine (0.86 mL, 7.3 mmol) was then added dropwise by a syringe and the resulting solution was stirred overnight. The mixture was neutralized with HCl (2 mol/L) and extracted with EtOAc, dried over Na_2SO_4 and evaporated in vacuum. Pure 3-(benzyloxy)-4-hydroxybenzaldehyde **10** was obtained after column chromatography purification (petroleum ether (60–90 °C): EtOAc = 3:1, v/v). mp 114.3–114.7 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 9.82 (s, 1H), 7.51 (d, 1H, J = 5.0 Hz), 7.40–7.46 (m, 6H), 7.06 (d, 1H, J = 10.0 Hz), 6.26 (s, 1H), 5.18 (s, 2H).

3-(Benzyloxy)-4-hydroxybenzaldehyde **10** (1 g, 4.38 mmol) and potassium carbonate (0.91 g, 6.57 mmol) in DMF (15 mL) was stirred at 40 °C for 10 min. Ethyl bromide (0.65 mL, 12.0 mmol) was added through the septum after the mixture was raised to 70 °C. The reaction mixture was then stirred at this temperature until TLC analysis indicated the completion of the reaction, then the mixture was quenched by water, extracted with EtOAc, dried over Na_2SO_4 and concentrated. Pure white 3-(benzyloxy)-4-ethoxybenzaldehyde **6** was obtained by crystallization (petroleum ether (60–90 °C): EtOAc = 9:1, v/v).

Compound **7** (4 g, 7.66 mmol) was dissolved in anhydrous THF (40 mL), and the mixture was stirred in an ice bath under nitrogen atmosphere, *t*-BuOK (1.31 g, 11.7 mmol) was added, then 3-(benzyloxy)-4-ethoxybenzaldehyde **6** (1 g, 3.90 mmol) was dissolved in anhydrous THF (10 mL) and added through the septum. The mixture was stirred at room temperature for 4 h. TLC analysis indicated the completion of the reaction, then the mixture was quenched by water, extracted with EtOAc, dried over Na_2SO_4 and concentrated under reduced pressure. Pure compound **sit-11** was obtained in 78% yield after column chromatography purification (petroleum ether (60–90 °C): EtOAc = 10:1, v/v). mp 135.8–136 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.49 (d, 2H, J = 5.0 Hz), 7.37–7.41 (m, 2H), 7.32–7.33 (m, 1H), 7.12 (d, 1H, J = 5.0 Hz), 7.05–7.07 (m, 1H), 6.81–6.93 (m, 3H), 6.70 (s, 2H), 5.19 (s, 2H), 4.13 (q, 2H, J = 5.0 Hz), 3.91 (s, 6H), 3.86 (s, 3H), 1.46 (t, 3H, J = 5.0 Hz). ^{13}C NMR ($CDCl_3$, 101 MHz): δ 153.4, 149.2, 148.7, 137.7, 137.3, 133.3, 130.4, 128.5, 127.9, 127.8, 127.3, 126.8, 120.5, 113.7, 112.9, 103.3, 71.5, 64.6, 60.9, 56.1, 14.9; MS (m/z): 421 (M^+); ESI-HRMS (m/z): Calcd. for $C_{26}H_{29}O_5$ $(M+H)^+$: 421.2015, Found: 421.2043; IR (KBr) (υ_{max}, cm^{-1}): 2966, 2934, 2835, 2357, 1581, 1508, 1274, 1242, 1130.

2.1.5. 2-Ethoxy-5-(3,4,5-trimethoxyphenethyl)phenol (*sit-2*)

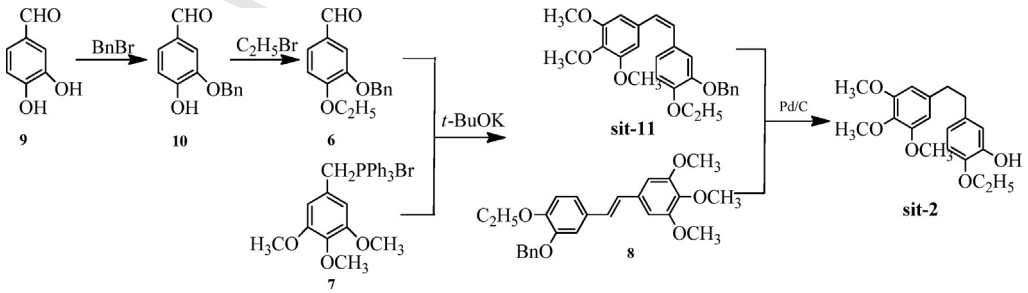
A mixture of compound **sit-11** (1.0 g, 2.38 mmol) and palladium-carbon (10%, 0.2 g) in MeOH (25 mL) was stirred at room temperature for 3 h under hydrogen atmosphere. Palladium-carbon was filtered, the filtrate was dried over Na_2SO_4 and concentrated. Compound **sit-2** was obtained in 92% yield. mp 69.3–69.6 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.81 (s, 1H), 6.75 (d, 1H, J = 5.0 Hz), 6.62 (d, 1H, J = 5.0 Hz), 6.38 (s, 2H), 5.63 (s, 1H), 4.09 (q, 2H, J = 5.0 Hz), 3.83 (s, 9H), 2.82 (s, 4H), 1.43 (t, 3H, J = 5.0 Hz). ^{13}C NMR ($CDCl_3$, 101 MHz): δ 153.0, 145.6, 144.0, 137.6, 136.2, 134.9, 119.7, 114.6, 111.5, 105.4, 64.6, 60.8, 56.0, 38.4, 37.3, 14.9; MS (m/z): 333 (M^+); ESI-HRMS (m/z): Calcd. for $C_{19}H_{25}O_5$ $(M+H)^+$: 333.1702; Found: 333.1741; IR (KBr) (υ_{max}, cm^{-1}): 3345, 2975, 2930, 2867, 1592, 1526, 1464, 1425, 1247, 1115, 1003, 869.

2.1.6. 2-Ethoxy-5-(3,4,5-trimethoxyphenethyl)phenyl sodium phosphate (*sit-8*)

A solution of phosphorus oxychloride (0.8 mL, 9.0 mmol) in CH_2Cl_2 (3 mL) was stirred in an ice bath. Compound **sit-2** (1 g, 3.01 mmol) was dissolved in CH_2Cl_2 (5 mL) and added. A solution of triethylamine (1.87 mL, 13.5 mmol) in CH_2Cl_2 (3 mL) was added dropwise after the mixture was stirring for 5 min. TLC analysis indicated the completion of the reaction, then the mixture was quenched by water, extracted with CH_2Cl_2, dried over Na_2SO_4 and concentrated. The viscous oil was cooled in an ice bath, neutralized to 8–10 (pH) with 2 mol/L sodium hydroxide and the mixture was stirred for 8 h at 70 °C, then filtered while hot, the filtrate was dried over Na_2SO_4 and concentrated. Pure white compound **sit-8** was obtained in 50% yield by re-crystallization from hot EtOH. mp 140.3–141.0 °C. ^{1}H NMR (D_2O, 500 MHz): δ 7.38 (s, 1H), 6.84 (d, 1H, J = 10.0 Hz), 6.65 (d, 1H, J = 5.0 Hz), 6.54 (s, 2H), 4.04 (q, 2H, J = 5.0 Hz), 3.74 (s, 6H), 3.67 (s, 3H), 2.78–2.81 (m, 4H), 1.33 (t, 3H, J = 5.0 Hz). ^{13}C NMR (D_2O, 101 MHz): δ 152.1, 146.7, 143.7, 139.0, 135.1, 134.7, 121.7, 120.3, 114.2, 105.9, 65.4, 60.7, 55.8, 48.9, 37.4, 36.5, 14.1. ^{31}P NMR (D_2O, 500 MHz): δ 3.20(s). MS (m/z): 456 (M^+). ESI-HRMS (m/z): calculated for $C_{19}H_{23}Na_2O_8P$ $(M+Na)^+$: 479.0824, found: 479.0813. IR (KBr) (υ_{max}, cm^{-1}): 3242, 2599, 2239, 1982, 1589, 1505, 1092, 988.

2.1.7. (Z)-5-(4-Ethoxy-3-nitrostyryl)-1,2,3-trimethoxybenzene (*sit-6*)

A mixture of 4-hydroxy-3-nitrobenzaldehyde **11** (1 g, 6.0 mmol) and potassium carbonate (1.24 g, 9 mmol) in DMF (15 mL) was stirred at 40 °C for 10 min. Ethyl bromide (0.9 mL, 12.0 mmol) was added through the septum after the mixture was raised to 70 °C. The reaction mixture was then stirred at this temperature until TLC analysis indicated the completion of the reaction, then the mixture was quenched by water, extracted with EtOAc, dried over Na_2SO_4 and concentrated. Pure white 4-ethoxy-3-nitrobenzaldehyde **12** was obtained by re-crystallization (petroleum ether (60–90 °C): EtOAc = 9:1, v/v). mp 46.4–46.9 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 9.93 (s, 1H), 8.33 (s, 1H), 8.06–8.08 (m, 1H), 7.21 (d, 1H, J = 10.0 Hz), 4.30 (q, 2H, J = 5.0 Hz), 1.53 (t, 3H, J = 5.0 Hz). Compound **7** (4 g, 7.66 mmol) was dissolved in anhydrous THF (40 mL), and the mixture was stirred in an ice bath under nitrogen atmosphere, *n*-butyl lithium (2.5 mol/L in hexanes, 2.6 mL) was added, then 4-ethoxy-3-nitrobenzaldehyde **12** (1 g, 5.13 mmol) was dissolved in anhydrous THF (10 mL) and added through the septum. The mixture was stirred at room



Scheme 2. New method for the synthesis of **sit-2** and **sit-11**.

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temperature for 12 h. TLC analysis indicated the completion of the reaction, then the mixture was quenched by water, extracted with EtOAc, dried over Na_2SO_4 and concentrated under reduced pressure. Pure compound **sit-6** was obtained in 50% yield after column chromatography purification (petroleum ether (60–90 °C): EtOAc = 10:1, v/v). mp 101.8–102.4 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 7.77 (s, 1H), 7.39–7.41 (m, 1H), 6.92 (d, 1H, J = 10.0 Hz), 6.57 (d, 1H, J = 10.0 Hz), 6.43–6.47 (m, 3H), 4.15 (q, 2H, J = 5.0 Hz), 3.85 (s, 3H), 3.72 (s, 6H), 1.46 (t, 3H, J = 5.0 Hz). ^{13}C NMR ($CDCl_3$, 101 MHz): δ 153.2, 151.0, 139.9, 137.7, 134.4, 131.8, 131.2, 129.5, 126.9, 125.8, 114.1, 105.9, 65.5, 61.0, 56.0, 14.5. MS (m/z): 359 (M^+). ESI-HRMS (m/z): calculated for $C_{19}H_{22}NO_6$ $(M+H)^+$: 360.1447, found: 360.1438. IR (KBr) (υ_{max}, cm^{-1}): 2957, 2835, 1735, 1621, 1577, 1530, 1503, 1458, 1429, 1428, 1412, 1349, 1333, 1164, 1129, 1037, 1005, 968, 930, 856.

2.1.8. 2-Ethoxy-5-(3,4,5-trimethoxyphenethyl)benzenamine (*sit-3*)

A solution of compound **sit-6** (1.0 g, 2.79 mmol) and palladium-carbon (10%, 0.2 g) in MeOH/EtOAc (25 mL, 1:1.5, v/v) was stirred at room temperature for 3 h under hydrogen atmosphere. Palladium-carbon was filtered off, the filtrate was dried over Na_2SO_4 and concentrated. Compound **sit-3** was obtained in 90% yield. mp 82.9–83.4 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 6.70 (d, 1H, J = 5.0 Hz), 6.59 (d, 1H, J = 5.0 Hz), 6.51–6.53 (m, 1H), 6.39 (s, 2H), 4.04 (q, 2H, J = 5.0 Hz), 3.83 (s, 9H), 2.75–2.83 (m, 4H), 1.42 (t, 3H, J = 5.0 Hz). ^{13}C NMR ($CDCl_3$, 101 MHz): δ 153.1, 146.7, 145.9, 137.6, 136.2, 135.2, 134.4, 128.1, 122.7, 119.7, 111.9, 64.3, 60.9, 56.1, 38.5, 37.2. MS (m/z): 331 (M^+). ESI-HRMS (m/z): calculated for $C_{19}H_{25}NaNO_4$ $(M+Na)^+$, 354.1681, found: 354.1691. IR (KBr) (υ_{max}, cm^{-1}): 3434, 3350, 3050, 2986, 2978, 2934, 2837, 1620, 1587, 1517, 1507, 1474, 1455, 1421, 1392, 1325, 1290, 1237, 1226, 1179, 1128, 1048, 1021, 1007, 973, 952, 921, 843.

2.1.9. 2-Amino-N-(2-ethoxy-5-(3,4,5-trimethoxyphenethyl)phenyl)-3-hydroxypropanamide (*sit-9*)

A mixture of compound **sit-3** (1 g, 3.02 mmol), Fmoc-L-serine (1.03 g, 3.15 mmol), DCC (0.65 g, 3.15 mmol), HOBT (0.43 g, 3.15 mmol) in DMF (20 mL) was stirred at room temperature for 5 h under nitrogen atmosphere. TLC analysis indicated the completion of the reaction, then the mixture was diluted by EtOAc (10 mL), dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was dissolved in CH_2Cl_2/MeOH (1:1, 10 mL), NaOH solution (2.5 mL, 2 mol/L) was added. The reaction mixture was stirred at room temperature for 24 h. After cooling, the solvents was quenched by saturated salt water, extracted with CH_2Cl_2, dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography (petroleum ether (60–90 °C): EtOAc = 1:2, v/v) to afford the compound **sit-9** in 45% yield. mp 162.0–162.5 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 9.92 (s,1H), 8.29 (s, 1H), 6.82–6.83 (m, 1H), 6.78 (d, 1H, J = 5.0 Hz), 6.40 (s, 2H), 4.07(q, 2H, J = 5.0 Hz), 4.03 (q, 1H, J = 5.0 Hz), 3.88 (q, 1H, J = 5.0 Hz), 3.83 (d, 9H, J = 5.0 Hz), 3.73 (s, 1H), 2.83 (s, 4H), 2.57 (s, 2H), 1.44 (t, 3H, J = 5.0 Hz). ^{13}C NMR ($CDCl_3$, 101 MHz): δ 171.2, 153.0, 146.2, 137.7, 136.2, 134.3, 127.2, 123.9, 119.7, 111.2, 105.5, 100.0, 64.5, 60.8, 56.6, 56.1, 38.6, 37.6, 29.7, 14.9. MS (m/z): 418 (M^+). ESI-HRMS (m/z): calculated for $C_{22}H_{31}N_2O_6$ $(M+H)^+$, 419.2182, found: 419.2170. IR (KBr) (υ_{max}, cm^{-1}): 3366, 3283, 2927, 2012, 1670, 1593, 1552, 1507, 1473, 1386, 1329, 1290, 1230, 1185, 1127, 1057, 1011, 972, 890, 871, 854.

2.1.10. 2-Amino-N-(2-ethoxy-5-(3,4,5-trimethoxyphenethyl)phenyl)acetamide (*sit-10*)

A mixture of compound **sit-3** (1 g, 3.02 mmol), Fmoc-glycine (0.94 g, 3.15 mmol), BOP (1.40 g, 3.15 mmol) in DMF (20 mL) was stirred at 60 °C for 2 h under nitrogen atmosphere. TLC analysis indicated the completion of the reaction, then the mixture was quenched by saturated $NaHCO_3$ (20 mL), extracted with CH_2Cl_2, dried over Na_2SO_4 and concentrated under reduced pressure. The crude product was dissolved in MeOH (15 mL) and NaOH solution (2.5 mL, 2 mol/L) was added. The reaction mixture was stirred at room temperature for 3 h until the TLC analysis indicated the completion of the reaction. After cooling, the solvents was quenched by saturated NaCl solution, extracted with CH_2Cl_2, dried over Na_2SO_4 and concentrated. The residue was purified by column chromatography (petroleum ether (60–90 °C): EtOAc = 1:1, v/v) to afford the compound **sit-10** in 50% yield. mp 92.1–93.9 °C. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 9.79 (s,1H), 8.38 (s, 1H), 6.77–6.84 (m, 2H), 6.42 (s, 2H), 4.08 (q, 2H, J = 10.0 Hz), 3.84 (d, 9H, J = 5.0 Hz), 3.56 (s, 1H), 2.84 (s, 4H), 2.19 (s, 2H), 1.45 (t, 3H, J = 10.0 Hz). ^{13}C NMR ($CDCl_3$, 101 MHz): δ 170.4, 153.0, 146.0, 137.8, 136.1, 134.4, 127.6, 123.5, 119.5, 111.1, 100.0, 64.4, 60.9, 56.1, 45.5, 38.7, 37.7, 14.9. MS (m/z): 388 (M^+). ESI-HRMS (m/z): calcd. for $C_{21}H_{29}N_2O_5$ $(M+H)^+$, 389.2076, found: 389.2076. IR (KBr) (υ_{max}, cm^{-1}): 3402, 3271, 2998, 2933, 2835, 1674, 1592, 1540, 1458, 1366, 1334, 1289, 1254, 1235, 1183, 1130, 1079, 1037, 1004, 976, 921, 859.

2.1.11. (Z)-1,2,3-Trimethoxy-5-(4-methoxy-3-nitrostyryl) benzene (*sit-7*)

Following the synthetic method from Pettit et al. [11], compound **sit-7** was obtained in 25% yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.03 (s, 1H), 7.68 (d, 1H J = 5 Hz), 7.11 (d, 1H, J = 5 Hz), 7.00 (q, 2H, J = 5 Hz), 6.75 (s, 2H), 4.01(s, 3H), 3.94 (s, 6H), 3.90 (s, 3H).

2.1.12. 2-Methoxy-5-(3,4,5-trimethoxyphenethyl)benzenamine (*sit-4*)

A solution of compound **sit-7** (1.0 g, 2.90 mmol) and palladium-carbon (10%, 0.2 g) in MeOH/EtOAc (25 mL, 1:1.5, v/v) was stirred at room temperature for 3 h. Palladium-carbon was filtered off, the filtrate was dried over Na_2SO_4 and concentrated. Compound **sit-4** was obtained in 90% yield. ^{1}H NMR (500 MHz, $CDCl_3$), δ 6.71 (d, 1H, J = 10 Hz), 6.58 (d, 1H, J = 5 Hz), 6.55 (d, 1H, J = 10 Hz), 6.39 (s, 2H), 3.84 (s, 12H), 2.80 (s, 4H), 1.58(s, 2H).

2.1.13. (Z)-2-Methoxy-5-(3,4,5-trimethoxystyryl)benzenamine (*sit-5*)

A solution of compound **sit-7** (1.0 g, 2.90 mmol) and zinc powder (1.89 g, 29 mmol) in AcOH (30 mL) was stirred at room temperature for 6 h. The mixture was filtered and the filtrate was dried over Na_2SO_4 and concentrated. The residue was purified from crystallization (petroleum ether (60–90 °C): EtOAc = 9:1, v/v) to afford compound **sit-5** in 73% yield. ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.95 (s, 1H), 6.90 (d, 3H, J = 10 Hz), 6.80 (d, 1H, J = 5 Hz), 6.72 (s, 2H), 3.90 (s, 12H).

2.2. Cell growth conditions and anti-proliferative assay

To better characterize drug-induced cytotoxicity of these compounds (**sit-1** to **sit-11**) in contrast with CA-4, some human cancer cells like human hepatocellular liver carcinoma cell (HepG2), human cholangiocarcinoma (QBC939), human breast cancer cells (SK-BR-3), human colon cancer cell (HCT-8) and human gastric cancer cell (MKN45) obtained from the Cell Bank of Chinese Academy of Science were treated. All of these cells were maintained in RPMI 1640 medium with 10% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO_2. All cells were seeded into 96-well flat-bottomed culture plates in triplicates separately with 10 μg/ml WA or GsA for 44 h. 500 μg/mL 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) was added and cells were then incubated for another 4 h. The IC_{50} values were calculated through the determination of lactate dehydrogenase (LDH) in cell culture supernatant using GraphPad Prism5.0 software.

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2.3. Molecular modeling

Energy minimization: Minimum energy conformations of all 11 CA-4 analogs and CA-4 were calculated using the minimize module of Sybyl-X 2.0 [12]. The force field was calculated with MMFF94 at an 8 Å cutoff for non-bonded interactions, and the atomic point charges were also calculated using MMFF94. Minimizations were achieved using the consecutive steepest descent method for the first 100 steps, conjugate gradient (Powell) and quasi-Newton (BFGS; named for its originators, and approximates the inverse of the Hessian matrix) energy minimization steps until the root-mean-square (RMS) of the gradient became less than 0.005 kcal mol^{-1} Å.

Docking calculations: The Surflex-Dock [13] module implemented in the Sybyl program was used for the docking studies. All CA-4 analogs were docked into a tubulin crystal structure (PDB ID: 3UT5) by an empirical scoring function and a patented search engine in Surflex-Dock. Protomol, a representation of a ligand making every potential interaction with the binding site, was applied to guide molecular docking. Protomols could be established by three manners: (1) Automatic: Surflex-Dock finds the largest cavity in the receptor protein; (2) Ligand: a ligand in the same coordinate space is used as the receptor; (3) Residues: residues in the receptor are specified [13]. In this study, the automatic docking was applied. Other parameters were established by default in the software. Surflex-Dock scores (total scores) were expressed in $-\lg K_d$ units to represent binding affinity.

3. Results and discussion

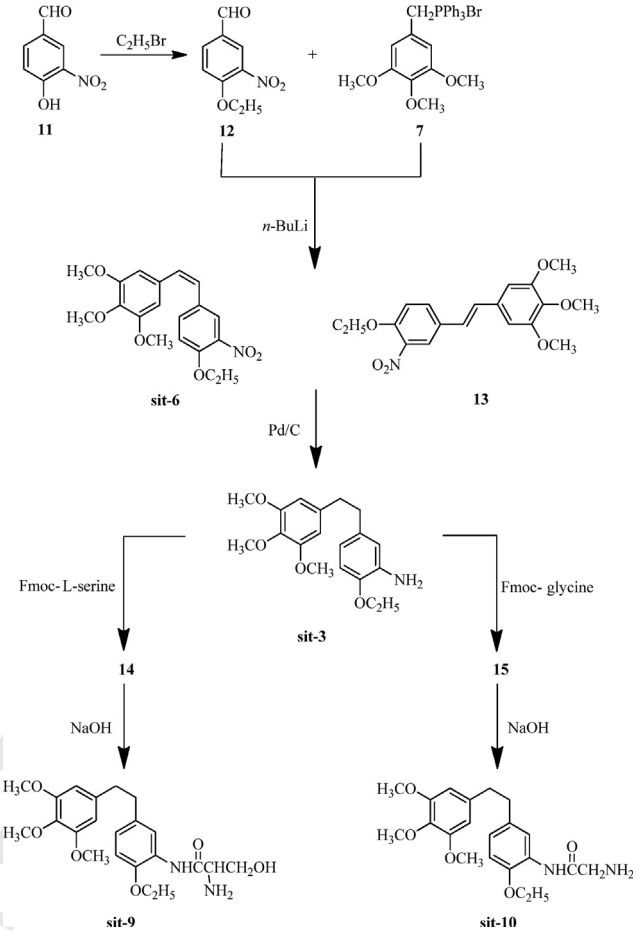
3.1. Chemistry

The synthesis of diphenylethane derivates (**sit-2**, **sit-3**, **sit-6**, **sit-8**, **sit-9**, **sit-10**, **sit-11**) have been reported by our group before (Scheme 1) [14]. For the construction of this series of diphenylethane derivatives, the synthesis of 3-(benzyloxy)-4-ethoxybenzaldehyde **6** was the main challenge. Firstly, the synthetic sequence was long. Secondly, the use of expensive, unstable and toxic metal reducers and the need for slow increase of temperature from ice bath to room temperatures in the procedures have limited the product yields. With our continuing interesting in preparing various CA-4 derivatives, we herein demonstrate a simpler route for the preparation of various diphenylethane derivates with 3,4-dihydroxybenzaldehyde **9** using the Wittig reaction as a key step as illustrated in Scheme 2. 3-(Benzyloxy)-4-hydroxybenzaldehyde **6** was obtained by reaction of commercially material 3,4-dihydroxybenzaldehyde **9** with benzyl bromide [15]. Subsequent alkylation of the intermediate aldehyde **10** gave 3-(benzyloxy)-4-ethoxybenzaldehyde **6**.

The modified synthesis of 3-(benzyloxy)-4-hydroxybenzaldehyde **6** required only 2 steps compared to 5 steps in the original method. And the total yield of compound **6** increased from 30% to 63%. A simple, facile, high yield, less cumbersome and environmental friendly synthesis of 3-(benzyloxy)-4-ethoxybenzaldehyde **6** was established.

The aldehyde **6** was reacted with triphenyl(3,4,5-trimethoxybenzyl)phosphonium bromine **7** and potassium *tert*-butoxide (*t*-BuOK) to furnish the Wittig product 5-(3-(benzyloxy)-4-ethoxyphenethyl)-1,2,3-trimethoxybenzene **sit-11**. Diphenylethene **sit-11** was hydrogenated under hydrogen balloon conditions using palladium-carbon as a catalyst to afford the final product **sit-2** in an overall yield of 43%.

The synthesis of the amino derivatives of **sit-3**, **sit-6**, **sit-9** and **sit-10** are outlined in Scheme 3. 4-Ethoxy-3-nitrobenzaldehyde **12** was obtained from the alkylation of commercial compound 4-hydroxy-3-nitrobenzaldehyde **11**. Then **sit-6** was prepared from



Scheme 3. Synthesis of sit-3, sit-9 and sit-10.

12 *via* the Wittig reaction. **Sit-3** was easy to synthesis following the general method. DCC and BOP were used to promote the coupling of **sit-3** and Fmoc-L-Ser or Fmoc-Gly to produce **sit-9** and **sit-10**.

3.2. In vitro *anti-proliferative activities*

The MTT assay was carried out to investigate the anti-proliferative activity of all synthesized CA-4 analogs (**sit-1** to **sit-11**) in human cervix carcinoma (HeLa). CA-4 was used as a positive control in the assay. The IC_{50} values for **sit-1**, **sit-2**, **sit-3**, **sit-4** and **sit-8** were 0.22 μmol/L, 0.11 μmol/L, 0.20 μmol/L, 0.16 μmol/L and 0.57 μmol/L, respectively (Table 1). This result demonstrated that analogs **sit-1**, **sit-2**, **sit-3** and **sit-4** have significant effects *in vitro*, when compared with the lead compound CA-4 (the IC_{50} value of CA-4 is 0.40 μmol/L). The anti-proliferative activities of **sit-1**, **sit-2** and **sit-3** were also tested in human hepatocellular liver carcinoma cell (HepG2), human cholangiocarcinoma (QBC939), human breast cancer cells (SK-BR-3), human colon cancer cell (HCT-8) and human gastric cancer cell (MKN45). The IC_{50} value of each analog shows that they all have some effect on each cancer cell *in vitro*, as shown in Table 2.

3.3. Structure–activity relationship study and molecular modeling

The double bond in Part A of CA-4 was hydrogenated to afford the final product **sit-1**. The anti-proliferative effect of **sit-1** was 2-fold more than that of CA-4, which suggested that the single bond is better than the double bond in Part A for the cytotoxicity in human cancer cells. The IC_{50} value for **sit-4** (a diphenylethane

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Table 1
Structure, purity, yield and *in vitro* antiproliferative activity of CA-4 analogs on HeLa.

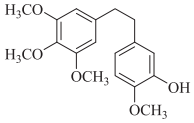
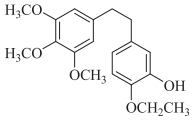
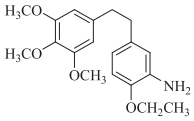
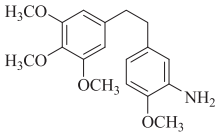
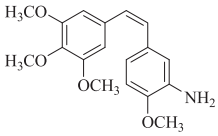
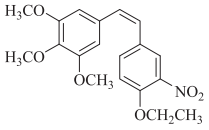
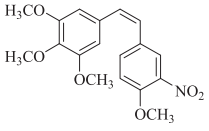
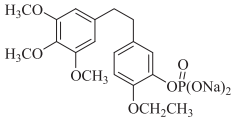
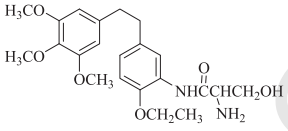
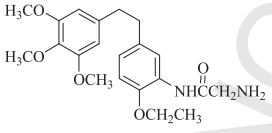
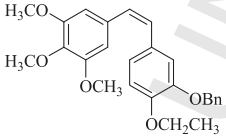
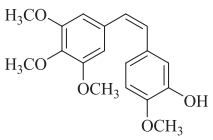
Compound	Structure	Purity (%)	Total Yield (%)	IC_{50} value (μmol/L)	Clog *P*
sit-1		99	32	0.22	3.07
sit-2		99	20/43	0.11	3.60
sit-3		99	62	0.20	3.46
sit-4		96	79	0.16	
sit-5		90	73	No effect	
sit-6		99	50	No effect	4.10
sit-7		90	25	No effect	
sit-8		99	50	0.57	
sit-9		97	45	No effect	1.16
sit-10		98	50	No effect	2.01
sit-11		99	22/48	No effect	5.56
CA-4		98	35	0.40	3.32

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Table 2
Cytotoxicity against five cancer cell lines by compounds **sit-1**, **sit-2** and **sit-3**.

IC_{50} value (μmol/L)	HepG2	QBC939	SK-BR-3	HCT-8	MKN45
sit-1	0.30	0.23	No effect	0.85	0.89
sit-2	0.13	0.12	0.24	0.43	0.58
sit-3	0.19	0.24	0.28	0.43	1.05

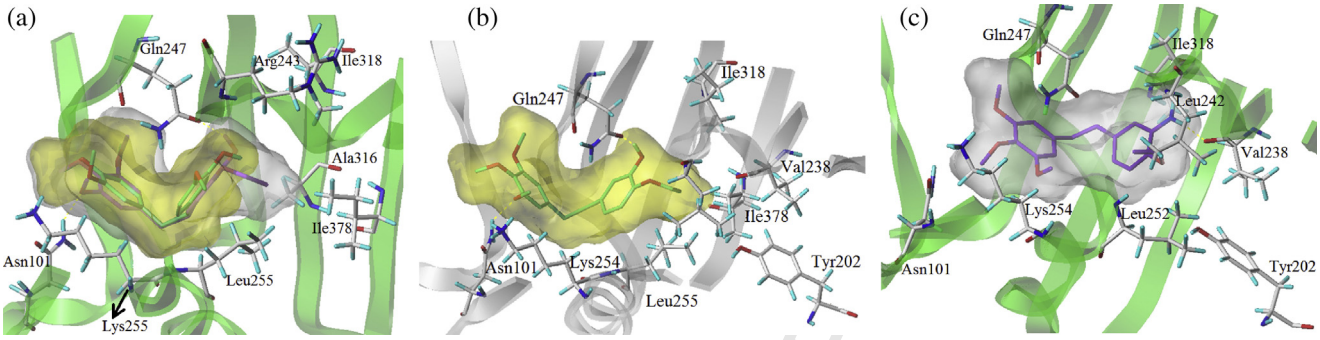

Fig. 2. Binding conformations of the analogs at the active site of tubulin. (a) Binding model of **sit-1** (methoxy derivative, its molecular surface was shown in yellow) and **sit-2** (ethoxy derivative, its molecular surface was shown in white). This docking result makes a clear explanation of why the ethoxy is better than the methoxy group for anti-proliferative effect. (b) and (c) are binding structures of **sit-2** (hydroxyl derivative) and **sit-3** (amino derivative), respectively. Both hydroxyl derivative and amino derivative have only one hydrogen bond with the receptor (Asn101).

derivative) was 0.16 μmol/L, but sit-5 (a diphenylethene derivative) has no effect in HeLa cells. This result further validated the above conclusion.

The methoxy group in Part B of **sit-1** was replaced by an ethoxy group to afford **sit-2**. The IC_{50} value of **sit-2** for each human cancer cells was near half of that of **sit-1** in Table 2. It suggested the ethoxy group is better than the methoxy group for anti-proliferative effect.

The Part C in **sit-2** is a hydroxyl group, and in **sit-3** is an amino group. Both compounds have some effect in those six human cancer cells (Table 2). The anti-proliferative activities of **sit-2** and **sit-3** in HepG2, SK-BR-3, HCT-8 are similar. The anti-proliferative effect of **sit-2** was 2-fold more than that of **sit-3** in HeLa and QBC939. There is no significant difference between hydroxyl and amino groups in Part C for the anti-proliferative effect. But the nitro group (**sit-6** and **sit-7**) or benzyloxy group (**sit-11**) can abolish the anti-proliferative effect (Table 1).

The colchicine binding pocket was used in the study of binding affinities of our analogs. The total score (a default scoring function in Sybyl) is an indication of the binding affinity of a ligand to its receptor. The scores of CA-4 and **sit-1** are similar with values of 7.04 and 6.96, respectively. And their anti-proliferative effects are also similar. The amino acids residues around the Part B in CA-4 are hydrophobic residues, which suggested the bioactivity can be increased if the hydrophobicity of Part B in these analogs was enhanced (Fig. 2). This docking result makes a clear explanation of why the ethyoxyl is better than the methoxyl group for anti-proliferative effect. Both the hydroxyl and the amino groups in Part C can form one hydrogen bond with the receptor (Fig. 2), but the nitro group or benzyloxy group cannot form any hydrogen bond with the receptor. It also can explain the difference of the anti-proliferative effect of analogs **sit-2**, **sit-3**, **sit-6**, **sit-7** and **sit-11**.

As CA-4 does not show *in vivo* efficacy due to its poor water solubility, compound **sit-8**, **sit-9** and **sit-10** were synthesized as the pro-drugs of **sit-2**. The Clog *P* values (calculated using Sybyl) of these analogs showed the water-soluble phosphate or amino acid derivative can improve the water solubility (data shown in Table 1).

In this paper, we designed and synthesized 11 CA-4 analogs, tested the anti-proliferative effect of each analog, and studied the structure–activity relationship of those analogs. These results will be useful for the design of new CA-4 analogs that are structurally related to those used in the current SAR study.

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