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Synthesis of combretastatin A-4 *O*-alkyl derivatives and evaluation of their cytotoxic, antiangiogenic and antitelomerase activity

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Dedicated to Professor G. Asensio, University of Valencia, on the occasion of his 65th birthday

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ABSTRACT

We here report the synthesis and biological evaluation of several combretastatin A-4 derivatives alkylated at the phenol hydroxyl group. Some of these derivatives contain an (*E*)-arylalkene fragment reminiscent of that present in some natural stilbenes like resveratrol. The cytotoxicities towards one human healthy kidney embryonic and two tumoral cell lines were determined. In addition, the ability of these compounds to inhibit the production of the vascular endothelial growth factor (VEGF) was measured. Finally, the expression of genes controlling the production of telomerase was measured. Some of the compounds were found to have an activity comparable or higher than that of combretastatin A-4 in at least one of the aforementioned biological properties. The compounds with the (*E*)-arylalkene fragment were in general terms more active than the simple *O*-alkyl derivatives. However, no clear structure/activity correlations were perceived when comparing the observed compound activities across the three biological properties. This points out the existence of marked differences between the mechanisms responsible for their cytotoxicity.

1. Introduction

It is widely known that cancer, one leading cause of death in developed countries,[1] may be induced by a plethora of both external and internal factors, including genetic mutations. Accordingly, a number of types of therapeutic attack has been investigated.[2,3] One of these involves the use of cytotoxic drugs, which exert their effect in many cases by means of inducing cell apoptosis.[4] A second type of therapeutic strategy against cancer is based on the use of compounds with vascular-targeting properties. These may be due to their ability to either inhibit the formation of new blood vessels (antiangiogenic agents) or else to promote the destruction of existing ones (antivascular agents).[5,6] Tumor angiogenesis is a very complex process and involves the tight interplay of many factors.[7] One of these is a protein called vascular endothelial growth factor (VEGF), a key regulator of angiogenesis which promotes endothelial cell survival, proliferation and migration while increasing vascular permeability.[8] In fact, overexpression in the production of VEGF has been reported to occur in various types of tumors.[9] Not unexpectedly, VEGF has become one further key target molecule in cancer therapy.[10,11]

A third therapeutic line follows the path of the chromosomal telomeres.[12] Telomeres are the terminal zones of chromosomes and display a special structure that fulfills at least two essential functions: (a) they must be recognized as functional domains, thus distinguishing them from random chromosomal breaks that would stimulate the onset of repair mechanisms; (b) they must prevent these DNA ends from fusing with other DNA ends.[13] To comply with these functions, an appropriate amount of repetitive DNA sequences (telomeres) must be added to the ends of the chromosomes. This task is fulfilled by a special type of ribonucleoprotein complex called telomerase, an enzyme with reverse transcriptase activity. The expression of this enzyme is restricted or absent in normal human somatic cells and so telomeres progressively shorten during cell lifespan.[12] This triggers a DNA damage response which culminates in cell senescence or apoptosis.[14]

Shortening of telomeres during cell division provides a barrier for tumor progression. Indeed, cancer cells have evolved the ability to overcome senescence by using mechanisms capable of maintaining telomere lengths, such as expressing telomerase, which enables them to divide indefinitely. This uncontrolled cell growth

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is a key feature of cancer malignancy and has been found to correlate with telomerase reactivation.[15] For this reason, and since telomerase has been detected in about 90% of all malignant tumors,[12] drugs with ability to inhibit telomerase activity are potentially useful weapons in the fight against cancer, aging and other diseases, including some related to premature telomere shortening.[16]

The emergence of resistances to existing drugs has led to a continuous need of developing new bioactive compounds that overcome such problems. Even though first observed in the case of antibiotics, resistances have been reported to therapies with various types of cytotoxic,[17] antiangiogenic[18] and antitelomerase agents.[19] The discovery and investigation of new members of these compound classes therefore constitutes an important goal in chemistry and pharmacology. Among the new drug types, the combretastatin family, which belongs like the well-known resveratrol to the stilbene class of natural products,[20] has acquired an outstanding status in the last years and their members have been found utility in various pharmacological applications.[21] The knowledge of the chemistry, biology, and medical potential of this particular compound class, discovered about 30 years ago in the African tree *Combretum caffrum*, has continued to advance.[22] Preclinical and clinical developments over the last decade have been rapidly accelerating for drugs such as combretastatin A-4 (**1a**), most particularly in the form of its phosphate prodrug CA4P (**1b**), and for combretastatin A-1 (**1c**) as promising cancer vascular-disrupting and ophthalmology drugs (Fig. 1).[22,23] Indeed, these encouraging developments have stimulated a variety of efforts devoted to the synthesis and biological evaluation of numerous combretastatin structural modifications.[24] Recent reports include SAR studies that provide varying levels of cancer cell growth inhibition.[25]

2. Research purpose

In the present paper, we are disclosing our results in the synthesis and biological evaluation of a number of *O*-alkyl derivatives of combretastatin A-4 **1a**. These are arranged in two series as shown in Fig. 2. One includes compounds **2a**–**e** having *O*-alkyl residues of the allyl and benzyl type. The second series encompasses compounds **2f**–**n**, which contain (*E*)-5-arylpent-4-enyl residues with various aryl groups. This part of the structure displays an arylalkene fragment which shows similarity to a part of the structure of antiangiogenic stilbenes of the resveratrol type (Fig. 1).[20,26] Accordingly, it may be expected that these compounds could not only exhibit cytotoxicity but also antiangiogenic activity. Thus, we here present the results of our measurements of the cytotoxicities of compounds **2a**–**n** but also of their ability to inhibit the production of the VEGF. Finally, and in order to cover all three aforementioned lines of therapeutic attack, we also have tested their ability to inhibit telomerase activity. In our belief, the observed results might be possibly helpful in aiding to clear the mechanisms of action of these compounds.

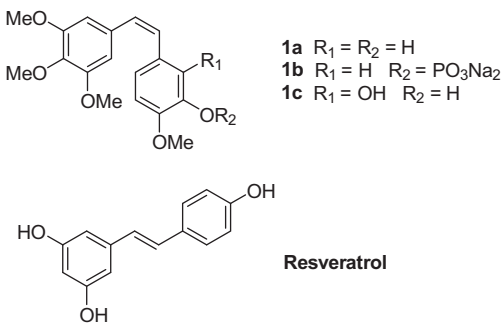


Figure 1. Structures of some combretastatins (**1a**–**1c**) and resveratrol.

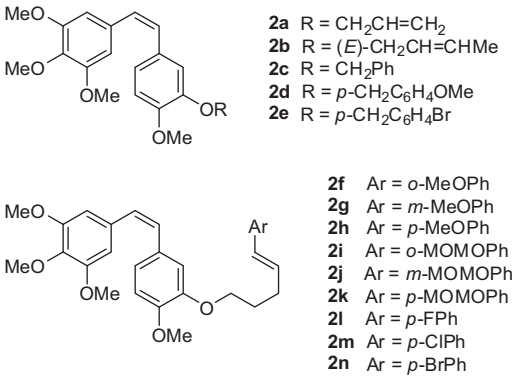


Figure 2. Structures of combretastatin A-4 *O*-alkyl derivatives **2a**–**2n** used in this study (MOM = $MeOCH_2$).

3. Chemical results

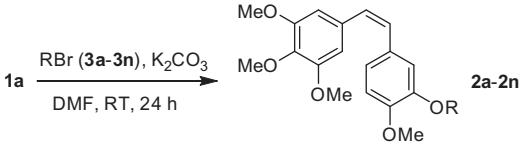
Combretastatin A-4 **1a**, the starting material for all compounds discussed here, was prepared according to a literature procedure.[27] Conversion into *O*-alkyl derivatives **2a**–**2n** was performed as depicted in Scheme 1 using bromides **3a**–**3n** (Scheme 2) as the alkylating reagents. Yields are given in the experimental part.

From the alkyl bromides mentioned in Scheme 1, **3a**–**3e** are commercially available. Bromides **3f**–**3n** were synthesized by means of ruthenium-catalyzed cross metathesis[28] between the commercially available bromide **4** and styrene derivatives **5**–**13** (Scheme 2). Yields are given in the experimental part.

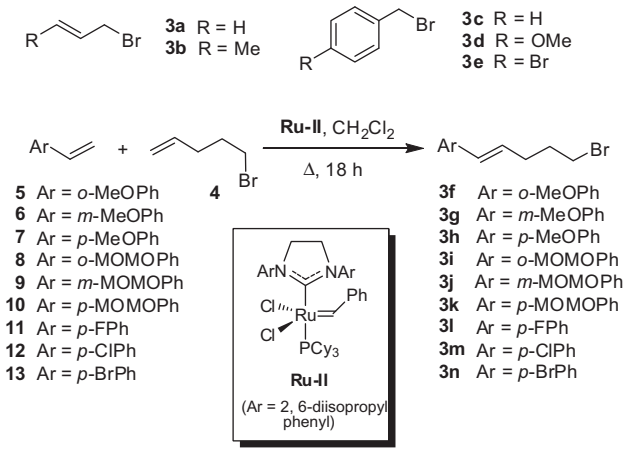
4. Biological results

4.1. Cytotoxicity of combretastatine derivatives

We first carried out a measurement of the cytotoxicity of the synthetic combretastatin derivatives **2a**–**2n**. To this purpose, MTT assays were performed using two tumoral cells, the human colon HT-29 and the breast adenocarcinoma MCF-7 cell lines, as well as one normal cell line, the human embryonic kidney cell line, HEK-293.[29] Cytotoxicity values, expressed as the compound concentration (μmol/L) that causes 50% inhibition of cell growth (IC_{50}), are shown in Table 1. The observed values are in the low to medium micromolar range, with compound **2i** showing the lowest values, not very different of those of combretastatin A-4 (CoA4) for these two particular cell lines. In addition, it is worth mentioning that some of the synthetic compounds are much more toxic for tumoral cells than for normal ones, an obviously desirable feature. This can be better appreciated with the α and β coefficients, obtained by dividing the IC_{50} values of the normal cell line by those of one or the other tumoral cell line (see footnote in Table). The highest value of either coefficient, the highest the therapeutic safety margin of the compound in the corresponding cell line. Thus, combretastatin A-4 shows high values of both coefficients, most particularly in the case of the MCF-7 cell line. This turns out also



Scheme 1. Synthesis of combretastatin A-4 *O*-alkyl derivatives **2a**–**2n**.



Scheme 2. Synthesis of alkyl bromides **3f**–**3n**.

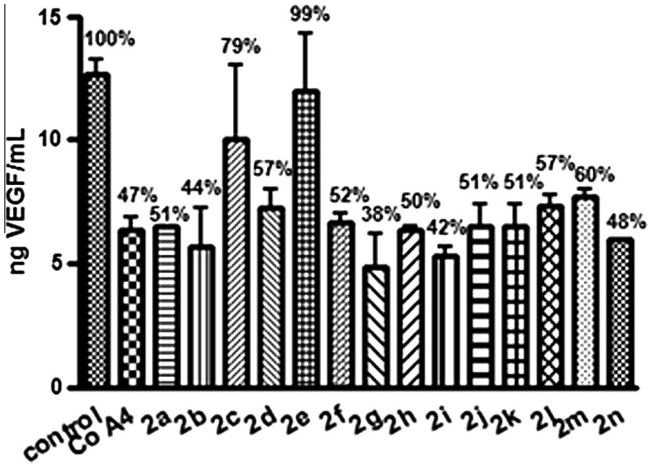


Figure 3. VEGF protein production from HT-29 cells treated with DMSO (control), combretastatin A-4 (CoA4) and derivatives **2a**–**n** (at least three measurements were performed in each case). Bars represent mean values of VEGF expression (in ng/mL) and error bars indicate standard errors of the mean. The statistical significance was evaluated using one-sample *t*-tests (P <0.001).

Table 1
Cytotoxicity of combretastatin A-4 derivatives **2a**–**2n**[a]

Compound	HT-29	MCF-7	HEK-293	α[b]	β[c]
CoA4	*4.2 ± 0.5*	*1 ± 0.2*	25 ± 3	*5.9*	*25*
2a	29 ± 3	12.0 ± 0.4	43.5 ± 0.8	1.5	3.6
2b	161 ± 5	*5.4 ± 0.4*	135 ± 13	0.8	*25*
2c	39 ± 6	59 ± 2	15 ± 3	0.4	0.3
2d	47 ± 5	48 ± 1	64 ± 7	1.4	1.3
2e	108 ± 1	29 ± 1	45 ± 4	0.4	1.6
2f	86 ± 6	21 ± 2	>400	*>4.6*	*>19*
2g	25 ± 3	41 ± 3	39 ± 2	1.6	1.0
2h	47 ± 7	16 ± 3	88 ± 6	1.9	*5.5*
2i	*9.6 ± 0.2*	*4.4 ± 0.8*	106 ± 9	*11*	*24.1*
2j	59 ± 7	8 ± 3	30 ± 2	0.5	3.7
2k	*7 ± 0.4*	42 ± 7	25 ± 4	3.6	0.6
2l	98 ± 8	16 ± 2	115 ± 5	1.2	*7.2*
2m	18 ± 3	40 ± 5	111 ± 3	*6.2*	2.8
2n	*11 ± 2*	37 ± 8	55 ± 5	*5.0*	1.5

Values of α and β have been rounded off to a decimal figure. The lowest IC_{50} values (for tumoral cells) and the highest values of the α and/or β coefficients (>4) have been highlighted in italics.

[a] IC_{50} values include those of combretastatin A-4 itself CoA4 and are expressed as the compound concentration (μmol/L) that causes 50% inhibition of cell growth, and are the average (±SD) of three different measurements (described in Section 6).

[b] α = IC_{50} (HEK-293)/IC_{50} (HT-29).

[c] β = IC_{50} (HEK-293)/IC_{50} (MCF-7).

to be the case of compounds **2f** and **2i**. Compounds **2m** and **2n** show a good selectivity only in the case of the HT-29 line (α >4) whereas compounds **2b**, **2h** and **2l** show a good selectivity in the specific case of the MCF-7 line (β >4).

4.2. Effect of combretastatin A-4 derivatives on VEGF production

According to that discussed in the Introduction section, we next investigated whether combretastatin derivatives **2a**–**n** were able to inhibit or at least decrease the production of the VEGF protein in HT-29 tumoral cells. Figure 3 shows the results of VEGF production obtained by means of ELISA measurements after treatment of HT-29 tumoral cells with combretastatin A-4 and with compounds **2a**–**n** dissolved in DMSO, which was the control substance in all experiments (in all cases, concentrations values below IC_{50} were used). With the control substance, the observed VEGF production was standardized to 100%, the other values being then referred to it. Thus, compound **2g** caused the highest degree of inhibition in the VEGF production, which underwent a reduction to 38% of the control value. This is an even stronger effect than that caused by combretastatin A-4, where VEGF production was reduced to 47% of the control value. Similarly strong effects were observed in the cases of compounds **2b** (44%) and **2i** (42%). The remaining compounds showed less favourable values, with compound **2e** being essentially inactive (99%).

While the results depicted in Figure 3 point out that compounds **2a**–**n** cause inhibition of the VEGF production, they do not say anything about the precise phase of the VEGF generation process with which they interfere. In order to deepen into the knowledge of this issue, we proceeded to determine whether the compounds under study were able to control protein production at the transcriptional level. With this idea in mind, we performed a reverse transcriptase/polymerase chain reaction (RT-PCR) analysis. For this purpose, we selected six of the combretastatin A-4 derivatives with the highest anti-VEGF activities (**2b**, **2g**, **2i**, **2j**, **2k** and **2n**). As shown in Figure 4, treatment of HT-29 tumoral cells with these derivatives and with combretastatin A-4 in DMSO did in fact cause a reduction of the transcription of VEGF mRNA as compared with control cells (the values of these are standardized to 100%). The most active derivatives turned out to be **2b** and **2k**, which proved able to reduce the expression of the corresponding gene to less than 70% of the control value. These effects are thus stronger than that of combretastatin A-4 itself, which only causes a slight reduction of the transcription level of VEGF mRNA (91%). Interestingly, compound **2g**, which showed the highest degree of inhibition of VEGF production (Fig. 3), leaved the transcription of VEGF mRNA practically unaltered (97%).

Table 2 present the same results depicted in Figures 3 and 4 for the aforementioned six compounds (**2b**, **2g**, **2i**, **2j**, **2k** and **2n**) although in the form of percentages of *inhibition* of VEGF production (100—% VEGF production) and of gene expression (100—% gene expression): thus, the highest values correspond to the strongest inhibitory effect. There is a visible lack of parallelism between the data of protein inhibition and those of gene inhibition. This suggests that these combretastatin A-4 derivatives exert the control of VEGF production at a phase different from that of gene transcription, perhaps during the posttranslational stage.

4.3. Effect of combretastatin A-4 derivatives on telomerase production

We were also interested in designing compounds with the ability to inhibit the expression of telomerase in tumoral cells. The role of this important ribonucleoprotein has been referred to

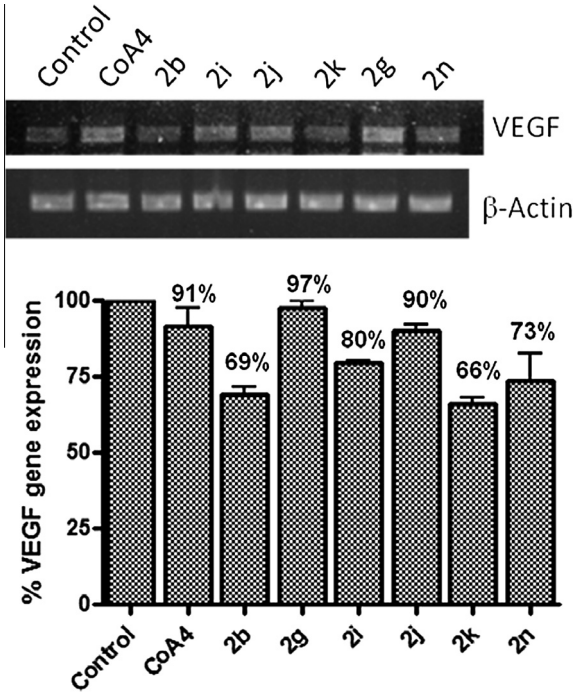


Figure 4. Agarose gel profile of products resulting from RT-PCR amplification. The total RNA of HT-29 cells previously treated with the appropriate combretastatin A-4 derivative was isolated, converted into cDNA, and amplified by PCR as described in Section 6 (primers used for the RT-PCR are shown in Table 3). Gene expression of VEGF and β-actin was quantified using the Image J program and normalized to that of the housekeeping gene β-actin. At least three measurements were performed in each case. Bars shown represent mean activations of VEGF gene expression and error bars indicate standard errors of the mean. The statistical significance was evaluated using one-sample *t*-tests (P <0.001).

Table 2
Inhibition of VEGF protein and of gene expression by combretastatin A-4 and some of its *O*-alkyl derivatives[a]

Compound	% Protein inhibition[b]	% Gene inhibition[c]
CoA4	53	9
2b	56	31
2g	62	3
2i	58	20
2j	49	10
2k	49	34
2n	52	27

[a] Concentrations used were below the IC_{50} values.
[b] Values obtained by subtracting from 100 the percentages of VEGF secretion values in Fig. 3.
[c] Values obtained by subtracting from 100 the percentages of VEGF gene inhibition values in Fig. 4.

in Section 1.[12–16] Human telomerase contains an RNA component (hTR) that serves as a template for the addition of the repeat nucleotide sequences and a protein subunit (hTERT) which catalyzes the nucleotide polymerization process. In addition, there are other associated protein factors, the role of which has not yet been completely elucidated. Human telomerase is regulated during development and differentiation, mainly through transcriptional control of the hTERT gene, the expression of which is restricted to cells that exhibit telomerase activity. This indicates that hTERT is the rate limiting factor of the enzyme complex.[16a] For the expression of the hTERT gene, two transcriptional factors called c-Myc and Sp1, among others, have been found to play an important role through upregulation of the mRNA encoding the hTERT protein subunit of telomerase.[30] Thus, and as a preliminary study of the potential anti-telomerase activity of combretastatin derivatives, we have investigated their ability to inhibit the expression of the hTERT and *c-myc* genes.

In order to determine whether *O*-alkyl derivatives of combretastatin A-4 were able to regulate the expression of the hTERT and *c-myc* genes, we have performed a RT-PCR analysis using HT-29 tumoral cells. For that purpose, we selected the same group of derivatives (**2b**, **2g**, **2i**, **2j**, **2k** and **2n**) previously investigated for their antiangiogenic activity. The results, depicted in Figure 5, show that treatment of HT-29 cells with combretastatin A-4 and the aforementioned derivatives dissolved in DMSO leads in fact to various degrees of reduction in the transcription of hTERT and

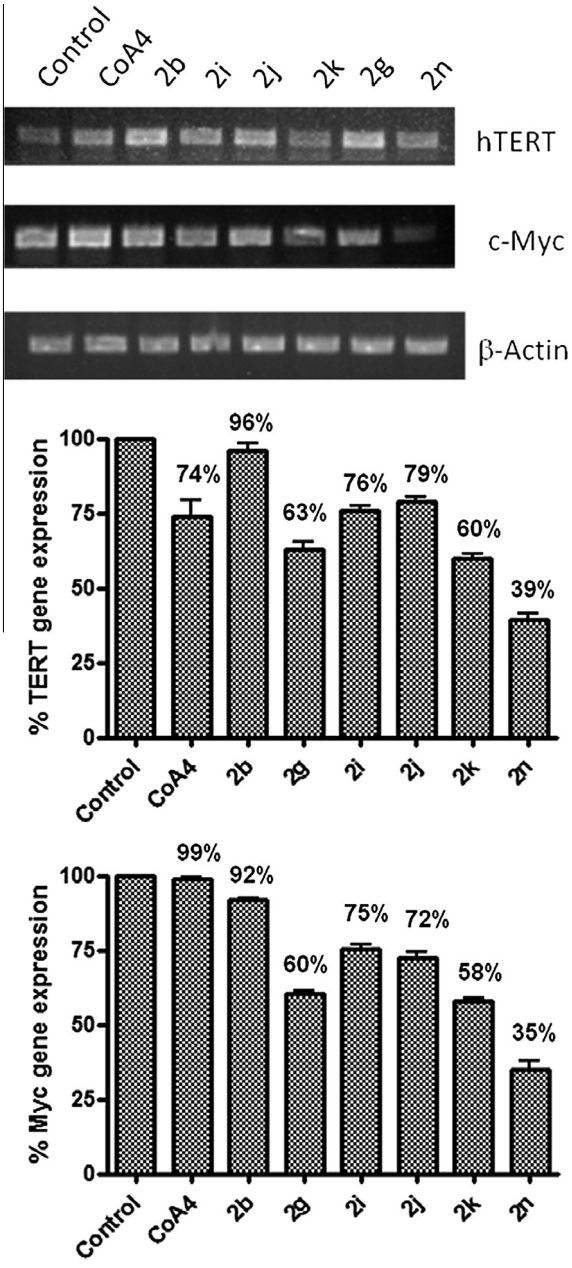


Figure 5. Agarose gel profile of products resulting from RT-PCR amplification. The total RNA of HT-29 cells previously treated with the appropriate compound was isolated, converted into cDNA, and amplified by PCR as described in Section 6 (primers used for the RT-PCR are shown in Table 3). Gene expression of hTERT, *c-myc* and β-actin was quantified using the Image J program and normalized to that of the housekeeping gene β-actin. At least three measurements were performed in each case. Bars shown represent mean activations of hTERT and *c-myc* gene expression and error bars indicate standard errors of the mean. The statistical significance was evaluated using one-sample *t*-tests (P <0.001).

c-myc mRNA as compared with control cells. The most active derivative was found to be **2n**, which proved able to reduce the expression of both genes to less than 40% of the control value. For the sake of comparison, combretastatin A-4 caused a reduction to 74% of the control value in the case of the hTERT gene and proved practically inactive in the case of the *c-myc* gene.

Comparison of the results for the expression of the hTERT gene with those of the *c-myc* gene reveals a good correlation between them (Fig. 5). We may conclude therefore that the compounds under study downregulate the expression of the hTERT gene by lowering the transcription of the *c-myc* gene.

5. Summary

A series of *O*-alkyl derivatives of combretastatin A-4 has been prepared and evaluated in relation to three types of biological properties: cytotoxicity, antiangiogenesis and telomerase inhibition. One healthy (human embryonic kidney, HEK-293) and two tumoral cell lines (human colon HT-29 and breast adenocarcinoma MCF-7) were used for the assays.

No clear correlations are perceived between structure and activity in the compounds under study. For instance, the strongest cytotoxicities (lowest IC_{50} values) and the highest α or β values are found almost exclusively among the compounds containing the (*E*)-arylalkene fragment, that is, within the **2f**–**2n** group (Table 1). However, the observed IC_{50} values do not bear a close relationship with the α/β coefficients. Only in the case of compound **2i** was an appreciable cytotoxicity towards both tumoral cell lines *accompanied by high α and β values*. In other cases (e.g., **2f**, **2h**, **2n**), these desirable features were observed in only one of the two cell lines.

The inhibition of the VEGF production, as a measure of the antiangiogenic activity, did not show a very marked relation with the structural type, either. Except for **2c** and **2e**, which were clearly less active, all other compounds displayed comparable activities, with **2g** being the most active. The most outstanding aspect here was that some of the compounds (**2b**, **2g** and **2i**) proved even more active in this property than combretastatin A-4. Again, these belong to the **2f**–**2n** group. From these, **2i** also showed favourable cytotoxicity indexes, as commented above.

The ability to inhibit the expression of the hTERT and *c-myc* genes was also found in the investigated combretastatin A-4 derivatives. The profile was, however, clearly different from that observed in the two other biological properties. For example, the strongest activity was found here in compound **2n**, with compounds **2b**, **2g** and **2i**, which had favourable antiangiogenic features, being much less active. Nonetheless, it is worth noting that several of the compounds displayed a higher activity than combretastatin A-4. Once again, the compounds of the **2f**–**2n** group were found more active than the simple *O*-alkyl derivatives.

In summary, some of the investigated combretastatin A-4 *O*-alkyl derivatives show an activity comparable or higher than that of combretastatin A-4 itself in at least one of the three examined biological properties. While it seems that compounds containing the (*E*)-arylalkene fragment (**2f**–**2n**) display in general stronger activities than the simple *O*-alkyl derivatives (**2a**–**2e**), no clear structure/activity correlations were perceived, however, when comparing the observed compound activities across the three biological properties. This point out the existence of marked differences between the mechanisms responsible for their cytotoxicity.

6. Materials and methods

6.1. Chemistry: general procedures

General features. NMR spectra were recorded at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) in $CDCl_3$ solution at 25 °C, if not otherwise indicated, with $CDCl_3$ signals as internal reference. ^{13}C NMR signal multiplicities were determined with the DEPT pulse sequence. Mass spectra were run in the electrospray (ESMS) mode and, in some cases, in the electron impact mode (EIMS). Reactions which required an inert atmosphere were carried out under dry N_2 with flame-dried glassware. Commercial reagents were used as received. Dichloromethane was freshly distilled from CaH_2. Column chromatography was performed on a silica gel column (60–200 μm) with elution with the indicated solvent mixtures.

6.2. Reaction conditions

6.2.1. Styrene derivatives

Styrene derivatives **5**–**7** and **11**–**13** are commercially available. Styrenes **8**–**10** were prepared as reported[31] by means of Wittig methylenation of the appropriate commercial benzaldehydes (yields in the range 60–70%):

*Compound **8**:* oil; ^{1}H NMR (500 MHz) δ 7.54 (1H, dd, J = 7.7, 1.5 Hz), 7.26 (1H, td, J = 7.7, 1.5 Hz), 7.20–7.10 (2H, m), 7.04 (1H, td, J = 7.5, 1 Hz), 5.80 (1H, dd, J = 17.7, 1.5 Hz), 5.33 (1H, dd, J = 11, 1.5 Hz), 5.25 (2H, s), 3.53 (3H, s); ^{13}C NMR (125 MHz) δ 154.4, 127.6 (C), 131.5, 128.8, 126.4, 121.9, 114.8 (CH), 114.5, 94.7 (CH_2), 56.0 (CH_3).

*Compound **9**:* oil; ^{1}H NMR (500 MHz) δ 7.26 (1H, t, J = 7.9 Hz), 7.12 (1H, br t, $J \sim 2$ Hz), 7.08 (1H, br d, $J \sim 7.6$), 6.97 (1H, ddd, J = 7.9, 2.4, 1 Hz), 6.71 (1H, dd, J = 17.5, 10.8 Hz), 5.77 (1H, dd, J = 17.5, 1 Hz), 5.27 (1H, dd, J = 10.8, 1 Hz), 5.21 (2H, s), 3.51 (3H, s); ^{13}C NMR (125 MHz) δ 157.6, 139.2 (C), 136.7, 129.5, 120.1, 115.7, 114.0 (CH), 114.3, 94.5 (CH_2), 56.0 (CH_3).

*Compound **10**:* oil; ^{1}H NMR (500 MHz) δ 7.38 (2H, br d, $J \sim 8.5$ Hz), 7.05 (2H, br d, $J \sim 8.5$ Hz), 6.71 (1H, dd, J = 17.6, 11 Hz), 5.77 (1H, d, J = 17.6 Hz), 5.21 (2H, s), 5.20 (1H, d, J = 11 Hz), 3.52 (3H, s); ^{13}C NMR (125 MHz) δ 157.0, 131.6 (C), 136.2, 127.3 (×2), 116.3 (×2) (CH), 112.0, 94.4 (CH_2), 55.9 (CH_3).

6.2.2. Synthesis of bromides 3f–3n by means of cross metathesis

Representative example: a solution of bromide **4** (60 μL, 75 mg, ca. 0.5 mmol) and styrene **6** (235 mg, 1.75 mmol) in dry, degassed CH_2Cl_2 (50 mL) was treated with **Ru-II** catalyst (150 mg, 0.175 mmol). The reaction mixture was then stirred at reflux under N_2 for 24 h. Subsequently, the mixture was treated with DMSO (600 μL)[32] and stirred overnight at room temperature. The reaction mixture was then evaporated under reduced pressure, and the residue was carefully chromatographed on silica gel (elution with hexane–Et_2O, 400:1).[33] This gave **3g** (55 mg, 43% based on **4**) together with the stilbene derivative generated by homodimerization of **6** (major product). In the other examples, yields were in the range 35–50% except for bromides **3l**–**3n**, which could not be purified and were obtained only in admixture with variable percentages of the homodimerization products. The mixtures were then used for the alkylation step.

*Compound **3f**:* oil; ^{1}H NMR (500 MHz) δ 7.43 (1H, br d, $J \sim 7.8$ Hz), 7.22 (1H, br t, $J \sim 7.8$ Hz), 6.94 (1H, t, J = 7.8 Hz), 6.88 (1H, br d, $J \sim 7.8$ Hz), 6.79 (1H, d, J = 16 Hz), 6.20 (1H, dt, J = 16, 7 Hz), 3.87 (3H, s), 3.48 (2H, t, J = 7 Hz), 2.42 (2H, br q, $J \sim 7$ Hz), 2.07 (2H, br quint, $J \sim 7$ Hz); ^{13}C NMR (125 MHz) δ 156.4, 126.4 (C), 129.2, 128.1, 126.5, 126.0, 120.6, 110.9 (CH), 33.2, 32.4, 31.8 (CH_2), 55.5 (CH_3); HR EIMS *m/z* (rel int.) 254.0351 (M^+, 40), 239 (M^+−Me, 100). Calcd for $C_{12}H_{15}$ ^{79}BrO, 254.0306.

*Compound **3g**:* oil; ^{1}H NMR (500 MHz) δ 7.23 (1H, t, J = 7.8 Hz), 6.96 (1H, br d, $J \sim 7.8$ Hz), 6.91 (1H, br t, $J \sim 2$ Hz), 6.79 (1H, dd, J = 7.8, 2.5 Hz), 6.44 (1H, d, J = 16 Hz), 6.18 (1H, dt, J = 16, 7 Hz), 3.83 (3H, s), 3.47 (2H, t, J = 7 Hz), 2.40 (2H, br q, $J \sim 7$ Hz), 2.05 (2H, br quint, $J \sim 7$ Hz); ^{13}C NMR (125 MHz) δ 159.8, 138.9 (C), 131.2, 129.5, 128.8, 118.7, 112.8, 111.4 (CH), 33.1, 32.2, 31.2 (CH_2), 55.2 (CH_3); HR EIMS *m/z* (rel int.) 254.0336 (M^+, 80), 175

(M^+–Br, 100), 147 (M^+–Br–C_2H_4, 98). Calcd for $C_{12}H_{15}$ ^{79}BrO, 254.0306.

Compound ***3h****:* oil; ^{1}H NMR (500 MHz) δ 7.30 (2H, br d, $J \sim 8.5$ Hz), 6.86 (2H, br d, $J \sim 8.5$ Hz), 6.41 (1H, d, $J = 16$ Hz), 6.04 (1H, dt, $J = 16$, 7 Hz), 3.82 (3H, s), 3.46 (2H, t, $J = 7$ Hz), 2.38 (2H, br q, $J \sim 7$ Hz), 2.05 (2H, br quint, $J \sim 7$ Hz); ^{13}C NMR (125 MHz) δ 158.9, 128.6 (C), 130.7 (×2), 127.1 (×2), 126.3, 114.0 (CH), 33.2, 32.4, 31.3 (CH_2), 55.3 (CH_3); HR EIMS *m/z* (rel int.) 254.0362 (M^+, 45), 175 (M^+–Br, 12), 147 (M^+–Br–C_2H_4, 100). Calcd for $C_{12}H_{15}$ ^{79}BrO, 254.0306.

Compound ***3i****:* oil; ^{1}H NMR (500 MHz) δ 7.45 (1H, br d, $J \sim 7.8$ Hz), 7.19 (1H, br t, $J \sim 7.8$ Hz), 7.10 (1H, br d, $J \sim 7.8$ Hz), 6.98 (1H, br t, $J \sim 7.8$ Hz), 6.81 (1H, d, $J = 16$ Hz), 6.18 (1H, dt, $J = 16$, 7 Hz), 5.23 (2H, s), 3.52 (3H, s), 3.49 (2H, t, $J = 7$ Hz), 2.42 (2H, br q, $J \sim 7$ Hz), 2.07 (2H, br quint, $J \sim 7$ Hz); ^{13}C NMR (125 MHz) δ 154.1, 127.4 (C), 129.2, 128.1, 126.4, 125.8, 122.0, 115.0 (CH), 94.8, 33.2, 32.4, 31.7 (CH_2), 56.1 (CH_3); HR EIMS *m/z* (rel int.) 284.0428 (M^+, 60), 239 (M^+–CH_2OMe, 100). Calcd for $C_{13}H_{17}^{79}BrO_2$, 284.0412.

Compound ***3j****:* oil; ^{1}H NMR (500 MHz) δ 7.23 (1H, t, $J = 7.8$ Hz), 7.05 (1H, br t, $J \sim 2$ Hz), 7.01 (1H, br d, $J \sim 7.8$ Hz), 6.92 (1H, br dd, $J \sim 7.8$, 2.2 Hz), 6.43 (1H, d, $J = 15.8$ Hz), 6.18 (1H, dt, $J = 15.8$, 6.8 Hz), 5.20 (2H, s), 3.50 (3H, s), 3.47 (2H, t, $J = 6.8$ Hz), 2.39 (2H, br q, $J \sim 6.8$ Hz), 2.05 (2H, br quint, $J \sim 6.8$ Hz); ^{13}C NMR (125 MHz) δ 157.5, 139.0 (C), 131.1, 129.5, 128.9, 119.8, 115.1, 113.7 (CH), 94.4, 33.0, 32.2, 31.2 (CH_2), 55.9 (CH_3); HR EIMS *m/z* (rel int.) 284.0416 (M^+, 100), 254 (M^+–CH_2O, 36), 175 (M^+–CH_2O–Br, 65). Calcd for $C_{13}H_{17}$ ^{79}BrO_2, 284.0412.

Compound ***3k****:* oil; ^{1}H NMR (500 MHz) δ 7.29 (2H, br d, $J \sim 8.5$ Hz), 6.99 (2H, br d, $J \sim 8.5$ Hz), 6.41 (1H, d, $J = 15.9$ Hz), 6.05 (1H, dt, $J = 15.9$, 6.8 Hz), 5.18 (2H, s), 3.49 (3H, s), 3.46 (2H, t, $J = 6.8$ Hz), 2.37 (2H, br q, $J \sim 6.8$ Hz), 2.04 (2H, br quint, $J \sim 6.8$ Hz); ^{13}C NMR (125 MHz) δ 156.5, 131.5 (C), 130.6, 127.1 (×2), 126.8, 116.3 (×2) (CH), 94.5, 33.1, 32.3, 31.2 (CH_2), 55.9 (CH_3); HR EIMS *m/z* (rel int.) 284.0353 (M^+, 85), 254 (M^+–CH_2O, 76), 147 ($C_5H_8Br^+$, 100). Calcd for $C_{13}H_{17}$ ^{79}BrO_2, 284.0412.

6.2.3. Synthesis of combretastatin A-4 derivatives 2a–2n

Representative example: A solution of **1a** (63 mg, 0.2 mmol) in dry DMF (3 mL) was treated under N_2 in the dark at room temperature with K_2CO_3 (70 mg, ca. 0.5 mmol) and stirred for 1 h. Subsequently, **3a** (52 μL, 0.6 mmol) was added and the stirring was continued for 24 h under the same conditions. The reaction mixture was then poured into saturated aqueous NH_4Cl and extracted three times with Et_2O (3 × 15 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and evaporated under reduced pressure. Column chromatography of the residue on silica gel (elution with hexane–EtOAc, 9:1) provided **2a** (50 mg, 70%). In the other cases, yields were in the range 40–70%. Bromides **3l**–**3n** were used in excess as the mixtures with the homodimerization products (see above). **Caution:** *reaction, work-up and purification procedures should be performed under minimization of exposure to light, due to the ease of photoinduced Z/E isomerization in the combretastatin moiety.*

Compound ***2a****:* solid, mp 107–108 °C; ^{1}H NMR (500 MHz) δ 6.86 (1H, dd, $J = 8.3$, 1.7 Hz), 6.84 (1H, d, $J = 1.7$ Hz), 6.79 (1H, d, $J = 8.3$ Hz), 6.52 (2H, s), 6.50 (1H, d, $J = 12.2$ Hz), 6.46 (1H, d, $J = 12.2$ Hz), 5.95 (1H, ddt. $J = 17.2$, 11.4, 5.5 Hz), 5.27 (1H, dq, $J = 17.2$, 1.5 Hz), 5.20 (1H, dq, $J = 11.4$, 1.5 Hz), 4.40 (2H, dt, $J = 5.5$, 1.5 Hz), 3.86 (3H, s), 3.84 (3H, s), 3.71 (6H, s); ^{13}C NMR (125 MHz) δ 153.0 (×2), 148.7, 147.5, 137.2, 133.0, 129.8 (C), 133.1, 129.7, 128.8, 122.3, 113.9, 111.3, 106.0 (×2) (CH), 117.9, 69.7 (CH_2), 60.9, 56.0 (×3) (CH_3); HR ESMS *m/z* 379.1518 (M+Na^+). Calcd for $C_{21}H_{24}NaO_5$, 379.1521.

Compound ***2b****:* oil; ^{1}H NMR (500 MHz) δ 6.86 (2H, m), 6.74 (1H, d, $J = 8.5$ Hz), 6.53 (2H, s), 6.50 (1H, d, $J = 12.1$ Hz), 6.44 (1H, d, $J = 12.1$ Hz), 5.75-5.60 (2H, m), 4.30 (2H, br d, $J \sim 5.8$ Hz), 3.85 (3H, s), 3.84 (3H, s), 3.72 (6H, s), 1.70 (3H, br dd, $J \sim 6.5$, 1.5 Hz); ^{13}C NMR (125 MHz) δ 153.0 (×2), 148.7, 147.6, 137.2, 133.0, 129.8 (C), 130.8, 129.7, 128.7, 125.9, 122.0, 113.6, 111.1, 106.0 (×2) (CH), 69.5 (CH_2), 60.9, 56.0 (×3), 17.7 (CH_3); HR ESMS *m/z* 393.1677 (M+Na^+). Calcd for $C_{22}H_{26}NaO_5$, 393.1678.

Compound ***2c****:* solid, mp 83–84 °C; ^{1}H NMR (500 MHz) δ 7.35–7.25 (5H, br m), 6.90–6.85 (2H, m), 6.80 (1H, d, $J = 8.3$ Hz), 6.51 (2H, s), 6.47 (1H, d, $J = 12.2$ Hz), 6.43 (1H, d, $J = 12.2$ Hz), 4.94 (2H, s), 3.87 (3H, s), 3.85 (3H, s), 3.71 (6H, s); ^{13}C NMR (125 MHz) δ 153.0 (×2), 148.9, 147.7, 137.2, 136.9, 133.0, 129.8 (C), 133.1, 129.6, 128.4 (×2), 127.7, 127.2 (×2), 122.4, 114.5, 111.5, 106.0 (×2) (CH), 70.9 (CH_2), 60.9, 56.0, 55.9 (×2) (CH_3); HR ESMS *m/z* 429.1674 (M+Na^+). Calcd for $C_{25}H_{26}NaO_5$, 429.1678.

Compound ***2d****:* solid, mp 62–63 °C; ^{1}H NMR (500 MHz) δ 7.24 (2H, br d, $J \sim 8.7$ Hz), 6.95–6.85 (3H, m), 6.78 (2H, br d, $J = 8.7$ Hz), 6.52 (2H, s), 6.47 (1H, d, $J = 12.2$ Hz), 6.43 (1H, d, $J = 12.2$ Hz), 4.87 (2H, s), 3.85 (3H, s), 3.84 (3H, s), 3.81 (3H, s), 3.71 (6H, s); ^{13}C NMR (125 MHz) δ 159.4, 153.0 (×2), 149.0, 147.8, 137.2, 133.0, 129.9, 129.1 (C), 129.7, 129.0 (×2), 128.8, 122.4, 114.7, 113.9 (×2), 111.5, 106.0 (×2) (CH), 70.7 (CH_2), 60.9, 55.9 (×3), 55.3 (CH_3); HR ESMS *m/z* 459.1783 (M+Na^+). Calcd for $C_{26}H_{28}NaO_6$, 459.1784.

Compound ***2e****:* oil; ^{1}H NMR (500 MHz) δ 7.44 (2H, br d, $J \sim 8.4$ Hz), 7.18 (2H, br d, $J = 8.4$ Hz), 6.88 (1H, dd, $J = 8.3$, 1.7 Hz), 6.83 (1H, d, $J = 1.7$ Hz), 6.80 (1H, d, $J = 8.3$ Hz), 6.50 (2H, s), 6.44 (2H, s), 4.87 (2H, s), 3.86 (3H, s), 3.85 (3H, s), 3.71 (6H, s); ^{13}C NMR (125 MHz) δ 153.0 (×2), 149.0, 147.4, 137.2, 136.0, 133.0, 129.8, 121.6 (C), 131.5 (×2), 129.5, 128.8, 128.7 (×2), 122.8, 114.6, 111.5, 106.0 (×2) (CH), 70.2 (CH_2), 60.9, 56.0 (×3) (CH_3); HR ESMS *m/z* 507.0782 (M+Na^+). Calcd for $C_{25}H_{25}$ ^{79}$BrNaO_5$, 507.0783.

Compound ***2f****:* oil; ^{1}H NMR (500 MHz) δ 7.41 (1H, dd, $J = 7.7$, 1.5 Hz), 7.19 (1H, td, $J = 7.7$, 1.5 Hz), 6.91 (1H, td, $J = 7.7$, 1 Hz), 6.90–6.85 (3H, m), 6.78 (1H, d, $J = 8$ Hz), 6.74 (1H, br d, $J \sim 15.8$ Hz), 6.53 (2H, s), 6.50 (1H, d, $J = 12.2$ Hz), 6.45 (1H, d, $J = 12.2$ Hz), 6.22 (1H, dt, $J = 15.8$, 6.8 Hz), 3.90 (2H, t, $J = 6.8$ Hz), 3.86 (3H, s), 3.84 (6H, s), 3.70 (6H, s), 2.36 (2H, br q, $J \sim 7$ Hz), 1.94 (2H, br quint, $J \sim 7$ Hz); ^{13}C NMR (125 MHz) δ 156.3, 153.0 (×2), 148.8, 148.1, 137.2, 133.0, 130.0, 126.8 (C), 130.4, 129.8, 128.8, 127.9, 126.5, 125.2, 122.0, 120.7, 113.9, 111.5, 110.8, 106.0 (×2) (CH), 68.4, 29.8, 28.8 (CH_2), 60.9, 56.1, 56.0 (×2), 55.5 (CH_3); HR ESMS *m/z* 513.2247 (M+Na^+). Calcd for $C_{30}H_{34}NaO_6$, 513.2253.

Compound ***2g****:* oil; ^{1}H NMR (500 MHz) δ 7.21 (1H, t, $J = 7.9$ Hz), 6.94 (1H, br d, $J \sim 7.7$ Hz), 6.90–6.85 (3H, m), 6.78 (1H, d, $J = 8.6$ Hz), 6.76 (1H, dd, $J = 8.3$, 2.5 Hz), 6.53 (2H, s), 6.50 (1H, d, $J = 12$ Hz), 6.45 (1H, d, $J = 12$ Hz), 6.38 (1H, br d, $J \sim 15.8$ Hz), 6.23 (1H, dt, $J = 15.8$, 6.8 Hz), 3.88 (2H, t, $J = 6.7$ Hz), 3.86 (3H, s), 3.84 (3H, s), 3.82 (3H, s), 3.70 (6H, s), 2.34 (2H, br q, $J \sim 7$ Hz), 1.92 (2H, br quint, $J \sim 7$ Hz); ^{13}C NMR (125 MHz) δ 160.0, 153.0 (×2), 148.8, 148.0, 139.2, 137.2, 133.0, 130.0 (C), 130.4, 130.1, 129.7, 129.4, 128.8, 122.1, 118.7, 113.8, 112.5, 111.5 (×2), 106.0 (×2) (CH), 68.2, 29.4, 28.7 (CH_2), 60.9, 56.0 (×3), 55.2 (CH_3); HR ESMS *m/z* 513.2255 (M+Na^+). Calcd for $C_{30}H_{34}NaO_6$, 513.2253.

Compound ***2h****:* oil; ^{1}H NMR (500 MHz) δ 7.28 (2H, br d, $J \sim 8.5$ Hz), 6.90–6.85 (4H, m), 6.79 (1H, d, $J = 8.2$ Hz), 6.54 (2H, s), 6.50 (1H, d, $J = 12$ Hz), 6.46 (1H, d, $J = 12$ Hz), 6.36 (1H, br d, $J \sim 15.8$ Hz), 6.09 (1H, dt, $J = 15.8$, 6.8 Hz), 3.89 (2H, t, $J = 6.7$ Hz), 3.86 (3H, s), 3.85 (3H, s), 3.82 (3H, s), 3.71 (6H, s), 2.33 (2H, br q, $J \sim 7$ Hz), 1.92 (2H, br quint, $J \sim 7$ Hz); ^{13}C NMR (125 MHz) δ 158.7, 153.0 (×2), 148.8, 148.0, 137.2, 133.0, 130.6, 130.0 (C), 129.8, 129.7, 128.7, 127.5, 127.1 (×2), 122.0, 113.9 (×2), 113.7, 111.4, 106.0 (×2) (CH), 68.2, 29.4, 28.8 (CH_2), 60.9, 56.0 (×3), 55.2 (CH_3); HR ESMS *m/z* 513.2256 (M+Na^+). Calcd for $C_{30}H_{34}NaO_6$, 513.2253.

Compound ***2i****:* oil; ^{1}H NMR (500 MHz) δ 7.44 (1H, dd, J = 7.9, 1.8 Hz), 7.16 (1H, td, J = 7.9, 1.8 Hz), 7.07 (1H, dd, J = 8, 1 Hz), 6.97 (1H, td, J = 7.6, 1 Hz), 6.90–6.85 (2H, m), 6.78 (1H, d, J = 8 Hz), 6.75 (1H, br d, $J \sim$ 15.8 Hz), 6.53 (2H, s), 6.50 (1H, d, J = 12 Hz), 6.45 (1H, d, J = 12 Hz), 6.22 (1H, dt, J = 15.8, 6.8 Hz), 5.20 (2H, s), 3.90 (2H, t, J = 6.8 Hz), 3.86 (3H, s), 3.84 (3H, s), 3.70 (6H, s), 3.48 (3H, s), 2.38 (2H, br q, $J \sim$ 7 Hz), 1.94 (2H, br quint, $J \sim$ 7 Hz); ^{13}C NMR (125 MHz) δ 154.0, 153.0 (×2), 148.8, 148.1, 137.2, 133.0, 130.0, 127.7 (C), 130.5, 129.8, 128.8, 127.9, 126.4, 125.0, 122.0, 121.9, 114.9, 113.8, 111.5, 106.0 (×2) (CH), 94.8, 68.3, 29.8, 28.8 (CH_2), 60.9, 56.1, 56.0, 55.9 (×2) (CH_3); HR ESMS m/z 543.2362 (M+Na$^+$). Calcd for $C_{31}H_{36}NaO_7$, 543.2359.

Compound ***2j****:* oil; ^{1}H NMR (500 MHz) δ 7.21 (1H, t, J = 7.8 Hz), 7.02 (1H, br s), 7.00 (1H, br d, $J \sim$ 8 Hz), 6.90–6.85 (3H, m), 6.78 (1H, d, J = 8.4 Hz), 6.54 (2H, s), 6.50 (1H, d, J = 12 Hz), 6.46 (1H, d, J = 12 Hz), 6.38 (1H, br d, $J \sim$ 15.7 Hz), 6.23 (1H, dt, J = 15.7, 6.8 Hz), 5.19 (2H, s), 3.88 (2H, t, J = 6.6 Hz), 3.86 (3H, s), 3.84 (3H, s), 3.70 (6H, s), 3.49 (3H, s), 2.34 (2H, br q, $J \sim$ 7 Hz), 1.91 (2H, br quint, $J \sim$ 7 Hz); ^{13}C NMR (125 MHz) δ 157.5, 153.0 (×2), 148.8, 148.0, 139.2, 137.2, 133.0, 129.9 (C), 130.3, 130.2, 129.7, 129.4, 128.8, 122.0, 119.8, 114.8, 113.8, 113.7, 111.4, 106.0 (×2) (CH), 94.4, 68.2, 29.4, 28.7 (CH_2), 60.8, 55.9 (x 4) (CH_3); HR ESMS m/z 543.2359 (M+Na$^+$). Calcd for $C_{31}H_{36}NaO_7$, 543.2359.

Compound ***2k****:* oil; ^{1}H NMR (500 MHz) δ 7.26 (2H, br d, $J \sim$ 8.5 Hz), 6.97 (2H, br d, $J \sim$ 8.5 Hz), 6.90–6.85 (2H, m), 6.78 (1H, d, J = 8.2 Hz), 6.53 (2H, s), 6.50 (1H, d, J = 12.3 Hz), 6.45 (1H, d, J = 12.3 Hz), 6.36 (1H, br d, $J \sim$ 15.7 Hz), 6.10 (1H, dt, J = 15.7, 6.8 Hz), 5.17 (2H, s), 3.88 (2H, t, J = 6.8 Hz), 3.86 (3H, s), 3.84 (3H, s), 3.70 (6H, s), 3.48 (3H, s), 2.32 (2H, br q, $J \sim$ 7 Hz), 1.91 (2H, br quint, $J \sim$ 7 Hz); ^{13}C NMR (125 MHz) δ 156.4, 153.0 (×2), 148.8, 148.0, 137.2, 133.0, 131.8, 130.0 (C), 129.8, 129.7, 128.8, 128.1, 127.1 (×2), 122.0, 116.3 (×2), 113.8, 111.4, 106.0 (×2) (CH), 94.5, 68.3, 29.4, 28.8 (CH_2), 60.9, 56.0 (x 4) (CH_3); HR ESMS m/z 543.2360 (M+Na$^+$). Calcd for $C_{31}H_{36}NaO_7$, 543.2359.

Compound ***2l****:* oil; ^{1}H NMR (500 MHz) δ 7.30 (2H, m), 6.98 (2H, m), 6.90–6.85 (2H, m), 6.79 (1H, d, J = 8.3 Hz), 6.53 (2H, s), 6.50 (1H, d, J = 12.1 Hz), 6.45 (1H, d, J = 12.1 Hz), 6.37 (1H, br d, $J \sim$ 15.8 Hz), 6.14 (1H, dt, J = 15.8, 6.8 Hz), 3.88 (2H, t, J = 6.8 Hz), 3.86 (3H, s), 3.84 (3H, s), 3.70 (6H, s), 2.33 (2H, br q, $J \sim$ 7 Hz), 1.91 (2H, br quint, $J \sim$ 7 Hz); ^{13}C NMR (125 MHz) δ 162.9/161.0 [d, $^1J_{C-F} \sim$ 244 Hz], 153.0 (×2), 148.8, 148.0, 137.2, ~133.9 [d, $^4J_{C-F}$ - ~ 3 Hz], 133.0, 129.9 (C), 129.7, 129.5, 129.3, 128.8, ~127.4 [d, $^3J_{C-F} \sim$ 8 Hz] (×2), 122.1, 115.3/115.2 [d, $^2J_{C-F} \sim$ 21 Hz], (×2), 113.7, 111.4, 106.0 (×2) (CH), 68.2, 29.3, 28.7 (CH_2), 60.9, 56.1, 56.0 (×2) (CH_3); HR ESMS m/z 501.2052 (M+Na$^+$). Calcd for $C_{29}H_{31}FNaO_5$, 501.2053.

Compound ***2m****:* oil; ^{1}H NMR (500 MHz) δ 7.26 (4H, br s), 6.88 (1H, dd, J = 8.2, 1.7 Hz), 6.85 (1H, d, J = 1.7 Hz), 6.79 (1H, d, J = 8.2 Hz), 6.53 (2H, s), 6.50 (1H, d, J = 12 Hz), 6.45 (1H, d, J = 12 Hz), 6.36 (1H, br d, $J \sim$ 15.8 Hz), 6.21 (1H, dt, J = 15.8, 6.8 Hz), 3.87 (2H, t, J = 6.8 Hz), 3.85 (3H, s), 3.84 (3H, s), 3.70 (6H, s), 2.34 (2H, br q, $J \sim$ 7 Hz), 1.91 (2H, br quint, $J \sim$ 7 Hz); ^{13}C NMR (125 MHz) δ 153.0 (×2), 148.8, 147.9, 137.2, 136.2, 133.0, 132.5, 129.9 (C), 130.5, 129.7, 129.3, 128.8, 128.5 (×2), 127.2 (×2), 122.1, 113.7, 111.4, 106.0 (×2) (CH), 68.2, 29.4, 28.6 (CH_2), 60.9, 56.0 (×3) (CH_3); HR ESMS m/z 517.1761 (M+Na$^+$). Calcd for $C_{29}H_{31}{}^{35}ClNaO_5$, 517.1758.

Compound ***2n****:* oil; ^{1}H NMR (500 MHz) δ 7.41 (2H, br d, J = 8.5 Hz), 7.20 (2H, br d, J = 8.5 Hz), 6.87 (1H, dd, J = 8.2, 1.8 Hz), 6.85 (1H, d, J = 1.8 Hz), 6.79 (1H, d, J = 8.2 Hz), 6.53 (2H, s), 6.50 (1H, d, J = 12 Hz), 6.45 (1H, d, J = 12 Hz), 6.35 (1H, br d, $J \sim$ 15.8 Hz), 6.22 (1H, dt, J = 15.8, 6.8 Hz), 3.87 (2H, t, J = 6.8 Hz), 3.85 (3H, s), 3.84 (3H, s), 3.70 (6H, s), 2.33 (2H, br q, $J \sim$ 7 Hz), 1.90 (2H, br quint, $J \sim$ 7 Hz); ^{13}C NMR (125 MHz) δ 153.0 (×2), 148.8, 147.9, 137.2, 136.6, 133.0, 131.5, 129.9 (C), 131.4 (×2), 130.7, 129.7, 129.4, 128.8, 127.5 (×2), 122.1, 113.7, 111.4, 106.0 (×2) (CH), 68.2, 29.4, 28.6 (CH_2), 60.9, 56.1, 56.0 (×2) (CH_3); HR ESMS m/z 561.1248 (M+Na$^+$). Calcd for $C_{29}H_{31}{}^{79}BrNaO_5$, 561.1253.

6.3. Biological procedures

6.3.1. Reagents and cell culture

Cell culture media were purchased from Gibco (Grand Island, NY, USA) and Biowhittaker (Walkersville, MD, USA). Fetal bovine serum (FBS) was a product of Harlan-Seralab (Belton, UK). Supplements and other chemicals not listed in this section were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). Plastics for cell culture were supplied by NUNC (Roskilde, Denmark). Combretastatin derivatives **2a**–**n** (samples purified by HPLC) were dissolved in DMSO at a concentration of 10 mg/mL and stored at −20 °C until use.

Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing glucose (1 g/L), glutamine (2 mM), penicillin (50 IU/mL), streptomycin (50 μg/mL) and amphoterycin (1.25 μg/mL), supplemented with 10% FBS.

6.3.2. Cell proliferation assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO) dye reduction assay in 96-well microplates was used, as previously described.[34] Some 5×10^3 cells of HT-29, MCF-7 or HEK-293 cells in a total volume of 100 μL of their respective growth media were incubated with serial dilutions of the tested compounds. After 3 days of incubation (37 °C, 5% CO_2 in a humid atmosphere), 10 μL of MTT (5 mg/ml in PBS) were added to each well and the plate was incubated for further 4 h (37 °C). The resulting formazan was dissolved in 150 μL of 0.04 N HCl/2-propanol and read at 550 nm. All determinations were carried out in triplicate. The IC_{50} values in Table 1 mean the concentration of compound yielding a 50% of cell survival.

6.3.3. RT-PCR analysis

HT-29 cells at 70–80% confluence were collected after serum starvation for 24 h. Cells were incubated with combretastatin derivatives in DMSO (see Figs. 4 and 5) for 48 h. Cells were collected and the total cellular RNA from HT-29 cells was isolated using Ambion RNA extraction Kit according to the manufacturer's instructions. The cDNA was synthesized by MMLV-RT with 1–21 μg of extracted RNA and oligo(dT)15 according to the manufacturer's instructions. Gene-specific PCR primers (see Table 3) were then added for amplification. PCR products were analysed by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining under UV transillumination. The sequences of primers used in the RT-PCR are listed in Table 3. The PCR conditions were as follows: VEGF at 94 °C for 30 s, at 58 °C for 1 min, and at 72 °C for 1 min 50 s; and β-actin at 94 °C for 30 s, at 58 °C for 50 s, and at 72 °C for 50 s; hTERT: 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min 30 s; c-Myc: 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min 50 s.

Table 3
Primers used and sizes of the PCR products

VEGF	Sense: 5′-CCTGATGAGATCGAGTACATCTT-3′ Antisense: 5′-ACCGCCTCGGCTTGTCAC-3′	379
hTERT	Sense: 5′-CGGAAGAGTGTCTGGAGCAA-3′ Antisense: 5′-GGATGAAGCGGAGTCTGGA-3′	145
c-Myc	Sense: 5′-AAGTCCTGCGCCTCGCAA-3′ Antisense: 5′-GCTGTGGCCTCCAGCAGA-3′	249
β-Actin	Sense: 5′-TCATGAAGTGTGACGTTGACATC CGT-3′ Antisense: 5′-CGTAGAAGCATTTGCGGTGCAC GATG-3′	287

Analysis of β-actin was used to monitor RNA integrity and accuracy of loading.[35]

6.3.4. ELISA analysis

HT-29 cells at 70–80% confluence were collected after serum starvation for 24 h. Cells were incubated with combretastatin derivatives in DMSO (see Fig. 3) for 72 h. Culture supernatants were collected and VEGF secreted by HT-29 cells was determined using Invitrogen Human Vascular Endothelial Growth Factor ELISA Kit according to the manufacturer's instructions.

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Supplementary data

Supplementary data (graphical NMR spectra of compounds **2a**–**2n**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.09.064.

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