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Structural requirements for the interaction of combretastatins with tubulin: how important is the trimethoxy unit?†

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A series of combretastatins possessing both a trimethoxy unit and other substituents on ring A has been synthesised and tested for cytotoxicity and their ability to interact with the protein tubulin. All previous studies have indicated that the trimethoxy unit is essential for interaction with tubulin. The studies herein show that molecules possessing functionalities other than trimethoxy can also interact with tubulin. Importantly a trimethyl substituted agent **52a** has shown reduced cytotoxicity, but increased potency in its ability to inhibit the assembly of tubulin.

Introduction

The combretastatins are a group of stilbenes which have shown[1–10] promising antimitotic activity. Combretastatin A-4 (**1**) is a naturally occurring stilbene which was originally isolated[11] from the South African tree *Combretum caffrum*. This phenolic stilbene **1** has proved to be the most potent agent which can interact[9,11] with the colchicine site on tubulin. Furthermore combretastatin A-4 (**1**) has shown[12] the ability to shut down tumour vasculature at 10% of its maximum tolerated dose without affecting the normal vasculature. Owing to its promise during preclinical trials a phosphate derivative of combretastatin A-4 (**1**) has now entered Phase II clinical trials.

A major structural feature of several of the known antimitotic agents (combretastatin A-4 (**1**), colchicine (**2**), steganacin (**3**), podophyllotoxin (**4**), Fig. 1), which bind at the colchicine site on tubulin, is a trimethoxy aryl unit.[1,2] It has previously been noted that alteration of the oxygenation pattern from a trimethoxy motif on ring A of the combretastatins adversely affects their biological properties.[2–4] This methoxylation pattern of ring A appears to be critical for efficient binding to tubulin.[3–6,9,13–15] The functionalities on ring C of colchicine (**2**) and ring B of the combretastatins are the key atoms which interact with tubulin and inhibit its assembly.[14,15] Previous work has shown that small groups (*e.g.* methyl, methoxy, dimethylamino *etc.*) on the 4-position of the B ring of the combretastatins are essential for biological activity.[1,3,4,13] The trimethoxy unit on ring A is thought to make an additive contribution to the strength of binding of colchicine **2** to tubulin and serves as an anchor that maintains the whole molecule in the proper orientation within the binding locus.[14]

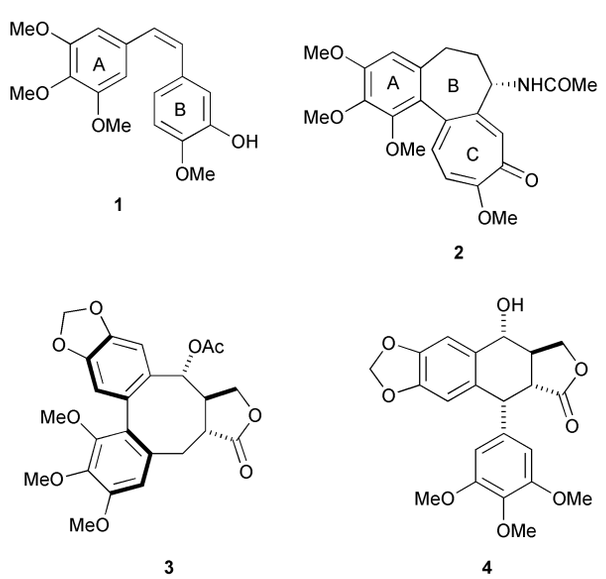

Fig. 1 Structures of combretastatin A-4 (**1**), colchicine (**2**), steganacin (**3**) and podophyllotoxin (**4**).

Our group has extensive[1,2,5–9,16–18] experience of the chemistry and biochemistry of the combretastatins and was not totally convinced of the necessity of a trimethoxyaryl group for biological activity. The trimethoxy unit on the naturally occurring combretastatins probably comes from the shikimate biosynthetic pathway. Shikimic acid provides sequentially phenylalanine, cinnamic acid and eventually trioxycinnamic acids. The trimethoxy unit of colchicine is thought[19] to be derived *via* this pathway. It is conceivable that these antimitotic natural products possess a trimethoxy aryl unit as a consequence of their biogenesis. Similar sized functionalities on the A ring of the combretastatins have not been explored and we considered it possible that such derivatives might have comparable biological activity to the natural products. Herein are described the synthesis and biological activities of a range of combretastatins where the trimethoxy motif has been substituted by other functionalities.

Results and discussion

Synthesis

A series of previously unknown trimethoxy substituted stilbenes were synthesised as well as a series of triethoxy, trifluoro, dimethyl, trimethyl and unsubstituted A ring combretastatins. In most cases a Wittig reaction of the appropriate phosphonium bromide with the required arylaldehyde afforded a

† Electronic supplementary information (ESI) available: experimental details. See http://www.rsc.org/suppdata/ob/b3/b306878a/

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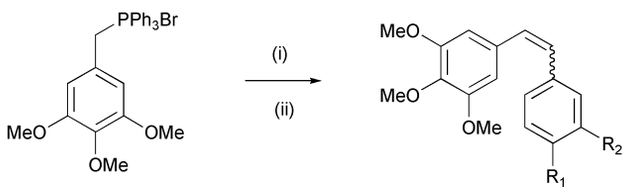
mixture of *Z*-(**a** series) and *E*-stilbenes (**b** series) which were separated by column chromatography. The stereochemistry of the olefins was determined from the coupling constants in the proton NMR (for *E* compounds $J \approx 16$ Hz, for *Z* compounds $J \approx 12$ Hz). In the case of silyl ether protected phenols, fluoride was used to generate the required carbinol. These methods produced the trimethoxy **6**–**11** (Scheme 1), triethoxy **13**–**18** (Scheme 2), trifluoro **22**–**25** (Scheme 3), dimethyl **28**–**36** (Scheme 4) and unsubstituted A ring combretastatins **38**–**41** (Scheme 5).

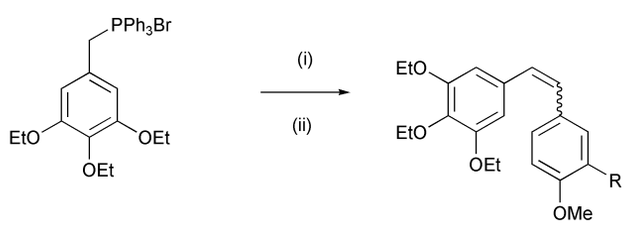
The trifluorostilbene **25** was prepared *via* cinnamic acid **26** using a Perkin-type condensation reaction (Schemes 6–8). The stereochemistry of this acid **26** was determined using UV spectroscopy. The extinction coefficients for stilbenes with the aryl groups *cis* to each other tend to be in the region of 10,000



5

6 R_1 = H; R_2 = OTBDMS
7 R_1 = Me; R_2 = Me
8 R_1 = OMe; R_2 = Br
9 R_1 =OMe; R_2 = NO_2
10 R_1 = OMe; R_2 = F
11 R_1 = H; R_2 = OH

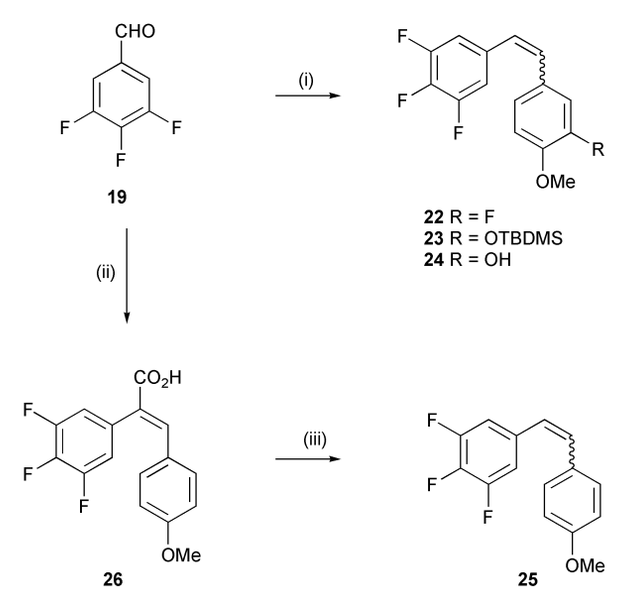
Scheme 1 Synthesis of trimethoxystilbenes **6**–**11**. *Reagents and conditions*: (i) *n*-BuLi–THF −20 °C; (ii) RCHO −20 °C to rt.



12

13 R = Br
14 R = H
15 R = F
16 R = Cl
17 R = OTBDMS
18 R = OH

Scheme 2 Synthesis of triethoxystilbenes **13**–**18**. *Reagents and conditions*: (i) *n*-BuLi–THF −20 °C; (ii) RCHO −20 °C to rt.



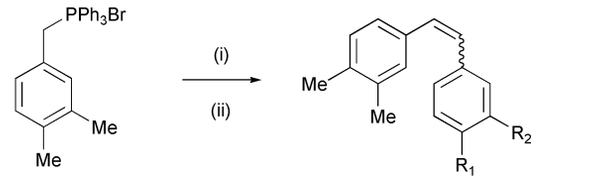
19

22 R = F
23 R = OTBDMS
24 R = OH

26

25

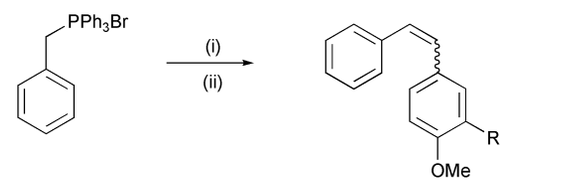
Scheme 3 Synthesis of trifluorostilbenes **22**–**25**. *Reagents and conditions*: (i) $ArCH_2PPh_3Br$–*n*-BuLi −20 °C; (ii) 4-methoxyphenylacetic acid–Ac_2O–Et_3N; (iii) Cu–quinoline 200 °C.



27

28 R_1 = Me; R_2 = H
29 R_1 = OMe; R_2 = NO_2
30 R_1 = OMe; R_2 = H
31 R_1 = OMe; R_2 = Br
32 R_1 = OMe; R_2 = F
33 R_1 = Me; R_2 = F
34 R_1 = Me, R_2 = NO_2
35 R_1 = OMe; R_2 = OTBDMS
36 R_1 = OMe; R_2 = OH

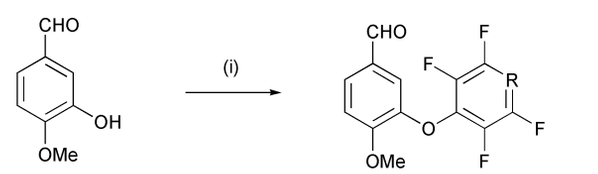
Scheme 4 Synthesis of dimethylstilbenes **28**–**36**. *Reagents and conditions*: (i) *n*-BuLi–THF −20 °C; (ii) RCHO −20 °C to rt.



37

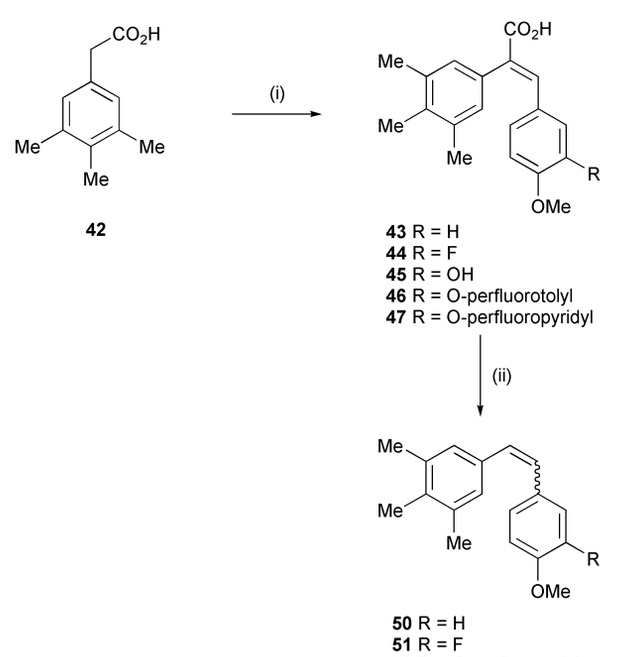
38 R = H
39 R = F
40 R = OTBDMS
41 R = OH

Scheme 5 Synthesis of unsubstituted stilbenes **38**–**41**. *Reagents and conditions*: (i) *n*-BuLi–THF −20 °C; (ii) RCHO −20 °C to rt.



48 R = CCF_3
49 R = N

Scheme 6 Synthesis of benzaldehydes **48** and **49**. *Reagents and conditions*: (i) 1 M NaOH–octafluorotoluene or pentafluoropyridine.

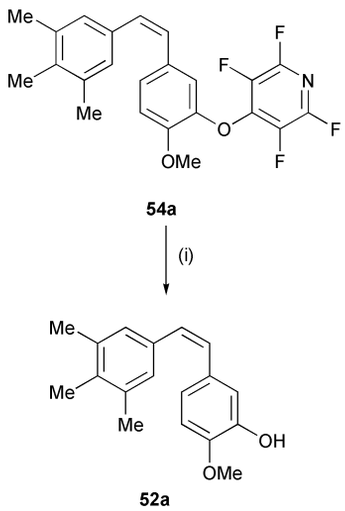


42

43 R = H
44 R = F
45 R = OH
46 R = O-perfluorotolyl
47 R = O-perfluoropyridyl

50 R = H
51 R = F
53 R = O-perfluorotolyl
54 R = O-perfluoropyridyl

Scheme 7 Synthesis of trimethylstilbenes **50**, **51**, **53** and **54**. *Reagents and conditions*: (i) RCHO–Ac_2O–Et_3N; (ii) Cu–quinoline 200 °C.



Scheme 8 Synthesis of trimethylstilbene **52**. *Reagents and conditions*: (i) NaOMe–DMF.

whilst their *trans* isomers have extinction coefficients of 30,000. NOESY spectra of similar acids (reduced to carbinols) have also elucidated the stereochemistry of this type of cinnamic acid.[18] Copper-mediated decarboxylation[18] of **26** afforded a separable *E*/*Z* mixture (4 : 5) of the trifluorostilbenes **25**. Similarly the trimethylstilbenes **50**,**51** were prepared from 3,4,5-trimethylphenylacetic acid (**42**) *via* the cinnamic acids **43**,**44**. However reaction of acid **42** with 3-hydroxy-4-methoxybenzaldehyde failed to produce the required cinnamic acid **45**. The perfluorinated aryl ethers **46**,**47** were prepared from reaction of acid **42** with the aldehydes **48**,**49**. Decarboxylation of **46**,**47** provided the *O*-protected *Z*-stilbenes **53a**,**54a**, respectively. Treatment of the perfluorotolyl ether **53a** with sodium methoxide failed to give phenol **52a**. However similar treatment of the perfluoropyridyl ether did afford the required phenolic stilbene **52a**.

Biochemistry

The sets of stilbenes were tested for cytotoxicity against the human leukaemia K562 cell line and for their ability to interact with tubulin using an assembly and colchicine displacement assay.

In the trimethoxy series it has been noted[3,7] that the 4-position on the B ring can be a small group (*e.g.* methoxy, methyl, ethyl or dimethylamino). However, no A ring trimethoxy stilbene with just a proton on the 4-position of the B ring had previously been tested for antimitotic activity. Phenol **11a** described here is similar to the lead compound combretastatin A-4 (**1**); it simply lacks the B ring 4-methoxy group. This phenol **11a** was, however, considerably less cytotoxic than combretastatin A-4 (**1**) and neither inhibited tubulin assembly or could displace colchicine from its binding site on tubulin. The dimethyl compound **7** showed moderate cytotoxicity, but failed to interact with tubulin. The halogenated compounds **8a**,**10a** showed potent cytotoxicity as well as the ability to interact with tubulin. Interestingly the fluoro derivative **10a**, although ten times less cytotoxic than combretastatin A-4 (**1**), was more potent in the tubulin assays.

Using an *in vitro* method to measure the potential ability of agents to interact with the vascular system, the halogenated stilbenes **8a**,**10a** were tested for their ability to inhibit the growth of human umbilical vein endothelial cells (HUVECs). This assay measures the permeability of HUVECS after treatment with possible antivascular agents. Increased permeability is indicative of the 'rounding up' of the cells which, *in vivo*, would lead to blockage of the tumour vasculature.[20] In this assay the fluoro compound **10a** was only slightly less active than combretastatin A-4 (**1**) (Table 1).

Table 1 Cytotoxicity (K562 cells, HUVECs/μM), inhibition of tubulin assembly (MA/μM), colchicine displacement (CD/μM) of trimethoxystilbenes **7**, **8**, **10 and 11**. ND means not determined

Compound	IC_{50} (K562)	IC_{50} MA	IC_{50} CD	IC_{50} HUVEC
1	0.001	0.175	3	0.0026
7a	0.04	2	>25	ND
7b	35.8	>10	>25	ND
8a	0.001	0.4	10	0.067
8b	7.83	>10	>25	ND
10a	0.01	0.085	2.8	0.004
11a	0.14	>10	>25	ND
11b	22	>10	>25	ND

Table 2 Cytotoxicity (K562 cells, HUVECs/μM), inhibition of tubulin assembly (MA/μM), colchicine displacement (CD/μM) of triethoxy stilbenes **13**–**16 and 18**. ND means not determined

Compound	IC_{50} (K562)	IC_{50} MA	IC_{50} CD	IC_{50} HUVEC
13a	0.6	>10	>25	ND
13b	>50	>10	>25	ND
14a	0.5	>10	>25	ND
14b	>50	>10	>25	ND
15a	0.044	1.25	>25	0.19
15b	>50	>10	>25	ND
16a	0.45	>10	>25	ND
16b	7	>10	>25	ND
18a	0.018	0.50	15.5	0.05
18b	0.2	>10	>25	ND

Table 3 Cytotoxicity (K562 cells/μM), inhibition of tubulin assembly (MA/μM), colchicine displacement (CD/μM) of trifluorostilbenes **22**–**25**

Compound	IC_{50} (K562)	IC_{50} MA	IC_{50} CD
22a	0.12	5.5	>25
22b	22	>10	>25
23a	>50	>10	>25
24a	0.13	4.5	>25
24b	5	>10	>25
25a	4.2	>10	>25
25b	40	>10	>25

Table 4 Cytotoxicity (K562 cells/μM), inhibition of tubulin assembly (MA/μM), colchicine displacement (CD/μM) of stilbenes **38**–**41**

Compound	IC_{50} (K562)	IC_{50} MA	IC_{50} CD
38a	5	>10	>25
38b	14	>10	>25
39a	2.5	>10	>25
39b	>50	>10	>25
40a	0.5	4.75	>25
40b	40	>10	>25
41a	0.5	2.5	>25
41b	1	>10	>25

In the triethoxy series, only the phenol **18a** and fluoro compound **15a** showed good cytotoxicity and the ability to interact with tubulin. The phenol **18a** was also particularly potent in the HUVEC assay (Table 2).

The unsubstituted **38**–**41** and trifluoro substituted stilbenes **22**–**25** were synthesised as lipophilic alternatives to the standard trimethoxy substituted compounds. None of these agents exhibited high cytotoxicity, although in the trifluoro series the B ring fluoro and hydroxy compounds **22a**, **24a** showed a small interaction with tubulin. Similarly, in the unsubstituted series, the phenol **41a** exhibited a slight interaction with tubulin (Tables 3 and 4).

The dimethyl substituted stilbenes, in general, showed poor cytotoxicity and little interaction with tubulin. However, the phenol **36a**, and the fluoro compound **32a** did exhibit consider-

able cytotoxicity to the K562 cell line and some ability to interact with tubulin (Table 5). In the trimethyl series all the agents **50a**,**51a**,**54a** were less cytotoxic than combretastatin A-4 (**1**). Interestingly, the phenolic compound **52a**, which has the B ring substitution pattern of combretastatin A-4 (**1**), is twenty-fold less toxic than combretastatin A-4 (**1**) in the cytotoxicity assay but more active in the tubulin assay (Table 6).

Cell cycle analysis was carried out on those agents which had shown some activity in the tubulin assays. Antimitotic agents tend to accumulate their DNA in the G_2/M phase of the cell cycle. All the compounds in Table 7 showed at least a moderate ability to block cells in the G_2/M phase. As well as combretastatin A-4 **1**, the fluoro and phenolic compounds **10a**,**15a**,**18a**,**51a**,**54a** and the stilbene **50a** showed potent ability to block cells in the G_2/M phase of the cell cycle.

Immunohistochemistry was used to visualise the effects of several of these agents on microtubules of VERO cells. In the control (Fig. 2a), the dark nuclei are clearly visible with intense staining occurring around the perinuclear area from which the microtubule network radiates out into the cytoplasm. When the cells were treated with combretastatin A-4 (**1**), a complete loss of fine structure was noticeable (Fig. 2b) and the staining became almost uniform over the cellular cytoplasm. The cells round-up and detach from their substratum. The immunohistochemistry sections for the trimethyl agents **50a**–**52a** reflect the results obtained in the cytotoxicity assays. The microtubule structures in the cells which have been treated with **50a** (Fig. 2c) and **51a** (Fig. 2d) are clearly visible and undamaged. However, in the cells treated with phenol **52a** (Fig. 2e), the microtubules show a similar disruption pattern to those treated with combretastatin A-4 (**1**). The triethoxy agents **15a** (Fig. 2f) and **18a** (Fig. 2g) also disrupt the microtubule structures in VERO cells. The microtubule structures (Fig. 2h) in the cells which have been treated with dimethyl phenol **36a** show some disruption but not to the extent of its trimethyl equivalent **52a**. Interestingly following treatment with **36a**, there appear to be several star-like structures present around the nuclei which may be asters. Asters are structures which are often seen when microtubules are allowed to repolymerise following treatment with a reversible antimitotic agent and it is from these structures that microtubule organising centres act as the nucleus for microtubule growth.

Table 5 Cytotoxicity (K562 cells, μM), inhibition of tubulin assembly (MA, μM), colchicine displacement (CD, μM) of dimethylstilbenes **28**–**34** and **36**

Compound	IC_{50} (K562)	IC_{50} MA	IC_{50} CD
28a	3.4	>10	>25
28b	>50	>10	>25
29a	1.8	>10	>25
29b	24	>10	>25
30a	1.6	>10	>25
30b	>50	>10	>25
31a	1.15	>10	>25
31b	>50	>10	>25
32a	0.09	6.57	>25
32b	2.5	>10	>25
33a	0.39	>10	>25
33b	>50	>10	>25
34a	1.5	>10	>25
34b	>50	>10	>25
36a	0.07	3.09	12.5

Table 6 Cytotoxicity (K562 cells/μM), inhibition of tubulin assembly (MA/μM), colchicine displacement (CD/μM) of trimethylstilbenes **50**–**52**

Compound	IC_{50} (K562)	IC_{50} MA	IC_{50} CD	IC_{50} HUVEC
50a	0.31	0.650	>25	>1
51a	0.14	0.700	10	0.46
52a	0.020	0.120	10	0.05

Conclusions

It is already well-documented that the *Z*-stilbenes are more potent microtubule interactors than their *E*-counterparts.[1–3,7,14] This fact is again borne out in this study. In this study the agents which have shown the greatest ability to interact with tubulin have been those possessing a 3-hydroxy substituent on the B ring. This again tallies with previous findings that small groups on the 4-position of the B ring along with a vacant or 3-hydroxy substituent are essential for microtubular interaction.

At present combretastatin A-4 (**1**) is in clinical trial as its water-soluble disodium phosphate salt. This phosphate has a short plasma half-life and dephosphorylates[10,21] to the phenol **1**. The discovery herein, that the B ring fluorinated stilbenes **10a**,**15a**,**51a** also show potent activities, may avoid this type of event.

The binding of colchicine (**2**) and combretastatin A-4 (**1**) to tubulin is reversible; so it is unlikely that a covalent bond is formed between agents **1**, **2** and the protein. One way in which the trimethoxy unit on combretastatin A-4 **1** may interact with its binding site on tubulin is through the oxygen atoms hydrogen bonding with the protein. This tallies with the results described herein for the triethoxy compounds **15a** and **18a**. However the microtubule disruption caused by the dimethyl **36a** and more significantly by the trimethyl substituted agent **52a**, indicates that hydrogen bonding of the A ring to tubulin is unlikely to be a significant mechanism of interaction.

What is particularly exciting about agent **10a** is that it is ten times less cytotoxic than combretastatin A-4 (**1**), but *more* active in depolymerising microtubules. Of the non-trimethoxy agents the trimethylphenol **52a** is again less toxic (twentyfold) than combretastatin A-4 (**1**) and again more active in inhibiting the assembly of tubulin.

Table 7 Cell cycle analysis of K562 cells treated with stilbenes **1**, **10**, **15**, **18**, **24**, **32**, **36**, **41**, **50**, **51 and 52**

		% cells with DNA content ≥ 2*n*		
Compound	% cells with DNA content < 2*n*	% cells in G_0–G_1 phase	% cells in S phase	% cells in G_2M phase
1	7	3	6	92
10a	5	5	16	79
15a	24	7	18	75
18a	4	3	6	92
24a	38	12	39	49
32a	28	10	33	57
36a	31	8	27	65
41a	25	8	24	68
50a	15	3	10	87
51a	23	6	17	77
52a	6	3	6	91

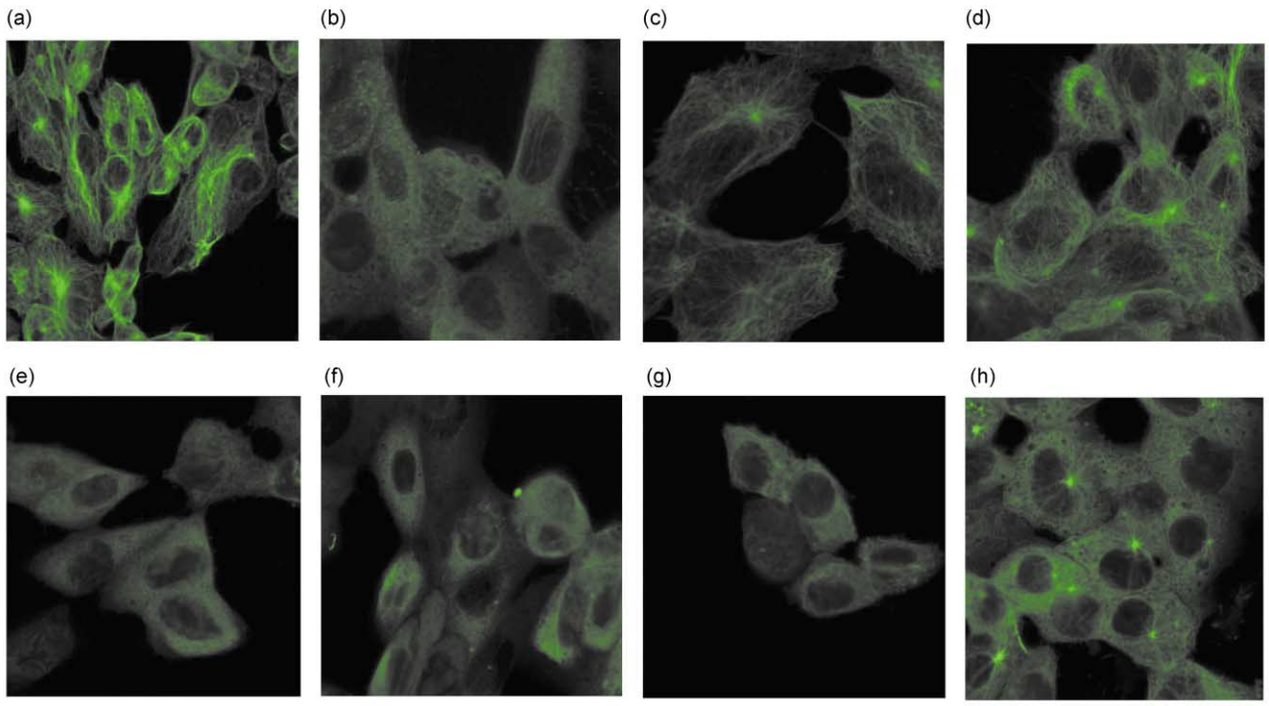


Fig. 2 (a) Immunohistochemistry sections of VERO cells [Control (DMSO)]. (b) After treatment with Combretastatin A-4 **1**. (c) After treatment with trimethylstilbene **50a**. (d) After treatment with trimethylstilbene **51a**. (e) After treatment with phenolic trimethylstilbene **52a**. (f) After treatment with triethoxystilbene **15a**. (g) After treatment with triethoxystilbene **18a**. (h) After treatment with phenolic dimethylstilbene **36a**.

Acknowledgements

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