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Discovery and hit to lead optimization of novel Combretastatin A-4

analogues: dependence of C-linker length and hybridization

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Abstract:

We have synthesized a large variety of CA-4 analogues having a non-isomerizable C-linker between the A- and B-aromatic rings. Most of them displayed a nanomolar level of cytotoxicity against a panel of human cancer cell lines and inhibited tubulin polymerization at a micromolar level. Among all these compounds, the most interesting compounds were undoubtedly *iso*CA-4 and structural analogues **18-20** as well as benzil derivatives **11** which displayed a comparable level of activity than that of CA-4. Moreover, it has been demonstrated that these drugs arrested cancer cells in the G₂/M phase of cellular cycle and induced apoptosis at very low concentrations. *In vitro* antivasular effects and the binding mode of the most active compounds was also investigated.

Key words: Combretastatin A-4, *iso*CA-4, cytotoxicity, tubulin, linker, apoptosis, binding.

1. Introduction:

Combretastatin A-4 (CA-4, Figure 1) **1**, a natural product originally isolated from the bark of the South African bush willow tree *Combretum caffrum* by Pettit in 1989 [1] exhibits strong antitubulin activity (IC₅₀ at a micromolar level) by binding to the colchicine binding-site of tubulin. [2, 3] CA-4 also exerts a highly potent cytotoxicity (GI₅₀ at a nanomolar level) against a variety of human cancer cells including multidrug resistant (MDR) cancer cell lines. [4] More importantly, CA-4 displays selective cytotoxicity toward tumour vessels by inhibiting the blood supply to the tumor, leading to cell death. [5] The major problems associated with this molecule were poor aqueous solubility [6, 7] and isomerization of the *Z*-alkene into the more stable but inactive *E*-isomer during storage, administration and metabolism. [8-10] The solubility problem was solved by the synthesis of the water-soluble phosphate CA-4P **2** and amino-serine AVE8062 **3** prodrugs which cause a rapid and extensive vascular shutdown in experimental tumor models. [11-13] CA-4P which has been developed by Oxigene Inc., in USA, is currently used for the treatment of thyroid cancers. [14] However, despite the efficiency of CA-4P, cardiovascular toxicity and neurotoxicity were dose-limiting for this molecule. [15] These drawbacks and the structural simplicity of CA-4 stimulate the search for new and more potent compounds with improved pharmacological properties. [16]

Figure 1. CA-4, its water-soluble prodrugs and structure activity relationships (SAR).

To avoid instability problems associated with the isomerization of the double bond, considerable effort has been done into modifying the *Z*-alkene double bond, most of the time by insertion of the CA-4 double bond in rings [17-21]. Others medicinal groups have attempted to replace the *Z*-double bond by linkers of different sizes, containing or not heteroatoms in their structure to increase the biological properties of such analogues. [22, 23]

This review will summarize our contribution since 2008, in acyclic linker modifications, with compounds having a six, four, two and one carbon atoms linker between the aromatic rings. The synthesis of such analogues will be presented and *in vitro* biological results, including cytotoxicity and inhibition of tubulin polymerization will be discussed in comparison to CA-4, as the reference. [24] After evaluation of cytotoxicity and antitubulin properties, the most potent derivatives will be considered for further investigations. In particular, the ability of these drugs to block the cellular cycle of cancer cells in the G₂/M phase will be studied and their apoptotic properties will be examined. The binding mode of selected drugs, able to occupy the colchicine-binding site, will be compared to CA-4 at the end of the manuscript.

2. Synthesis and biological evaluation of CA-4 analogues with aromatic rings joined by C-acyclic linkers of varying length and hybridization.

2.1 C6-linker: enediynes 4.

2.1.1 Synthesis. In order to preserve the (Z)-stereochemistry of the CA-4 stilbene double bond and to maintain the π -conjugated system, we first prepared a series of CA-4 analogues incorporating between the double bond and the aromatics two triple bonds. [25] Moreover, such analogues, having a enediyne moiety in their structure could benefit from this pharmacophore found in many anticancer agents, as for example the calicheamicin, esperamicin and dynemicin families. [26]

Scheme 1. "Synthesis of (Z)-enediynes **4a-d**.

"Reagents and conditions: (i) PdCl₂(PPh₃)₂ (5 mol%), CuI (10 mol%), (Z)-1,2-dichloroethylene (2 equiv), *n*-BuNH₂ (2 equiv), Et₂O, 20 °C. (ii) PdCl₂(PhCN)₂ (5 mol%), CuI (10 mol%), alkyne (1.2 equiv), piperidine.

Enediynes **4a-d** (Scheme 1) were synthesized in two steps from alkyne **5** readily prepared according to literature. [27, 28] A first Pd-coupling reaction between **5** and (Z)-1,2-dichloroethylene furnished the isomerically pure Z-chloroenyne **6** in 77% yield. [29] Based on previous work with this class of not photosensitive compounds, we showed that the C-Cl bond is not inert to further coupling reactions with terminal alkynes. [30, 31] As expected, (Z)-chloroenyne **6** reacted cleanly with a selection of terminal alkynes to afford stereoselectively the desired (Z)-enediynes **4a-d**.

2.1.2 Biological results. Enediynes **4a-d** were evaluated for their cytotoxic effects against K562 (human cervical carcinoma), MCF7 (human breast adenocarcinoma), MCF7R (multidrug resistant human breast cancer) cells and for tubulin polymerization inhibitory activities. However most of enediynes were poorly cytotoxic (GI₅₀ > 1000 nM) against the three tested cell lines. Enediyne **4d**, having the greatest resemblance to AVE8062 **3**, inhibited the tubulin polymerization (IC₅₀ = 68 μ M) but was almost 50-fold less active on tubulin than CA-4. One note that Wu described the synthesis of another series of (Z)-enediynes having various substituents on both aromatic rings. [32] Among the synthesized compounds, aniline **7** having *ortho*-substituents on aromatic rings displayed interesting anticancer properties with GI₅₀ of 10⁻⁷-10⁻⁶ M against various cancer cell lines and inhibited tubulin polymerization with an IC₅₀ value of 10 μ M. In the enediynes series, examination of the biological results revealed that the incorporation of two triple bonds does not play a role for maximal anticancer properties. Then, we decided to examine the role on biology of the incorporation of a single triple bond between both aromatic rings and the Z-double bond by preparing a series of (Z)-enyne having a C4-linker.

2.2 C4-linker: (Z)-enyne 8 and 9.

2.2.1 Synthesis. Enynes **8a-c** having a triple bond between the 3,4,5-trimethoxyphenyl A-ring and the *Z*-double bond were easily prepared from (*Z*)-chloroenyne **6** whereas enynes **9a-c** having the triple bond between the B-ring and the stilbene moiety were synthesized from (*Z*)-chloroenynes **10a-c**. [30] In the presence of Et₃N and a catalytic amount of PdCl₂(PPh₃)₂, (*Z*)-chloroenynes **6** and **10a-c** undergo rapid coupling with Grignard reagents [33-36] to afford stereoselectively the required enynes derivatives in good yields (Scheme 2).

Scheme 2. ^aSynthesis of enynes **8a-c** and **9a-c**.

^aReagents and conditions: (i) ArMgBr (2 equiv), PdCl₂(PPh₃)₂ (5 mol%), Et₃N (8 equiv), THF, rt.

2.2.2 Biological results. Enynes **8** and **9** were not cytotoxic (GI₅₀ > 1000 nM) and did not inhibit significantly tubulin polymerization (IC₅₀ > 65 μM). These results suggested that the insertion of a triple bond between the stilbene moiety and both A- or B-rings is not acceptable for a potent anticancer activity. One can note that contrary to enynes **8** and **9**, vinylogous CA-4 derivatives having an additional (*E*)-double bond between the A- or B-rings and the stilbene moiety [37] inhibited tubulin assembly at concentrations comparable or lower to that of CA-4.

2.3 C2-linker: benzil derivatives **11**.

It has been demonstrated, that phenstatin **12** [38-40] and chalcone **13**, [41-43] where the ethylene bridge of CA-4 was replaced by a carbonyl or a propenone function respectively, showed excellent antiproliferative and antitubulin properties (Figure 2).

Figure 2. Cytotoxicity against P388 (leukemia) cells and ITP of phenstatin **12** and chalcone **13**.

Phenstatin 12 C1-linker	Chalcone 13 C3-linker	Target benzil 11 C2-linker
GI ₅₀ /GI ₅₀ CA-4P = 8	GI ₅₀ /GI ₅₀ CA-4 = 9	
ITP: IC ₅₀ /IC ₅₀ CA-4 = 0.9	ITP: IC ₅₀ /IC ₅₀ CA-4 = 0.9	

With this idea in mind, we next focused our attention on the preparation of benzil derivatives **11** having two keto-groups as C2-linker which could be seen as hybrids of CA-4 and phenstatin **12**.

2.3.1 Synthesis. To this end, we have investigated the oxidation of various diarylalkynes with DMSO in the presence of catalytic amounts of transition-metal salts. We found that a variety of metal catalysts including, CuBr, InCl₃, MnBr₂ were effective to achieve this transformation. Among them, FeBr₃ holds certain supremacy and was used to successfully oxidize various diarylalkynes into benzil derivatives in DMSO under microwave irradiation. [44] However, under these conditions, phenol- or aniline-containing diarylalkynes were not transformed effectively into the required benzils. This difficulty was solved by replacing

FeBr₃ by PdI₂ [45] and thus a series of benzil derivatives **11** was prepared by this way at 140°C under a classical heating (Figure 3).

Figure 3. Synthesis, cytotoxicity and inhibition of tubulin polymerization (ITP) of selected benzils **11a-d**.

Compounds	Cytotoxicity GI ₅₀ (nM) ^a				ITP IC ₅₀ (μM) ^b
	HCT116	K562	H1299	MDA-MB231	
11a	300	-	-	-	21
11b	40	25	30	40	NA ^c
11c	35	25	30	40	1.5
11d	38	20	50	30	5.0
CA-4	1.8	3.6	5.0	3.0	1.0

^aGI₅₀ is the concentration of compound needed to reduce cell growth by 50% following cell treatment with the tested drug (average of three experiments). ^bITP, Inhibition of Tubulin Polymerization; IC₅₀ is the concentration of compound required to inhibit 50% of the rate of microtubule assembly (average of three experiments). ^cNot active.

2.3.2 Biological results. [46]

2.3.2.1 Antiproliferative activity. Diketones **11c** and **11d** having the greatest resemblance to CA-4 and AVE8062 as well as acetate **11b** showed potent nanomolar activity with GI₅₀ values ranging from 35 to 40 nM in HCT116 cancer cell lines (GI₅₀ CA-4 = 1.8 nM). These 1,2-diketones, except **11a** which displayed a lower level of cytotoxicity against HCT116 (GI₅₀ = 300 nM) cells, were next evaluated in K562 (chronic myelogenous leukemia), H1299 (non small lung human carcinoma) and MDA-MB-231 (human breast cancer) cell lines. Again, these compounds were found to be very effective with GI₅₀ values ranging from 20 to 50 nM which are comparable to that observed in the HCT116 cell line (Figure 3).

2.3.2.2 Antitubulin activity. Benzils **11** were next evaluated for their ability to inhibit tubulin polymerization. The results indicated that the benzil's antiproliferative activity correlated with the inhibition of tubulin assembly. Compounds **11c** and **11d** were efficacious in inhibiting tubulin assembly, with IC₅₀ values of 1.5 and 5.0 μM, respectively, comparable to that of CA-4 (IC₅₀ = 1.0 μM). One can note that, in the absence of esterases in the tubulin assay, the cytotoxic acetate prodrug **11b** was not transformed into its phenolic active form (see **11c** for comparison) and was found to be inactive on tubulin.

2.3.2.3 Effects on cell cycle distribution and apoptosis. Because molecules exhibiting activity on tubulin binding should cause the alteration of cell cycle parameters leading to a preferential G₂/M blockade, [16, 47, 48] the effect of **11c** and **11d** on cell cycle distribution was analyzed in HCT116 and H1299 cells cultured for 24 h at different concentrations. As shown in Table 1, a concentration dependent increase in the number of HCT116 and H1299 cells arrested in the G₂/M phase was observed with compounds **11c** and **11d**. It should be noted that in all cells treated with diketones **11c** and **11d** for 24 h, a sub-diploid DNA content was observed (data not shown) indicating that cells are undergoing apoptosis probably as a result of the cell cycle being arrested in the G₂/M phase.

Table 1. Flow cytometry analysis of compounds **11c** and **11d** in HCT116 and H1299 cancer cells^a

Benzil	Concentration (nM)	HCT116 (% G ₂ /M)	H1299 (% G ₂ /M)
DMSO	-	11.5	8.4
11c	10	11	11.4
	20	18.5	15
	50	22.8	27.7
11d	10	13	11.7
	20	15.9	12.2
	50	28	16.5

^aData represent percentage of cells in G₂/M phase of the cell cycle after 24 h of treatment with **11c** and **11d**. Data are representative of three independent experiments.

Therefore, the effect of diketones **11c** and **11d** at different concentrations (5, 50, 100 nM) on apoptosis induction in H1299 cells was studied using caspases -3 and -7 standard assays. [49] The enzymatic activity of both caspases was measured by monitoring the cleavage of Z-DEVD-R110, a fluorogenic substrate, in cancer cell lines. The results presented in Figure 4, show a significant concentration-dependent increase in proteolytic activity of caspases in the cells treated for 48 h with diketones **11c** and **11d**. There is a marked effect in apoptosis in H1299 cells using **11c** or **11d** at a low concentration of 50 nM. Taken together, the biological results suggested that the effects of benzils **11c** and **11d** on cellular cycle correlated well with their antitubulin activity and also induced apoptosis at low concentrations in the tested cell lines.

Figure 4. Apoptotic effects of benzils **11c** and **11d** in H1299 cells. The results are expressed in the percentage of apoptotic cells induced by different concentrations of **11c** and **11d** (evaluation after 48 h of treatment).

2.3.2.3 Effect of **11d** on human umbilical vein endothelial cell (HUVEC) organization.

In order to expand this study, the effects of compound **11d** on the proliferation of normal endothelial cells (HUVEC) were determined. After 72 h of incubation, **11d** showed cytotoxic activity at a nanomolar level (GI₅₀ = 40 nM) on the human umbilical

vein endothelial cells. Additionally, the ability of **11d** to disrupt a network of capillary-like tubular structures formed by endothelial cells on a matrigel matrix was evaluated (Figure 5).

Figure 5. Effects of benzil **11d** on newly formed vessels.

In vitro, vehicle or **11d** was added to cords formed by endothelial cells on Matrigel, 24 h after HUVEC seeding; images were taken 3 h after addition of the compound. (A) Control; (B) **11d** (100 nM).

Addition of **11d** to formed cords at a concentration of 100 nM and subsequent incubation rapidly disrupt the vascular network. It is interesting to note that this effect was visible after 3 h of treatment and at a dose of 100 nM which was not cytotoxic for such incubation time (data not shown). Altogether, these results indicated that benzil **11d** is identified as a promising lead in the development of new antivasculature compounds. From a SAR purpose, the biological data collected in this study show that the replacement of the (Z)-olefin in CA-4 by two carbonyl groups is well tolerated and led highly potent derivatives. It is important to note that the two carbonyls present in compounds **11** are important for potent anticancer properties. Indeed, dehydrocombretastatin A-4 also named erianin **14** (Figure 6), which was prepared from the CA-4 reduction [50] was found to be less cytotoxic than its parent CA-4 [51] and slightly less potent as an inhibitor of tubulin polymerization ($IC_{50} = 3.3 \mu M$ for erianin). [52]

Figure 6. Selected CA-4 analogues having a two atoms linker, erianin **14**, benzamide **15**, and ketones **16** and **17**.

^aHCT29 cells.

Similarly, the replacement of one carbonyl in benzil of type **11** by a NH group resulted in significant decrease in cytotoxicity and antitubulin properties as it was demonstrated with benzamide **15**. [53] Finally, desoxybenzoin analogues **16** and **17** were inactive [51] showing again the crucial role for activity of the two adjacent carbonyls may be for hybridization considerations.

2.4 C1-linkers

We next focused our attention on the synthesis of CA-4 analogues having various C1-linkers with Csp² and Csp³ at the A- and B-ring junction. Concerning Csp² hybridization, we first examined the synthesis and evaluation of di-substituted ethylenes **18-20**, trisubstituted ethylenes **21**, **22** and tetrasubstituted ethylenes **23**. Then restricted derivatives having the double bond inserted into the B-ring were examined with dihydronaphthalenes **30**, chromenes **32** and benzoxepins **33**. Concerning Csp³ hybridization, we also studied the synthesis and the evaluation of erianin analogues **37** and diarylmethane compounds **38**.

2.4.1 C1-linker: diarylethylenes 18-20.

2.4.1.1 Synthesis of 1,1-diarylethylenes 18-20.

We began the C1-linker program by the synthesis of 1,1-diarylethylene derivatives **18-20** having a C1-linker between the aromatic A and B-rings. It should be noted that compound **18a**, a structural phenstatin analogue [38] which is also the third isomer of the natural CA-4, named *iso*CA-4, [54] has never been studied despite the impressive number of synthetic analogues. From a synthetic point of view, the access to 1,1-diarylethylene derivatives **18-20** was envisioned in our lab according to several routes including, the Pd-coupling of *N*-tosylhydrazones [54-56] or vinylstannanes [57] with aryl halides or triflates, the addition of Grignard reagents to acetophenones followed by dehydration and Wittig olefination of phenstatin derivatives. [58] The synthesis of 1,1-diarylethylenes **18a-f** is described in Scheme 3.

Scheme 3. ^aSynthesis of selected diarylethylenes **18a-f**.

^aReagents and conditions: (i) ArMgBr, THF, -40 °C or ArLi, hexanes/toluene: 1/3. (ii) PTSA, (10 mol%), CH₂Cl₂, rt. (iii) K₂CO₃, MeOH, rt. (iv) Ac₂O, Pyr, CH₂Cl₂.

Diarylethylene derivatives **18b-e** were prepared from 3,4,5-trimethoxyacetophenone through addition of arylGrignard or aryllithium reagents. [59] Crude tertiary alcohols were then treated with a catalytic amount of PTSA in CH₂Cl₂ and furnished **18b-e** in good yields. *iso*CA-4 **18a** was obtained from alkaline desilylation of **18e** in MeOH in a good 94% yield.

Scheme 4. ^aSynthesis of selected nitrogen-containing diarylethylenes **19a-c**.

^aReagents and conditions: (i) ArMgBr, THF, -78 °C (ii) PCC, CH₂Cl₂, 20 °C (iii) Ph₃PCH₃Br, LiHMDS, THF, 0 °C to 20 °C (iv) Zn, AcOH, 20 °C (v) NaNO₂, NaN₃, HCl_{aq}, acetone, 0 °C.

Because in the CA-4 series it has been demonstrated that the C3'-OH group is amenable to modifications, [7, 59, 60] we have prepared other *iso*CA-4 analogues having C3'-nitrogen or C3'-carbon substituents. [61] Nitrogen-containing substrates **19a,b** were prepared from the condensation of 4-methoxy-3-nitrobenzaldehyde with 3,4,5-trimethoxyphenylmagnesium bromide in THF. The resulting alcohol was oxidized with PCC in CH₂Cl₂ and a further Wittig reaction afforded **19a** which underwent reduction of the nitro function, using Zn in AcOH to provide **19b** (Scheme 4). We also examined the replacement of the C3'-NH₂ group with an azide group affording *iso*N₃CA-4 **19c**.

A third series of *iso*CA-4 analogues **20a-j** with C-linked substituents the C3'-position was studied. Such compounds were prepared from diarylethylene **18g** [61] having an iodine atom on C3' (Scheme 5). Heck and Suzuki couplings afforded in good yields 3'-(*E*)-cinnamate **20a** and diaryl derivative **20d**, respectively. The preparation of acetylenic *iso*CA-4 analogues **20e** and **20g,h** was successfully achieved by Sonogashira-Linstrumelle couplings. For SAR considerations, the acetylenic triple bond of compound **20h** was hydrated using a catalytic amount of PTSA in EtOH under microwave irradiation. [62, 63] Under this metal-free procedure, arylketone **20i** was regioselectively obtained. When applying the same PTSA-protocol to *ortho*-methoxydiarylalkyne **20g**, a 5-*endo*-dig-cyclization process occurred to give [64, 65] **20j** in a good yield of 86%.

Scheme 5. "Synthesis of selected C3' substituted diarylethylenes **20a-j** from **18g**.

^aReagents and conditions: (i) Methyl acrylate, PdCl₂(PPh₃)₂, Et₃N, DMF, 60 °C; (ii) NaOH, MeOH, rt; (iii) DIBALH, THF, -78 °C; (iv) 4-MeOPhB(OH)₂, Pd(PPh₃)₄, NaHCO₃, DME-H₂O, reflux; (v) Alkyne, PdCl₂(PPh₃)₂, CuI, Et₃N, THF; (vi) PTSA, EtOH, microwave, 170 °C; (vii) TBAF, THF, rt.

2.4.1.2 Biological results.

Table 2. Cytotoxicity of selected compounds **18-20** against HCT116 cancer cells

Compounds	GI ₅₀ nM ^a	Compounds	GI ₅₀ nM ^a	Compounds	GI ₅₀ nM ^a	Compounds	GI ₅₀ nM ^a
<i>iso</i> CA-4 (18a)	2	19a	60	20d	300	20j	4500
18b	40	<i>iso</i> NH ₂ CA-4 (19b)	2	20e	730	CA-4	2
18c	80	<i>iso</i> N ₃ CA-4 (19c)	72	20f	30		
<i>iso</i> FCA-4 (18d)	7	20a	80	20g	1500		
18e	180	20b	80	20h	30		
18f	8	20c	45	20i	NA ^b		

^aA sample's concentration which produces a 50% reduction cell growth. ^bNot active.

2.4.1.2.1 Cytotoxicity. As mentioned in Table 2, several 1,1-diarylethylene derivatives retained potent growth inhibitory activity at a nanomolar level, and lowest GI₅₀ values were obtained with *iso*CA-4, *iso*FCA-4, *iso*NH₂CA-4 and ester **18f**. These values, and

particularly the one of *iso*CA-4 ($GI_{50} = 2$ nM), are comparable to that of CA-4 showing that the replacement of the (Z)-1,2-ethylene by a 1,1-ethylene bridge resulted in retention of cytotoxicity in these series of compounds. It is noteworthy that a fluorine atom, a NH_2 substituent or an acetate function on the C3'-position are well tolerated leading to potent compounds with GI_{50} values ranging from 2 to 8 nM. Moreover, we were pleased to observe that compounds **20h** and **20c** having on the C3'-position, a propyn-ol or a propen-ol chain respectively, displayed a high level of cytotoxicity (30-45 nM). These preliminary cytotoxic results led us to explore the potential of the more active 1,1-diarylethylenes against other cancer cell lines.

As shown in Table 3, all 1,1-diarylethylenes exhibited similar potent cytotoxic effects against the tested cancer cell lines. It should be noted that in the 1,1-diarylethylene series, *iso*CA-4, *iso*NH₂CA-4 and *iso*FCA-4 displayed GI_{50} of 3 to 8 nM, which was similar to that obtained for CA-4. Again, 1,1-diarylethylenes **20c** and **20h** having alkenyl or alkynyl chains on C3' were cytotoxic against all cancer cells.

Table 3. Cytotoxicity of selected 1,1-diarylethylenes **18-20** in various cancer cells^a

Compound	Cytotoxicity GI_{50} (nM) ^a							ITP ^b
	K562 ^b	U87 ^b	A549 ^b	MDA-MB435 ^b	MDA-MB23 ^b	H1299 ^b	HUVEC ^b	IC ₅₀ (μM)
<i>iso</i> CA-4	5	8	8	4.5	4	5	1.5	2
18b	18	28	20	25	10	-	38	2
<i>iso</i> FCA-4	4	7	7	8	3	-	5	4
<i>iso</i> NH ₂ CA-4	4	8	7	7	6	9	3	1.5
<i>iso</i> N ₃ CA-4	28	-	-	-	30	50	-	3
20a	80	-	-	-	500	70	-	2
20b	80	-	-	-	500	70	-	3
20c	2	-	-	-	5	5	-	2
20f	20	-	-	-	30	35	-	67
20h	35	-	-	-	7	25	-	2
CA-4 ^d	3.5	3	7	3	3.5	5	2.5	1

^a GI_{50} is the concentration of compound needed to reduce cell growth by 50% following 72 h cell treatment with the tested drug (average of three experiments). ^bK562, myelogenous leukaemia; U87, glioblastoma; A549, carcinomic alveolar basal epithelial; MDA-MB435, breast cancer and MDA-MB231 hormone-independent breast cancer; H1299 non-small cell lung carcinoma, HUVEC human umbilical vein endothelial cell, ITP, Inhibition of Tubulin Polymerization; IC₅₀ is the concentration of compound required to inhibit 50% of the rate of microtubule assembly (average of three experiments). ^cNot determined. ^dThe GI_{50} and IC₅₀ values (cytotoxicity and ITP respectively) for CA-4 was determined in this study.

2.4.1.2.2 Antitubulin activity.

The effect on *in vitro* polymerization of tubulin of 1,1-diarylethylenes was next examined. On the exception of terminal alkyne **20f**, all selected derivatives were excellent inhibitors of tubulin assembly with IC₅₀ values ranging from 1.5 to 6 μM (Table 3). As it was observed for benzil derivatives **11**, we conclude that the antiproliferative activity of the selected 1,1-diarylethylenes derives from an interaction with the colchicine site of tubulin and an interference with microtubule assembly.

2.4.1.2.3 Effects on cell cycle distribution and apoptosis.

The effect of the more potent diarylethylenes, *iso*CA-4, *iso*NH₂CA-4 and *iso*FCA-4 on cell cycle distribution at a concentration of 10 nM was evaluated on MDA-MB231, K562 and HCT116 cancer cell lines. As depicted in Figure 7, these compounds caused a massive cell accumulation in the G₂/M phase at a low concentration of 10 nM. Similarly, 1,1-diarylethylenes **20c** and **20h**, having alken-ol and alkyn-ol substituents on the C3'-position arrest the cellular cycle in the G₂/M phase at a low concentration on various cancer cell lines (data not shown). The effect of *iso*CA-4 on induction of apoptosis in HCT116, MDA-MB 231 and K562 cells was next studied. The results presented in Figure 8 show a significant dose-dependent increase in proteolytic activity of caspases-3 and -7 in the cells treated for 24 h with *iso*CA-4. A spectacular 12- to 15-fold increase in apoptosis was evidenced in K562

leukemia cells previously described as being resistant to apoptosis induction by a variety of agents including diphtheria toxin, camptothecin, cytarabine, etoposide, paclitaxel, staurosporine, and antifas antibodies. [66-71] Similar results were obtained with *isoFCA-4* and *isoNH₂CA-4* which induce apoptosis in the three tested cell lines. [58]

Figure 7. Effect of *isoCA-4* (blue), *isoNH₂CA-4* (pink) and *isoFCA-4* (green) on cell cycle distribution in various cancer cell lines at 10 nM (DMSO control in red).

Figure 8. Apoptotic effect of *isoCA-4* against HCT116, MDA-MDB231 and K562 cells.^a

^aThe results are expressed in the percentage of apoptotic cells detected following 24 h treatment with *isoCA-4* at different concentrations 1 nM (white), 5 nM (gray) and 10 nM (black).

2.4.1.2.4 Effect of *isoFCA-4*, *isoCA-4* and *isoNH₂CA-4* on HUVEC organization.

The effects of the more active compounds *isoFCA-4*, *isoCA-4* and *isoNH₂CA-4* on the proliferation of normal endothelial cells were evaluated.

Figure 9. Effects of *isoFCA-4*, *isoCA-4* and *isoNH₂CA-4* on newly formed vessels.

As shown in Figure 9, *iso*FCA-4, *iso*CA-4 and *iso*NH₂CA-4 disrupt the vessels-like structure and the integrity of the entire network at a concentration of 10 nM after a short reaction time of 3 h which was shown to be not toxic for HUVEC. [58] This preliminary result suggests that *iso*CA-4 and some 1,1-diarylethylene analogues such as *iso*FCA-4 and *iso*NH₂CA-4 may be new leads for the development of antivasular agents.

2.4.2 C1-linker: trisubstituted ethylenes **21** and **22**.

2.4.2.1 Synthesis and evaluation of trisubstituted olefins **21**. [72]

The encouraging biological results obtained with diaryl-substituted olefins **18-20** have encouraged us to study a novel series of trisubstituted olefins **21**. Such derivatives were prepared by palladium-catalyzed coupling of *N*-tosylhydrazones with aryl iodides (Scheme 6).

Scheme 6. Synthesis and biological evaluation of trisubstituted olefins **21**.

^aReagents and conditions: (i) PdCl₂(MeCN)₂ (5 mol%), dppp (10 mol%), Cs₂CO₃, dioxane, 90 °C. (ii) K₂CO₃, MeOH, rt.

The required *N*-Tosylhydrazone was readily prepared from 3,4,5-trimethoxyacetophenone in 3 steps: bromination using *N*-methylpyrrolidin-2-one hydrotribromide (MPHT) complex developed in our group, [73-74] substitution of the bromine atom by MeOH in the presence of Ag₂CO₃/BF₃·Et₂O [75] and finally hydrazone formation using *p*-toluenesulfonylhydrazide. Hydrazone was then coupled with various aryl iodides under our previous reported conditions [76] using PdCl₂(MeCN)₂/dppp as the catalytic system and Cs₂CO₃ as the base. The OTBDMS protecting group of **21c** was removed using K₂CO₃ in MeOH to yield phenol **21d**. Enol ethers **21a,b** and **21d** were tested as a mixture of (*E*)- and (*Z*)-isomers against HCT116, K562 and H1299 cells.

Table 4. Cytotoxicity against HCT116, K562 and H1299 cells and ITP of **21a,b** and **21d**.

Compound	Cytotoxicity GI ₅₀ (nM)			ITP
	HCT116	K562	H1299	IC ₅₀ (μM)
21a	80	40	43	3
21b	8	39	60	30
21d	24	32	25	2
<i>iso</i> CA-4 ^a	2	2.2	4	1.5

^aThe GI₅₀ and IC₅₀ values (cytotoxicity and ITP respectively) for *iso*CA-4 was determined in this study.

The results presented in Table 4 demonstrated that trisubstituted enol ethers **21** displayed excellent antiproliferative activity against the three tested cell lines. Interestingly, fluorine derivative **21b** which displayed the best cytotoxicity level against HCT116 cell line with a GI₅₀ value of 8 nM did not inhibit efficiently the tubulin polymerization (IC₅₀ = 30 μM). On the contrary, enolether derivatives **21a** and **21d** which were found to be slightly less cytotoxic against HCT116 cells were very effective in their ability to inhibit tubulin assembly with IC₅₀ values of 2 and 3 μM, respectively, comparable to that of *iso*CA-4. As it was previously reported for the most of antimetabolic agents, these tubulin polymerization inhibitors **21a** and **21d** arrested the majority of K562 cells in the G₂/M phase (data not shown) at a concentration of 50 nM. [72] The biological results obtained with trisubstituted olefins **21** prompted us to synthesize hybrid olefin **22** having in its structure the CA-4 and the *iso*CA-4 moieties to combine within a single substance the anticancer effects of both isomers (Scheme 7).

2.4.2.2 Synthesis and evaluation of hybrid molecule 22

Scheme 7. ^aSynthesis of targeted olefin **22**.

^aReagents and conditions: (i) $\text{PdCl}_2(\text{PPh}_3)_2$ (2 mol%), Bu_3SnH , (1.2 equiv), THF. (ii) I_2 (1 equiv), CH_2Cl_2 , 20 °C. (iii) ArZnCl (2.2 equiv), $\text{PdCl}_2(\text{PPh}_3)_2$ (5 mol%), THF. (iv) $n\text{-BuLi}$ (4.5 equiv), THF, -78 °C. (v) HCl 1M, 20 °C.

Triarylolefin **22** was prepared in five steps from diarylalkyne **25**. [77] We have benefited from the presence of an *ortho* bromine atom on the trimethoxyphenyl ring of **25** to achieve a regioselective hydrostannylation of the triple bond. [78-82] Under this *ortho*-directing effect (ODE) [79, 83], the reaction afforded exclusively α -vinyl stannane **26** in a good 92% yield. A further iodolysis of **26** led to vinyl iodide **27** which underwent a Negishi coupling to furnish triarylolefin **28**. [84] Finally, removal of the *ortho* bromo directing-group by halogen-metal exchange and subsequent hydrolysis, followed by MOM cleavage afforded triarylolefin **22** as a pure isomer. Unfortunately, olefin **22** was poorly cytotoxic against HCT116 cells with a GI_{50} values of 5 μM and did not inhibited the polymerization of tubulin significantly. We suppose that triarylolefin **22** is probably too bulky to bind to the tubulin on the colchicine binding-site to explain these disappointing results.

2.4.3 C1-linker: tetrasubstituted ethylenes 29.

2.4.3.1 Synthesis and evaluation of tetrasubstituted olefins 29

We envisioned synthesizing a series of tetrasubstituted olefins **29** having on C2, different uncongested substituents enabling a binding on β -tubulin. Olefins **29a-m** were selected to achieve this goal and their synthesis is described in Scheme 8.

Scheme 8. ^aSynthesis of tetrasubstituted olefins **29**.

^aReagents and conditions: (i) ArI (0.8 equiv), $\text{PdCl}_2(\text{MeCN})_2$ (5 mol%), dppp (10 mol%), Cs_2CO_3 (3 equiv), dioxane, 90 °C. (ii) PTSA, EtOH. (iii) Fe , HCl , EtOH, 100 °C.

Although the double bond in compounds **29** could be generated by a Wittig reaction, a more convergent approach was recently developed in our group. [76] We found that hindered polyoxygenated *N*-tosylhydrazones coupled with a variety of aryl iodides using PdCl₂(MeCN)₂ as a catalyst, dppp as a ligand and of Cs₂CO₃ as a base in dioxane at 90 °C. Under these optimized conditions, targeted tetrasubstituted olefins **29** were obtained in good yields (62-82%). Preliminary antiproliferative results reveal that olefins **29** displayed a poor level of cytotoxicity. The most active molecules in this series were **29b** and **29g** with GI₅₀ values of 300 and 250 nM, respectively. These compounds were not effective to inhibit tubulin assembly with IC₅₀ values superior to 10 μM.

2.4.4 C1-linker: conformationally restricted *iso*CA-4 analogues.

2.4.4.1 Synthesis and biological evaluation of dihydronaphthalene **30 and naphthalene **31** derivatives.** To increase binding affinity and then activity, conformational restriction by a cycle has been successfully envisioned by different groups with significative bioactive molecules **32** [85], **33** [86] and **34** [87] represented in Figure 10.

Figure 10. Cytotoxicity of restricted CA-4 analogues **32-34**.

^aCEM leukemia cells. ^bHBL100 human epithelial mammary cells. ^cNCI-H460 human non-small lung cancer cells.

Scheme 9. ^aSynthesis of dihydronaphthalenes **30** and naphthalenes **31**. [88]

^aReagents and conditions: (i) 3,4,5-trimethoxyphenyl iodide (1.1 equiv), Pd₂dba₃ (10 mol%), XPhos (40 mol%), LiOtBu (2.2 equiv), dioxane 90 °C in a sealed tube. (ii) Pd(OAc)₂ (5 mol%), PPh₃ (20 mol%), K₂CO₃ (2 equiv), BuOH, 100 °C. (iii) NaN₃ (2 equiv), CuI (10 mol%), DMEDA (15 mol%), sodium ascorbate (5 mol%), DMSO/H₂O: 5/1, 60 °C. (iv) KOH (5 equiv), Pd₂dba₃ (10 mol%), *t*BuXPhos (20 mol%), dioxane/H₂O: 1/1, 90 °C in a sealed tube.

These considerations led us to prepare a series of new restricted *iso*CA-4 analogues by inserting an additional ring between the 1,1-ethylene double bond and the B-ring. Thus, dihydronaphthalene **30** and naphthalene derivatives **31** were designed and their synthesis is reported in Scheme 9. As previously reported, the pivotal dihydronaphthalene **30a** was prepared by coupling its

corresponding *N*-tosylhydrazone with 3,4,5-trimethoxyiodobenzene under Pd-catalysis. Compound **30c**, having a NH₂ substituent was prepared from **30a** in a 60% yield, by using sodium azide as the amine source in the presence of CuI in EtOH. [89] Finally, phenol derivative **30d**, having the greatest resemblance to *iso*CA-4 was prepared in a 63% yield using KOH as nucleophile under Pd-catalysis. [90] After an extensive research, a DDQ-oxidation of dihydronaphtalene **30a** in CH₂Cl₂ at room temperature furnished naphthalene **31a** in an acceptable yield of 45%. As for **30a**, structural modifications on the C-Br bond of **31a** led to the desired naphthalene derivatives **31b-d**.

All dihydronaphtalene **30** and naphthalene derivatives **31** were evaluated for their cytotoxicity toward HCT116 cells and best molecules were tested against H1299, MDA-MB231 and K562 cancer cell lines (Table 5).

Table 5. Cytotoxicity of selected compounds against different human cancer cell lines and ITP.

Compounds	Cytotoxicity GI ₅₀ (nM)				ITP IC ₅₀ (μM)
	HCT116	H1299	MDA-MB231	K562
30a	320	-	-	-	-
30b	300	-	-	-	-
30c	7	30	85	180	2.1
30d	85	32	20	15	1.9
31c	55	90	60	85	2.0
<i>iso</i> CA-4 ^a	2	5	4	4	2.2

^aThe GI₅₀ and IC₅₀ values for *iso*CA-4 was determined in this study.

Naphtalene compounds were not cytotoxic against HCT116 (data not shown) except naphtylamine **31c** which was found to be a potent inhibitor of tubulin assembly (IC₅₀ = 2 μM). Nevertheless, **31c** had GI₅₀ values ranging from 55 to 90 nM which are approximately 20 times less active than its non-restricted *iso*NH₂CA-4 analogue. Dihydronaphtalene derivatives **30** were found to be more cytotoxic than their naphthalene congeners **31**. As observed in the *iso*CA-4 series, NH₂ and OH substitutions led to cytotoxic molecules as observed for **30c** and **30d**. These two drugs which retained a high level a cytotoxicity against HCT116 cells were also strongly cytotoxic against H1299, MDA-MB231 and K562 cancer cell lines with GI₅₀ values ranging from 7 to 180 nM. Compounds **30c** and **30d** showed a tubulin polymerization IC₅₀ similar to that of *iso*CA-4 (2.1 and 1.9 vs 2.0 μM). Next, to confirm that the mechanism of action of **30d** was antimitotic, we evaluated its effect on cell cycle. As shown in Figure 11, compound **30d** induced a prevalent block of cells in the G₂/M phase of the cell cycle at a concentration of 10⁻⁸ M.

Figure 11. Evaluation of G₂/M arrest in MDA-MB231, K562, HCT116 and H1299 cells exposed to **30d**.

Recently, Pinney reported the synthesis of benzosuberene derivatives [87, 91] as highly cytotoxic agents and showed that the double bond in *iso*CA-4 could be included in a 7-membered ring to maintain potent activity. Having achieving our goal with dihydronaphthalenes and naphthalene derivatives, we next envisioned to prepare and evaluate a novel series of dihydrobenzoxepin derivatives **32** having a 7-membered heterocyclic-ring.

Scheme 10. ^aSynthesis of benzoxepins **32a-d**.

^aReagents and conditions: (i) Grubbs II catalyst, CH₂Cl₂, rt. (ii) H₂, Pd/C, EtOAc. (iii) PCC, CH₂Cl₂. (iv) *N*-tosylhydrazide, EtOH, reflux. 3,4,5-trimethoxyphenyl iodide 1.1 equiv, Pd₂dba₃ (10 mol%), XPhos (40 mol%), LiOtBu (2.2 equiv), dioxane 90 °C, sealed tube. (v) Pd(OAc)₂ (5 mol%), PPh₃ (20 mol%), K₂CO₃ (2 equiv), BuOH, 100 °C. (vi) NaN₃ (2 equiv), CuI (10 mol%), DMEDA (15 mol%), sodium ascorbate (5 mol%), DMSO/H₂O: 5/1, 60 °C. (vii) KOH (5 equiv), Pd₂dba₃ (10 mol%), *t*BuXPhos (20 mol%), dioxane/H₂O: 1/1, 90 °C, sealed tube.

2.4.4.2 Synthesis and biological evaluation of benzoxepins 32.[92] The target molecules **32a-d** were prepared from isovanillin (Scheme 10). Chemical amendments of isovanillin afforded rapidly diene **33** which underwent ring-closing olefin metathesis

using Grubbs II catalyst. The resulting dihydrobenzoxepin-5-ol **34** was subjected to double bond reduction, PCC oxidation and *N*-tosylhydrazone formation to give **35** in good yields. A further palladium-catalyzed coupling reaction with 3,4,5-trimethoxyiodobenzene furnished the 9-bromo-8-methoxybenzoxepin **32a** which was transformed into functionalized benzoxepins **32b-d** as it was previously described for dihydronaphthalene **30a**.

Benzoxepin derivatives **32a-d** were evaluated against HCT116, H1299, K562 and MDA-MB-231 cells. The GI₅₀ values are shown in Table 6. Benzoxepin **32d**, having a OH substituent on C9 was the most active compound in all cancer cells with GI₅₀ values of 1.5-8 nM, comparable to that of *isoCA-4*. Derivative **32c** having on the C9 portion a NH₂ function, showed a lower but interesting level of cytotoxicity with GI₅₀ values ranging from 8 to 40 nM. On the exception of 9-bromobenzoxepin **32a**, compounds **32b-d** strongly inhibited tubulin polymerization with similar IC₅₀ values of 3.6-3.9 μM which were about two times greater than that of *isoCA-4*. Next, flow cytometry analysis indicated that the more potent benzoxepin **32b**, at a low concentration of 5 nM, arrested the cell cycle in the G₂/M phase in H1299, K562, HCT116 and MDA-MB-231 cells [92] as it was previously observed with other antitubulin drugs. Furthermore, **32b** was characterized as a strongly apoptotic agents at a low concentration of 5 nM with a marked effect in H1299 cells (data not shown).

Table 6. Cytotoxicity of benzoxepins **32a-d** against four cancer cell lines and IPT.

Compounds	HCT116	Cytotoxicity GI ₅₀ (nM)			IPT IC ₅₀ (μM)
		H1299	K562	MDA-MB231	
32a	170	ND ^a	^a	^a	NA ^b
32b	85	180	100	250	3.9
32c	22	30	40	8	3.6
32d	1.5	4.5	8	3	3.8
<i>isoCA-4</i>	2	5	5	4	2.2

^aND Not determined. ^bNA Not active

Having succeeded in the syntheses of bioactive 1,1-diarylethylene derivatives, with *isoCA-4* as lead compound, and in the preparation of restricted *isoCA-4* derivatives, we next envisioned to reduce the ethylene double bond for SAR considerations.

2.4.5 C1-linker: diarylethane **36** and diarylmethane **37** derivatives.

2.4.5.1 Synthesis of *isoerianin* analogues **36.** [93] On the basis of the previous bioisosteric considerations with diarylethylene compounds, we next hypothesized that compounds of type **36** having a 1,1-diarylethane scaffold could be as active as their 1,2-diarylethylene isomers (Scheme 11).

Scheme 11. Bioisosteric equivalence between natural erianin and *isoerianin* **36a**.

In particular, it would be of interest to compare the biological properties of compound **36a**, *isoerianin*, with its natural isomer and anticancer agent erianin. [94] Moreover, compounds of type **36** could be regarded as acyclic and simplified analogues of the antimitotic and antiproliferative podophyllotoxin. [95, 96] *Isoerianin* derivatives were prepared in a single step by reduction of 1,1-diarylethylene derivatives **28-30**. The water-soluble phosphate **36b** was obtained from *isoCA-4* by phenol dibenzylphosphorylation followed by concomitant H₂/Pd-hydrogenolysis and double-bond reduction.

2.4.5.1 Biological activities of *isoerianin* derivatives 36a-d.

2.4.5.1.1 Cytotoxicity and inhibition of tubulin polymerization. The cytotoxicities against five human cancer and HUVEC cell lines of selected *isoerianin* compounds are presented in Table 7. It is of interest to note that *isoerianin* exhibited similar or greater cytotoxic activity than erianin with GI₅₀ values ranging from 25 to 45 nM. Both enantiomers of *isoerianin* were separated by chiral HPLC and were found to display a similar level of cytotoxicity with GI₅₀ values of 26-29 nM against the HCT116 cancer cells (data not shown). Changing the C3'-OH by a phosphate group, as for water-soluble derivative **36b**, decreased cytotoxicity by 2-fold as compared to *isoerianin*. Interestingly, indole **36c** and C3'-NH₂-derivative **36d** are the most cytotoxic agents in the 1,2-diarylethane series **36**. Examination of antitubulin properties of *isoerianin* reveal that this compound displayed a potent activity (IC₅₀ = 3 μM) comparable to that of erianin (1.5 μM).

Table 7. Cytotoxic activity and inhibition of tubulin polymerization of selected *isoerianin* derivatives **37**.

Compounds	Cytotoxicity GI ₅₀ (nM)					HUVEC	IPT IC ₅₀ (μM)
	HCT116	K562	H1299	MDA-MB435	MDA-MB231		
<p>isoerianin</p>	28	25	40	35	35	45	3
<p>37b</p>	100	47	50	54	41	70	-
<p>37c</p>	29	32	42	33	32	15	20
<p>37d</p>	60	20	60	50	50	30	13
erianin	44	36	42	31	49	10	1.5

Figure 12. Effect of *isoerianin* on cell cycle distribution in K562 and H1299 cancer cell lines at 50 nM.

2.4.5.1.3 Effects on cell cycle distribution and apoptosis. We next investigated whether *isoerianin* influence the cell cycle in K562 and H1299 cells. As it was previously showed for *isoCA-4* derivatives, treatment with *isoerianin* for 24 h at a concentration of 50 nM blocked the cellular cycle in the G₂/M phase in K562 and H1299 cell lines (Figure 12). Furthermore, we studied the effect of *isoerianin*, on the apoptosis induction in K562 and H1299 cells. After 24 h of treatment with *isoerianin* at a concentration of 50 nM, a pronounced processing of pro-caspases was observed (Figure 13). At this concentration, *isoerianin* was 4-fold more active as apoptosis inducer in H1299 than in apoptosis resistant-K562 cells.[66] Similarly, C3'NH₂-derivative **36d** activate efficiently the caspases family leading to apoptosis [93] (data not showed). These results show that the reduction of the ethylene double-bond present in *isoCA-4* analogues had no deleterious impact on the apoptotic potent of such reduced-compounds **37**.

Figure 13. Apoptotic effect of *isoerianin* in HCT1299 and K562 cells.

2.4.5.1.4 Effect of *isoerianin* on HUVEC organization.

The effect of *isoerianin* on HUVECs was next evaluated at a concentration of 1 μ M. As observed in Figure 14, the tubes' structures were severely damaged when cells were exposed to *isoerianin* after 3 h of treatment. We found that *isoerianin* had equivalent activities in damaging vascular tubes at low concentrations than that of *isoCA-4*, *isoFCA-4*, *isoNH₂CA-4* as well as benzil derivative **11d**.

Figure 14. Effect of *isoerianin* on newly formed vessels.

2.4.5.2 C1-linker: diarylmethane derivatives **37**.

We next designed a series of diarylmethane compounds of type **37** based on *isoerianin* to examine the effects regarding removal of the methyl substituent on the C1 linker. The synthesis of diarylmethane derivatives **37a-e** is shown in Scheme 12.

Scheme 12. ^aSynthesis of diarylmethanes **37**.

Diarylmethanes **37** [40, 97, 98] were prepared from reduction of diarylcarbinols using either catalytic TfOH in *i*-PrOH [97] or dichloromethylsilane/sodium iodide [87] or by NaBH_4 -reduction of the aminophenstatin analogue. These diarylmethane derivatives were evaluated by an *in vitro* cytotoxicity assay which was carried out with a panel of human cancer cells. Unfortunately, all of these compounds were essentially not cytotoxic [93] and did not inhibit tubulin polymerization on the exception of phenol **37e** ($\text{IC}_{50} = 2.3 \mu\text{M}$). [98] These results demonstrate that the replacement of the ethyl group in *isoerianin* or ethylene group in *isoCA-4* by a methylene resulted in complete loss of cytotoxic activities indicating that an additional carbon atom (sp^3 or sp^2) on C1 between the A- and B-rings is critical for cytotoxicity.

3. Dihedral angles values between A- and B-rings and docking studies of bioactive analogues.

3.1 X-ray structures of *isoCA-4* and *isoerianin*.

Figure 15. X-ray structure of *isoCA-4*

The high purity of *isoCA-4* synthesized in our lab, enabled us to perform its X-ray structure determination. As shown in Figure 15, the X-ray crystallographic analysis for *isoCA-4* revealed that the A- and B-rings are inclined toward each other with a dihedral angle of 68° . [54] It is of interest to note that, a similar non-planarity was observed with tubulin binding ligands as CA-4, CA-1 and colchicine in which the dihedral angle values are of 53° [9, 100], 66° [101] and 53° [102] respectively. Similarly, and according to X-ray structure, we also found that the dihedral angle between the two aromatic rings in *isoerianin* is of the same order of magnitude with a value of 77° [93] (data not shown). These data and similar observations with efficient tubulin-binding ligands [103-105] indicate clearly that the conformational relationship of the A- and B-rings ($50^\circ < \text{dihedral angle} < 80^\circ$) is an important component of ligand binding to β -tubulin on the colchicine binding-site. The presumptive binding mode of the most bioactive tubulin ligands (e.g. *isoCA-4*, *isoerianin*, dihydronaphthalene **30d**, benzoxepin **32b** and diarylethylenes **20c**, **20h**) and the dihedral angles values between diaryl groups are presented in Table 8.

Table 8. Docking-derived superimposition of the most bioactive ligands and dihedral angle values between diaryl groups.

<p><i>isoCA-4</i>: $\alpha = 68^\circ$</p> <p>8a. Putative binding mode and superimposition of <i>isoCA-4</i> (blue), CA-4 (green), phenstatin (red) and colchicine (orange) in the colchicine binding site.</p>	<p><i>isoerianin</i>: $\alpha = 77^\circ$</p> <p>8b. Putative binding mode and superimposition of <i>isoerianin</i> (green), <i>isoCA-4</i> (blue) and colchicine (orange) in the colchicine binding-site.</p>	<p>dihydronaphthalene 30d $\alpha = 70^\circ$</p> <p>8c. Putative binding mode and superimposition of 30d (gray) and <i>isoCA-4</i> (blue)</p>
<p>benzoxepin 32b $\alpha = 75^\circ$</p> <p>8d. Putative binding mode and superimposition of 32b (gray) and <i>isoCA-4</i> (blue)</p>	<p>diarylethylene 20c: $\alpha = 75^\circ$</p> <p>8e Putative binding mode of 20c</p>	<p>diarylethylene 20h: $\alpha = 76^\circ$</p> <p>8f. Putative binding mode of 20h</p>

It is interesting to note that all of the selected ligands are structurally similar to CA-4 ($\alpha = 53^\circ$) and *isoCA-4* ($\alpha = 68^\circ$) with comparable dihedral angle values ranging from 68° to 77° . The presence of a σ -bond bridge in *isoerianin* ($\alpha = 77^\circ$) and the conformational restriction imposed to ligands by the formation of an additional ring (**30d** $\alpha = 70^\circ$) or heterocyclic system (**32b** $\alpha = 75^\circ$) does not modify significantly the dihedral angles between the A- and B-rings. As observed in figures **8a** and **8b** (Table 8), the superimposition of *isoCA-4*, *isoerianin*, and references CA-4, phenstatin and colchicine as they docked within the active site of 1saO, illustrates the similar nature of positioning of the 3,4,5-trimethoxy substituents in A-ring and also 3-hydroxy-4-methoxy substituents (B-ring). Similarly, conformationally restricted *isoCA-4* analogues such as dihydronaphthalene **30d** and benzoxepin **32b** show good superimposition with *isoCA-4* with the trimethoxyphenyl ring in contact with Cys241 and with the 3-hydroxy-substituent of the B-ring which forms a hydrogen bond with Val181 as it was previously reported with other CA-4 analogues. [106-108] Contrary to the above situation where Val181 was important for binding in the colchicine-binding site of tubulin, the hydrogen bond with Val181 was not observed for compounds **20c** and **20h** bearing an alkenyl or alkynyl substituent on C3'-position. Although the trimethoxyphenyl moiety remains anchored at the same place in proximity of Cys241, a polar cavity formed by Gln247, Tyr224, Gln11 and Lys254 accommodates the C3'-substituents of the B-ring. In details, **20c** with the backbone carbonyl of Gln247, whereas alkynol **20h** form a double hydrogen bond with Gln247 and Tyr224. Moreover, the 4'-methoxy

group of each molecule attached to the B-ring is seen to establish an additional extra hydrogen bond with Lys254 (not displayed in figure **8e** and **8f**). These results indicate that, due to the replacement of the C3'-OH group of *isoCA-4* by a larger alkenyl or alkynyl group in compounds **20c** and **20h**, the position of the latter ligands is variously shifted in the tubulin binding-site without alteration for activity. Thus, it can be assumed that the large polar cavity formed by Gln247, Tyr224, Gln11 and Lys254 can accommodate a wide range of new ligands allowing to complete the SAR.

4. Conclusions

This review has provided the synthesis and *in vitro* assays of new CA-4 analogues developed in our group with non-isomerizable carbon-linkers of different sizes and SAR are summarized in Figure 16. In the C6 and C4 linker series, enediyne **4** and enyne derivatives **8-9** were found to be inactive with $GI_{50} > 1000$ nM and did not inhibit tubulin assembly significantly. In the C2 linker class, benzils **11c** and **11d** which strongly inhibited the cell growth at a nanomolar level (20 nM $< GI_{50} < 50$ nM) are the most promising derivatives. In addition to their cytotoxic effects against various cancer cells, benzils derivatives showed *in vitro* antivasular effects in HUVEC cells by disrupting tubes formation. These compounds were also potent inhibitors of tubulin assembly, with micromolar IC_{50} values, and arrested HCT116 and H1299 cells in the G₂/M phase of the cell cycle. Moreover, a significant increase in proteolytic activity of caspase in cells treated with diketones **11c** and **11d** clearly demonstrates the potential of these drugs as apoptosis inducers.

Figure 16. SAR of combretastatin A-4 analogues having various C-linkers and advantages of *isoCA-4*.

In the C1 linker series, after a detailed investigation of SAR, we found that 1,1-diarylethylene compounds **18-20** (*isoCA-4* and analogues) as well as their reduced congeners *isoerianin* derivatives **36** were highly cytotoxic against a panel of cancer cells and

inhibited the tubulin assembly at low concentrations. We also demonstrate that it is possible to lock the 1,1-ethylene bond of these derivatives in a dihydronaphthalene, chromene or benzoxepin ring with no significant loss of cytotoxicities. All of these non-isomerizable compounds including *isoCA-4*, *isoerianin*, dihydronaphthalene, chromene and benzoxepin derivatives, cause G₂/M arrest and strongly induce apoptotic cell death in various cancer cell lines at low concentrations. *In vitro* antivasular effects of these drugs were tested using a human umbilical vein endothelial cell culture assay. Addition of these drugs to formed cords disrupted the integrity of the network after 3 h of treatment at doses which were not cytotoxic for such incubation time thereby demonstrating the anti-angiogenic effect of these new compounds.

For further *in vivo* investigations, we have selected as lead compound *isoCA-4* which is the most active compound and which displays the largest structural simplicity. To the end, we first have recently demonstrated that *isoCA-4* was poorly metabolized (<10%) after incubation in human liver microsomal fractions. Seven phase I metabolites resulting mainly from O-demethylation and hydroxylation of the aromatic rings were identified, synthesized and evaluated. [109] All of these metabolites display a moderate level of cytotoxicity (GI₅₀ ranging from 35 to 2000 nM) and did not inhibit tubulin assembly, indicating that the antiproliferative properties of *isoCA-4* are due to the parent drug itself since its metabolites are significantly less cytotoxic. Due to the poor water-solubility of *isoCA-4* and to avoid the tedious preparation of phosphate-prodrugs, we envisioned to investigate the anticancer activity of *isoCA-4* *in vivo* on the experimental LS174T human colon adenocarcinoma by incorporating *isoCA-4* in squalenoyl nanoassemblies. [110] As it was previously observed with gemcitabine-squalene nanoparticles which was found to display improved anticancer activity, comparatively to gemcitabine free [111], we hope to increase the pharmacokinetic parameters and the biodistribution, [112] as well as an improved intracellular penetration. These results will be published in due course.

Conflict Of Interest

None

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None

Abbreviations

Miscellaneous

VDA _s	Vascular disrupting agents
CA-4	Combretastatin A-4
ITP	Inhibition of tubulin polymerization
IC ₅₀	Concentration of compound required to inhibit 50% of the rate of microtubule polymerization
GI ₅₀	Concentration of compound needed to reduce cell growth by 50%
MDR	Multi-drugs resistant
rt	Room temperature
DNA	Desoxyribonucleic acid
HPLC	High Performance Liquid Chromatography

Chemistry

PCC	Pyridinium chlorochromate
PTSA	<i>Para</i> -toluenesulfonic acid
DMSO	Dimethyl sulfoxide
DDQ	2,3-Dichloro-5,6-dicyanobenzoquinone

Cancer Cells

HCT	Human colon tumor
SH-SY5Y	Neuroblastoma
MCF7	Human breast carcinoma
CA-46	Burkitt lymphoma
K562	Human cervical carcinoma
MCF7R	Multidrug resistant human breast cancer
K562	Chronic myelogenous leukemia
H1299	Non small lung human carcinoma
A549	Carcinomic alveolar basal epithelial
MDA-MB-435	Breast cancer
MDA-MB-231	Hormone-independent breast cancer
U87	Glioblastoma
NCI-H460	Non small lung human carcinoma
DU-145	Human prostate cancer
SK-OV-3	Human ovarian carcinoma
CEM	Leukemia cells
HBL100	Human epithelial mammary cells
NCI-H460	Human non-small lung cancer cells
LS174T	Human colon adenocarcinoma
HUVEC	Human umbilical vein endothelial cells

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