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Design, synthesis and biological evaluation of combretastatin A-4 sulfamate derivatives as potential anti-cancer agents†

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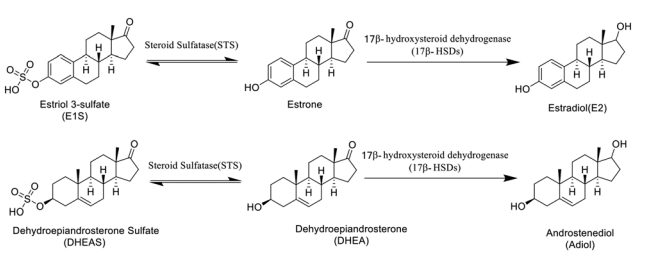
A series of combretastatin A-4 (CA-4) sulfamate derivatives were synthesized and their structure–activity relationship on tubulin, arylsulfatase and tumor cell antiproliferation inhibition was studied. Among them, compound **16a** showed excellent potency as well as CA-4 under the same conditions against six tumor cells including HTC-116, HeLa, HepG2, MGC803, MKN45 and MCF-7 cells, respectively. Molecular docking revealed that several important hydrogen bond interactions were formed between the sulfamate group of **16a** and the colchicine binding site of tubulin and steroid sulfatase respectively. Although compound **16a** was less active than CA-4 in regard to its *in vitro* activity as an inhibitor of tubulin polymerization, it was effective as an inhibitor of arylsulfatase. This novel combretastatin A-4 sulfamate derivative has the potential to be developed as a dual inhibitor of tubulin polymerization and arylsulfatase for cancer therapy.

The occurrence and development of cancer is a complex and long process, and cancer is one of the major causes for the increase in world mortality.[1–3] In particular, breast cancer is the most common cancer with high incidence rates in women, making up about one third of global cancer diagnoses for women, and it is one of the major health problems in the world.[4–7] In the past few decades, great progress was made in molecular-targeted anti-tumor drug research.[8] Tubulin and arylsulfatase are promising targets in cancer therapy. Tubulin is the fundamental unit of microtubules in the cell, and it plays a critical role in maintaining the shape, movement and intracellular material transportation in the cell. Tubulin targeted inhibitors are effective in the treatment of cancer.[9] There are three major binding pockets in tubulin, which were named after three different drugs, paclitaxel, vincristine and colchicine, respectively. Tubulin inhibitors binding to these pockets can arrest tumor cell mitosis and induce apoptosis.[9,10] Paclitaxel and vincristine are effective chemotherapy drugs widely used in the clinic, and some marketed drugs were developed targeting the paclitaxel and vincristine binding sites.[10] However, colchicine was not used in treating cancer due to its high toxicity, and studies on inhibitors targeting colchicine binding sites are one of the hotspots in antineoplastic drug research.[11]

Arylsulfatases are one kind of a protein family in charge of the hydrolysis of sulfonates, and they play a significant role in the hormonal regulation in cells, the degradation of cell components and signaling pathway regulation.[12] The abnormal activity of arylsulfatases in the body is closely related to the growth of tumors. Currently, there are four extensively studied arylsulfatases, namely lysosomal arylsulfatases (ARS-A and ARS-B), endoplasmic reticulum arylsulfatase (ARS-C) and extracellular arylsulfatase (Hsulf-1). In the clinic, the activity of ARS-A and ARS-B has become an important indicator in tumor diagnosis as the ARS activity in urine would significantly increase in all types of tumor patients.[13] ARS-C, also known as steroid sulfatase (STS), is majorly responsible for adjusting the balance of hormone levels (Scheme 1) by hydrolysis of estrone sulfate (E1S) and dehydroepiandrosterone sulfate (DHE-AS) in order to release



Scheme 1 The role of steroid sulfatase in hormone regulation.

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estrone and dehydroepiandrosterone (DHEA). The abnormal state of STS will imbalance the hormone levels in the body as well as promote the growth and proliferation of cancer cells. Steroid sulfatase (STS) inhibitors are an effective therapy for the treatment of hormone dependent cancer such as breast and cervical cancer by inhibiting the hydrolysis of E1S.[14] In the past few years, a lot of steroid sulfatase inhibitors (**1–5**, Fig. 1), including stilbene compounds, have been developed and they exhibit good inhibitory activity against breast cancer cells *via* inhibiting steroid sulfatase.[15] The sulfamate group is an essential pharmacophore for potency, and estrone-3-*O*-sulfamate (EMATE, **1**), the sulfamate derivative of estrone, is a typical representative of this kind of inhibitor.[16]

Combretastatin A-4 (CA-4, **6a**) and combretastatin A-1 (CA-1, **7**) are *cis*-stilbene type natural products originally isolated from the South African bushwillow tree *Combretum caffrum*. They inhibit tubulin polymerization by interacting with the colchicine-binding sites of tubulin, thus arresting the mitosis of tumor cells and inducing their apoptosis. In addition, CA-4 disrupts tumor vasculaures at a nontoxic dose resulting in tumor cells starving to death without affecting the healthy cell's blood supply.[17] Efforts directed toward the discovery of more potent and selective vascular disrupting agents continue on a global basis.[18] In recent years, a large number of structure modifications were made to find more potent CA-4 analogues,[19,20] and among them, AVE-8062A (**10**) and CA-4P (**11**) have entered phase II/III clinical trials (Fig. 1).[20] Structure–activity relationship studies indicate that the *cis*-conformation is crucial for potency. Our groups also have made a lot of progress in finding more potent CA-4 analogues with improved properties by using the twin drug, prodrug and fluorine modification strategies.[21]

In addition to the strong arylsulfatase inhibition, the anti-tumor mechanism of EMATE is also related to its weak tubulin inhibition activity (IC_{50} = 25.90 μM, Table 2). 2-Methoxyestradiol (2-ME), an endogenous metabolite of 17-estradiol, is also a weak tubulin inhibitor (IC_{50} ≈ 40 μM) and it can inhibit microtubule assembly and induce G2/M arrest and apoptosis in many actively dividing cell types while sparing quiescent cells.[22] 2-ME can also bind to the colchicine site of tubulin. Therefore, it has potent antineoplastic activity as an apoptosis inducer and an angiogenesis inhibitor. However, 2-ME is ineffective for arylsulfatase inhibition as compared to EMATE.[22] The

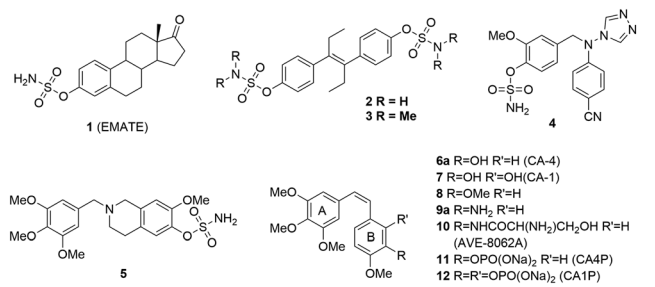


Fig. 1 Chemical structures of **1–12**.

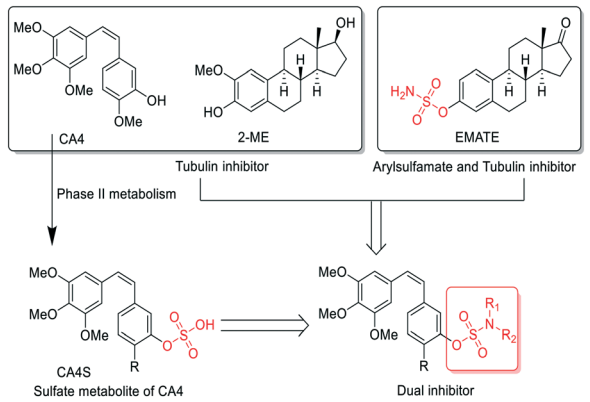
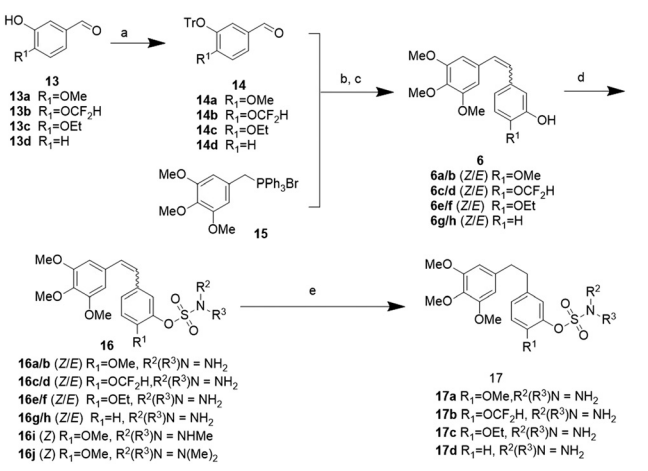


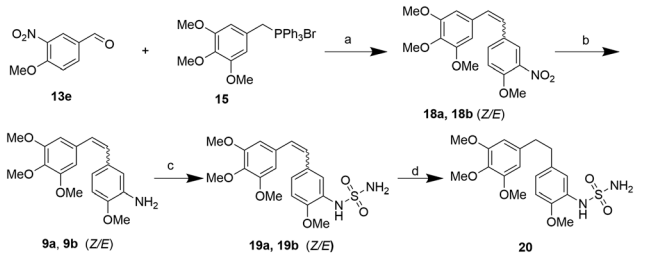
Fig. 2 The design of sulfamate derivatives of CA-4.

sulfamide group is a widely used pharmacophore in medicinal chemistry as it possess both hydrogen bond donors and acceptors. There are many reports about sulfamate modification of other molecular structures as a potent dual inhibitor of carbonic anhydrase and steroid sulfatase.[23] Inspired by the structure and the difference of activity in tubulin and sulfatase inhibition between EMATE and 2-ME, we speculated that sulfamate modification of CA-4 would lead to the foundation of effective dual inhibitors of both tubulin and steroid sulfatase. We hope to obtain a drug candidate with better potency, stability and pharmacokinetic profiles than CA-4 through the sulfamate derivatives of CA-4 and further structure–activity relationship (SAR) studies (Fig. 2). Herein, we reported the synthesis and biological evaluation of novel combretastatin analogues, while also discussing the molecular docking study and the SAR analysis.

Firstly, compound **16a**, the sulfamate modified CA-4 (**6a**), was synthesized and its antiproliferation activity was



Scheme 2 Synthetic route of combretastatin A-4 sulfamate derivatives. Reagents and conditions: a) Ph_3CCl(TrCl), THF, Et_3N, rt, 2 h; b) *n*-BuLi, THF, −78 °C, 30 min, then adding **14**/THF solution, −78 °C–RT, overnight; c) 37% HCl (aq.), toluene, rt, 2 h; d) NaH, DMF, RSO_2-Cl (R = NH_2, NHMe, $N(Me)_2$), 0 °C to RT, overnight; e) H_2 (1 atm), 10% Pd/C, EtOH, rt, 3 h.

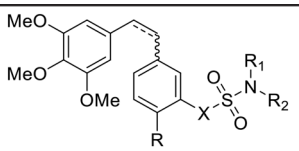


Scheme 3 Synthetic route of combretastatin sulfamide derivatives. Reagents and conditions: a) *n*-BuLi, THF, −78 °C, 30 min, then adding **13e**/THF solution, −78 °C–rt, overnight; b) Zn, CH_3COOH, 2 h, rt; c) NaH, DMF, NH_2SO_2Cl, 0 °C–rt, overnight; d) H_2 (1 atm), 10% Pd/C, EtOH, rt, 3 h.

compared with that of CA-4 as the positive control. To our delight, compound **16a** showed excellent anti-tumor activity with respect to CA-4 under the same conditions. Next, we designed a number of combretastatin A-4 sulfamate and sulfamide derivatives to explore their structure–activity relationship. The synthetic procedures are outlined in Schemes 2 and 3. The aldehyde intermediate (**13**) was purchased or prepared following a reported protocol.[21b] The hydroxyl group of **13** was protected with trityl to give **14**. A (*Z*/*E*)-stilbene mixture was obtained from the aldehyde intermediates (**14** or **13e**, in Schemes 2 and 3) and triphenyl(3,4,5-trimethoxybenzyl)phosphonium bromide (**15**) *via* the Wittig reaction, followed by deprotection of the trityl group or reduction of the nitro group to give 3-hydroxyl or 3-amino substituted (on the B ring) stilbenes (**6** and **9**, Schemes 2 and 3). Their *Z* and *E* isomers were separated by flash column chromatography. Compounds **6** and **9** were further reacted with sulfamoyl chloride respectively to achieve the corresponding sulfamate (**16**) and sulfamide (**19**) derivatives of stilbene. The diphenylethane sulfamate and sulfamide derivatives (**17** and **20**) were obtained from the Pd/C catalyzed hydrogenation of **16** and **19**.

The *in vitro* antiproliferative activity of these compounds were evaluated *via* the CCK-8 assay by using CA-4 and EMATE as the positive control. Six human tumor cell lines, including the HTC-116, HeLa, HepG2, MGC803, MKN45 and MCF-7, were tested. The results are summarized in Table 1. Among all the analogues, only compound **16i**, the monomethylated **16a**, showed comparable potency (IC_{50} = 3.4–11.3 nM) to **16a** (IC_{50} = 3.6–9.5 nM) and CA-4 (IC_{50} = 2.9–8.1 nM) on the six tumor cell lines, except that its inhibitory activity (IC_{50} = 0.53 μM) on MCF-7 cells is slightly less potent than that of **16a** (IC_{50} = 0.11 μM) and CA-4 (IC_{50} = 0.14 μM). As for compound **16j**, the dimethyl substituted **16a**, its potency was reduced although it showed stronger potency on HTC-116 cells (IC_{50} = 0.37 μM) than on the other five tumor cell lines (IC_{50} = 7.14–14.26 μM). The replacement of sulfamate with sulfamide (**19a**) also led to a potency decrease compared with **16a**. **19a** showed better potency (IC_{50} = 0.27, 0.75, 0.50 μM) on HeLa, HepG2, and MGC803 than on the other three cell lines (HCT-116, MKN45 and MCF-7, IC_{50} = 3.29, 9.56, 11.19 μM). The electronic and steric effects of R-substitution also has a great influence on potency. When the methoxyl group of **16a** was replaced with a hydrogen (**16g**, R = H), difluoromethoxy (**16c**,

Table 1 The *in vitro* antiproliferation activities of combretastatin A-4 sulfamate and sulfamide derivatives against on six human tumor cells[a]



Compounds				IC_{50} (mean ± SD, μM)[a]					
Entry	R	$R^1(R^2)N$	X	HTC-116	HeLa	HepG2	MGC803	MKN45	MCF-7
CA-4	—	—	—	0.0047 ± 0.0008	0.0029 ± 0.0001	0.0029 ± 0.0007	0.0060 ± 0.0006	0.0081 ± 0.0004	0.14 ± 0.02
EMATE	—	—	—	0.90 ± 0.04	0.63 ± 0.06	0.85 ± 0.05	0.48 ± 0.09	0.54 ± 0.12	0.07 ± 0.10
16a (*Z*)	OMe	NH_2	O	0.0050 ± 0.0005	0.0061 ± 0.0003	0.0036 ± 0.0002	0.0047 ± 0.0006	0.0095 ± 0.0004	0.11 ± 0.04
16b (*E*)	OMe	NH_2	O	1.57 ± 0.07	4.50 ± 0.07	0.87 ± 0.09	2.98 ± 0.07	10.52 ± 0.04	5.44 ± 0.05
16c (*Z*)	OCF_2H	NH_2	O	9.11 ± 0.04	7.82 ± 0.05	4.81 ± 0.06	9.00 ± 0.05	12.52 ± 0.04	0.29 ± 0.05
16d (*E*)	OCF_2H	NH_2	O	12.84 ± 0.03	8.42 ± 0.04	5.54 ± 0.05	9.24 ± 0.04	13.84 ± 0.04	13.79 ± 0.03
16e (*Z*)	OEt	NH_2	O	0.55 ± 0.07	0.48 ± 0.08	0.41 ± 0.05	0.27 ± 0.07	0.32 ± 0.07	0.30 ± 0.06
16f (*E*)	OEt	NH_2	O	9.12 ± 0.04	5.64 ± 0.05	2.63 ± 0.07	7.63 ± 0.05	8.05 ± 0.05	13.71 ± 0.03
16g (*Z*)	H	NH_2	O	9.50 ± 0.03	16.30 ± 0.03	9.36 ± 0.05	8.53 ± 0.04	15.32 ± 0.02	11.10 ± 0.04
16h (*E*)	H	NH_2	O	40.30 ± 0.04	19.00 ± 0.03	11.85 ± 0.03	27.03 ± 0.02	25.04 ± 0.02	27.44 ± 0.02
16i (*Z*)	OMe	NHMe	O	0.0113 ± 0.0003	0.0034 ± 0.0005	0.0039 ± 0.0010	0.0037 ± 0.0006	0.0073 ± 0.0003	0.53 ± 0.03
16j (*Z*)	OMe	$N(Me)_2$	O	0.37 ± 0.07	14.26 ± 0.04	9.96 ± 0.03	7.14 ± 0.03	8.95 ± 0.37	11.06 ± 0.04
19a (*Z*)	OMe	NH_2	NH	3.29 ± 0.77	0.27 ± 0.06	0.75 ± 0.06	0.50 ± 0.07	9.56 ± 0.09	11.19 ± 0.06
19b (*E*)	OMe	NH_2	NH	8.65 ± 0.04	0.38 ± 0.05	7.53 ± 0.06	6.19 ± 0.06	18.87 ± 0.03	17.09 ± 0.04
17a[b]	OMe	NH_2	O	2.63 ± 0.08	9.04 ± 0.04	1.06 ± 0.05	3.61 ± 0.08	11.87 ± 0.04	5.91 ± 0.06
17a[b]	OCF_2H	NH_2	O	26.48 ± 0.02	35.65 ± 0.01	15.88 ± 0.02	23.26 ± 0.02	71.38 ± 0.02	52.74 ± 0.03
17c[b]	OEt	NH_2	O	10.16 ± 0.03	2.930 ± 0.07	3.65 ± 0.07	3.59 ± 0.06	7.67 ± 0.06	14.75 ± 0.02
17d[b]	H	NH_2	O	52.49 ± 0.02	27.06 ± 0.03	12.03 ± 0.03	17.64 ± 0.04	25.70 ± 0.03	>100
20[b]	OMe	NH_2	NH	30.63 ± 0.04	2.926 ± 0.07	8.10 ± 0.05	8.21 ± 0.04	73.88 ± 0.05	20.13 ± 0.03

[a] The experiment was carried out using an EnoGeneCellTM counting Kit-8 (CCK-8) assay. [b] Saturated bond linked compound.

Table 2 The *in vitro* inhibitory activities of combretastatin A-4 sulfamate derivatives against arylsulfatase and tubulin

				IC_{50} ± SD (μM)	
Entry	R^1	$R^2(R^3)N$	X	Arylsulfatase[b]	Tubulin[c]
16a (*Z*)	OMe	NH_2	O	6.16 ± 0.55	6.60 ± 0.80
16b (*E*)	OMe	NH_2	O	12.86 ± 0.25	55.70 ± 6.00
16c (*Z*)	OCF_2H	NH_2	O	4.64 ± 1.42	72.60 ± 10.7
16e (*Z*)	OEt	NH_2	O	90.19 ± 7.83	3.10 ± 1.10
16g (*Z*)	H	NH_2	O	0.47 ± 0.01	>100
16i (*Z*)	OMe	NHMe	O	>100	1.80 ± 0.00
16j (*Z*)	OMe	$N(Me)_2$	O	>100	12.50 ± 1.50
17a[a]	OMe	NH_2	O	>100	>100
19a (*Z*)	OMe	NH_2	NH	>100	86.20 ± 6.40
EMATE	—	—	—	5.01 ± 0.01	25.90 ± 7.10
CA-4	—	—	—	>100	1.00 ± 0.20

[a] Saturated bond linked compound. [b] Steroid sulfatase. [c] Tubulin polymerization.

R = OCF_2H) or ethoxy (**16e**, R = OEt) group, their cellular potency decreased compared with that of **16a** and **CA-4**. In addition, it was observed that the *cis*-double bond linked compounds (**16a**, **16c**, **16e**, **16g** and **19a**) showed better cellular potency than the *trans*-double bond (**16b**, **16d**, **16f**, **16h** and **19e**) and saturated bond linked compounds (**17a–17d**, **20**). A CA-4 sulfate derivative (CA4S, Fig. 2) was identified as a phase II metabolite of CA4.[24] We have also synthesized it as a control; however, it is ineffective in tumor cell cytotoxicity assays (IC_{50} > 100 μM).

In order to verify the effectivity of these novel sulfamate derivatives of CA-4 on both arylsulfatase and tubulin, we tested their inhibitory activity on steroid sulfatase and tubulin polymerization with EMATE and CA-4 as the control, and the results are summarized in Table 2. The half inhibition rate (IC_{50}) of EMATE on steroid sulfatase and tubulin polymerization is 5.01 μM and 25.90 μM, respectively. The test inhibitory potency of combretastatin A-4 on tubulin (IC_{50} = 1.0 μM) is consistent with literature reports; however, it is ineffective on steroid sulfatase. When the hydroxyl of CA-4 was modified by sulfamide (**16a**), it showed a slightly lower potency (IC_{50} = 6.60 μM) on tubulin polymerization inhibition than CA-4 together with a comparable potency (IC_{50} = 6.16 uM) on steroid sulfatase to that of EMATE. By comparing the anti-proliferation activities and the relationship of the steroid sulfatase and tubulin inhibition potencies of CA-4 and **16a**, we can conclude that **16a** is a dual inhibitor, with the additional steroid sulfatase inhibition compensating for the lost contribution of tubulin polymerization inhibition to its anti-tumor activity. From the SAR analysis (described in the ESI†) based on arylsulfatase and tubulin polymerization inhibition (Table 2), we can conclude the following: 1) the *cis*-isomers show better activity for arylsulfatase and tubulin polymerization inhibition than the *trans*-isomers and saturated bond linked analogues. 2) The electronic and steric effects of R-groups are different on the two targets. Electron withdrawing groups are favorable for arylsulfatase inhibition but are unfavorable for tubulin inhibition at the same time. Meanwhile, bulky groups are favorable for tubulin inhibition but unfavorable for arylsulfatase inhibition. 3) The amino group of **16a** probably has a critical hydrogen bond interaction with the binding site of both targets. By comparing with **16a**, monomethyl substitution increased the potency while dimethyl substitution decreased the potency for tubulin polymerization inhibition. However, the substitution of the amino group of **16a** will lead to the loss of potency on arylsulfatase. 4) Replacing the sulfamate group with sulfamide decreases the potency on both targets. 5) Tubulin inhibition plays a dominant role over arylsulfatase inhibition in the anti-tumor activity in this kind of dual inhibition.

Compound (**16a**) was further selected for molecular docking studies in order to explore how the CA-4 sulfamate derivatives interact with tubulin and steroid sulfatase. The protein complexes tubulin (PDB: 5LYJ)[24] and human placental estrone/DHEA sulfatase (PDB: 1P49)[25] were used for simulation in Sybyl-X 2.0. The force field was Tripos with an 8 Å cutoff for non-bonded interactions, and the atomic point charges were also calculated with the Gasteiger–Huckel method. Minimization was achieved using the steepest descent method for the first 100 steps, followed by the Broyden–Fletcher–Goldfarb–Shanno (BFGS) method until the

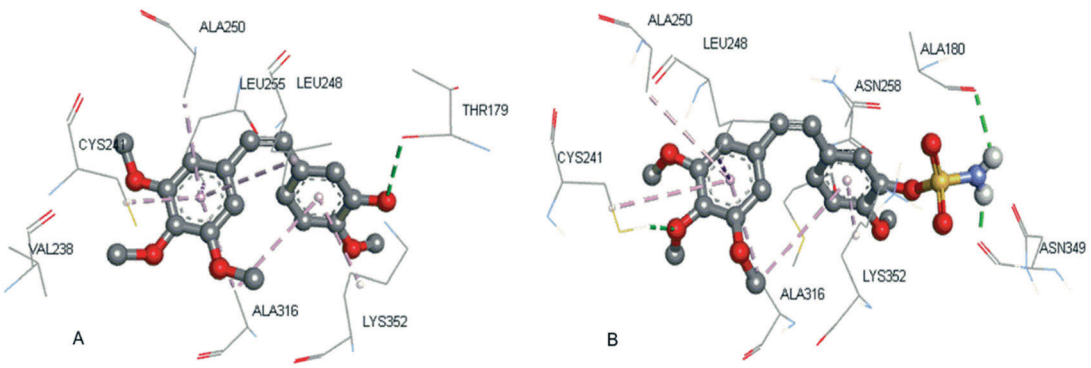


Fig. 3 The binding information of CA-4 in the co-crystal structure (A, PDB code: 5LYJ) and the docking result of **16a** (B).

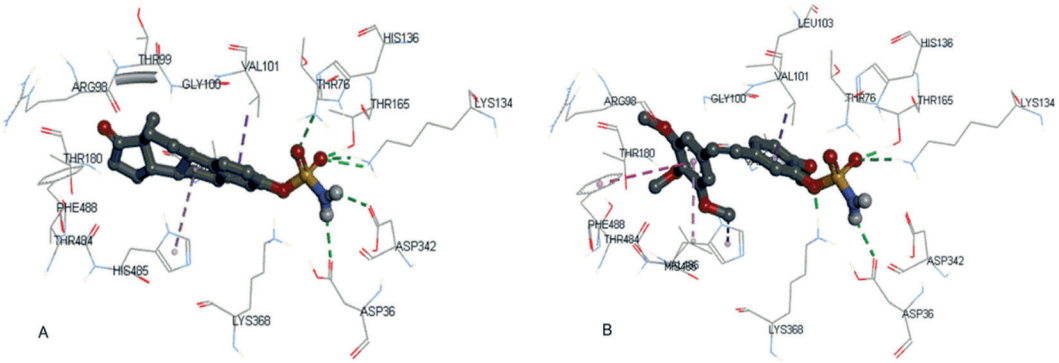


Fig. 4 The docking result of EMATE (A) and **16a** (B) with steroid sulfatase (PDB code: 1P49).

root-mean-square (RMS) of the gradient became <0.005 kcal/(mol Å). The Surflex-Dock module implemented in the Sybyl program was used for the docking study. The colchicine-binding site of tubulin was used and verified from the original ligand (CA-4) for docking (total score = 8.34, similarity = 0.73). The docking score and similarity of **16a** is 6.27 and 0.45, respectively. As shown in Fig. 3, the A/B ring binding mode of **16a** is almost identical to that of CA-4, both having van der Waals interaction with the same hydrophobic pocket formed by the amino acid residues VAL238, CYS241, LEU248, ALA250, LEU255, ALA316 and LYS352. The hydroxyl group of CA-4 formed a hydrogen bond with THR179, while the sulfamide group of **16a** formed two hydrogen bonds with ALA180 and ASN349.

Both EMATE and **16a** were docked into the same binding pocket of steroid sulfatase, and their docking scores were 6.16 and 5.34, respectively. As shown in Fig. 4, the hydrophobic part of EMATE and **16a** were superimposable, having van der Waals interaction with the hydrophobic pocket formed by the residues VAL101, LEU103, VAL486, HIS485, PHE488, *etc.* The sulfamate group of **16a** formed four important hydrogen bonds with the residues THR165, LYS134, ASP36 and LYS368 in the binding pocket. As for EMATE, there are six hydrogen bonds between the sulfamate group and the residues THR76, THR165, LYS134, ASP342 and ASP36. These important hydrogen bonding interactions explain why **16a** is less potent than EMATE, and the methylation of the sulfamate group (**16i** and **16j**) leads to the loss of potency for steroid sulfatase inhibition.

In summary, we have synthesized a few novel combretastatin sulfamate derivatives, and evaluated their activities on steroid sulfatase, tubulin and tumor cell proliferation inhibition. Among them, compound **16a** has a well-balanced sulfatase, tubulin and cellular inhibition potency. The SAR analysis and molecular docking study indicate that the sulfamate group is crucial for sulfatase inhibition and helpful to form additional hydrogen bonds with the colchicine-binding site of tubulin. These results highlighted that compound **16a** is a promising anticancer agent, and may be valuable in finding more effective treatments for cancer. To the best of our knowledge, this is the first sulfamate analogue of CA-4. Further biological investigation is being conducted and the progress will be reported in the future.

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Abbreviations

STS	Steroid sulfatase
E1S	Estrone sulfate
DHE-AS	Dehydroepiandrosterone sulfate
DHEA	Dehydroepiandro-sterone
CA-4	Combretastatin A-4
EMATE	Estrone-3-*O*-sulfamate
SAR	Structure–activity relationship

Author contributions

All the authors have given approval to the final version of this manuscript.

Conflicts of interest

The authors declare no competing financial interests.

Acknowledgements

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