



Título artículo / Títol article: Design and Synthesis of Pironetin Analogue/Combretastatin A-4 Hybrids and Evaluation of Their Cytotoxic Activity

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Revista: European Journal of Organic Chemistry, 2014(11)

Versión / Versió: Post-print autor

Cita bibliográfica / Cita bibliogràfica (ISO 690): TORIJANO-GUTIÉRREZ, Sandra, et al. Design and Synthesis of Pironetin Analogue/Combretastatin A-4 Hybrids and Evaluation of Their Cytotoxic Activity. European Journal of Organic Chemistry, 2014, vol. 2014, no 11, p. 2284-2296.

url Repositori UJI: <http://hdl.handle.net/10234/127145>

Design and Synthesis of Pironetin Analogue-Combretastatin A-4 Hybrids and Evaluation of Their Cytotoxic Activity

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ABSTRACT

We here describe the preparation of a series of hybrid molecules containing a combretastatin A-4 moiety and a pironetin analogue fragment. The cytotoxic activities of these compounds have been measured. Relations between structure and cytotoxicity are discussed. Some of the tested compounds showed cytotoxicity values of the same order of magnitude as the parental molecules, combretastatin A-4 and pironetin, and were less toxic than the latter for normal cells.

KEYWORDS

Combretastatin A-4; pironetin analogues; hybrid molecules; tubulin-active compounds; cytotoxicity.

HIGHLIGHTS

- Hybrid molecules containing a combretastatin A-4 moiety and a pironetin analogue fragment have been prepared and tested for cytotoxicity against a normal and two tumoral cell lines.

- Some of the tested compounds show cytotoxicity values of the same order of magnitude as the parental molecules, combretastatin A-4 and pironetin.
- The normal vs. tumoral cytotoxicity ratio of some of the tested compounds are higher than in the case of the parental molecules, that is, they are safer than the latter compounds.

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 various mechanisms of cell death [4]. Many of such drugs owe this property to interaction with the microtubule network. Microtubules are dynamic polymers that play a central role in a number of cellular processes, most particularly cell division, as they are key constituents of the mitotic spindle. Microtubules are constituted of a protein named tubulin, the functional form of which is a heterodimer formed through non-covalent binding of two monomeric constituents, called α - and β -tubulin. For cell division to occur in a normal way, microtubules must be in a constant state of formation and disruption, a process named microtubule instability [5]. Any molecule which influences microtubule instability will also influence the cell division process, not only of normal cells but also of tumoral ones. Therefore, it is not surprising that tubulin-binding molecules (TBM) constitute a most important class of anticancer agents. TBMs may be divided in two broad categories, those that bind to α -tubulin and those that bind to β -tubulin. The latter group is presently by far the most numerous and contains products which cause either disruption or stabilization of microtubules. Among the drugs that belong to this group, colchicine [6] and the combretastatins [7] (Fig. 1) exert their effects by causing disruption of microtubules. In contrast, another important representative of the same group, paclitaxel, was the first-described tubulin-binding drug that was found to stabilize microtubules [8]. Even though they display opposite effects, these drugs are known to bind to β -tubulin, whenever to different sites within this protein subunit [9-11].

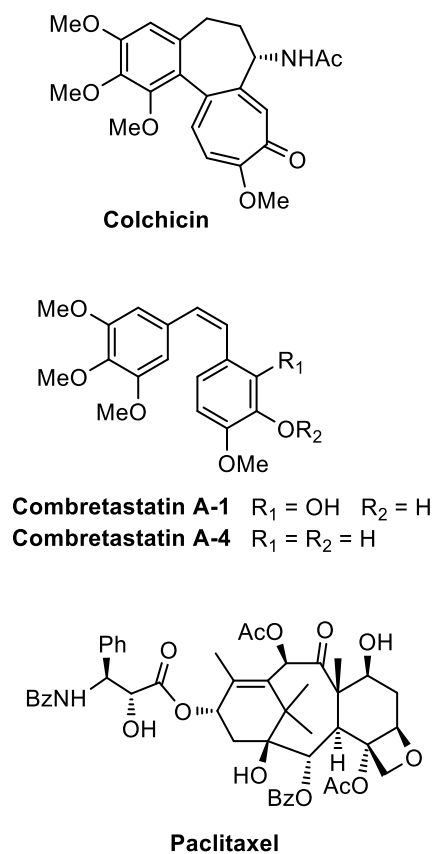


Figure 1. Structures of some natural products reported to selectively bind to β -tubulin.

The number of products that bind to α -tubulin is very small, the naturally occurring 5,6-dihydro- α -pyrone pironetin being the first-reported example [12], followed a short time later by the peptide-like hemiasterlin family [13] (Fig. 2). Pironetin is a potent inhibitor of tubulin assembly and has been found to arrest cell cycle progression in the G2/M phase [14].

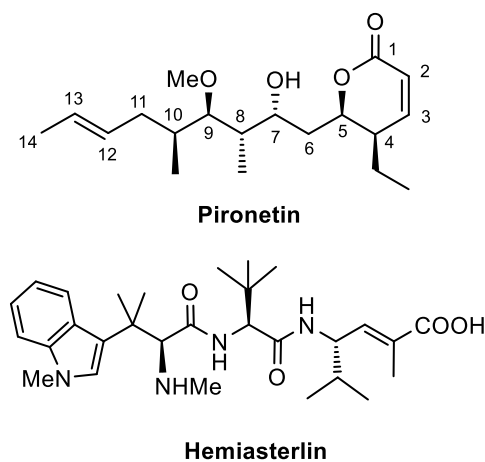


Figure 2. Structure of two natural products reported to selectively bind to α -tubulin.

Some structure-activity (SAR) studies on pironetin have been reported. These studies have shown that the presence of the conjugated C2–C3 double bond and of the hydroxyl group at C-9, either free or methylated, are essential for the biological activity. The presence of a (7*R*)-hydroxyl group also seems to be relevant. It has been suggested that the Lys352 residue of the α -tubulin chain adds in a Michael fashion to the conjugated double bond of pironetin, therefore forming a covalent bond with C-3 of the dihydropyrone ring (Fig. 3). In addition, it has been suggested that the Asn258 residue of α -tubulin holds the pironetin molecule through two hydrogen bonds to the dihydropyrone carbonyl and the methoxyl oxygen atoms [14].

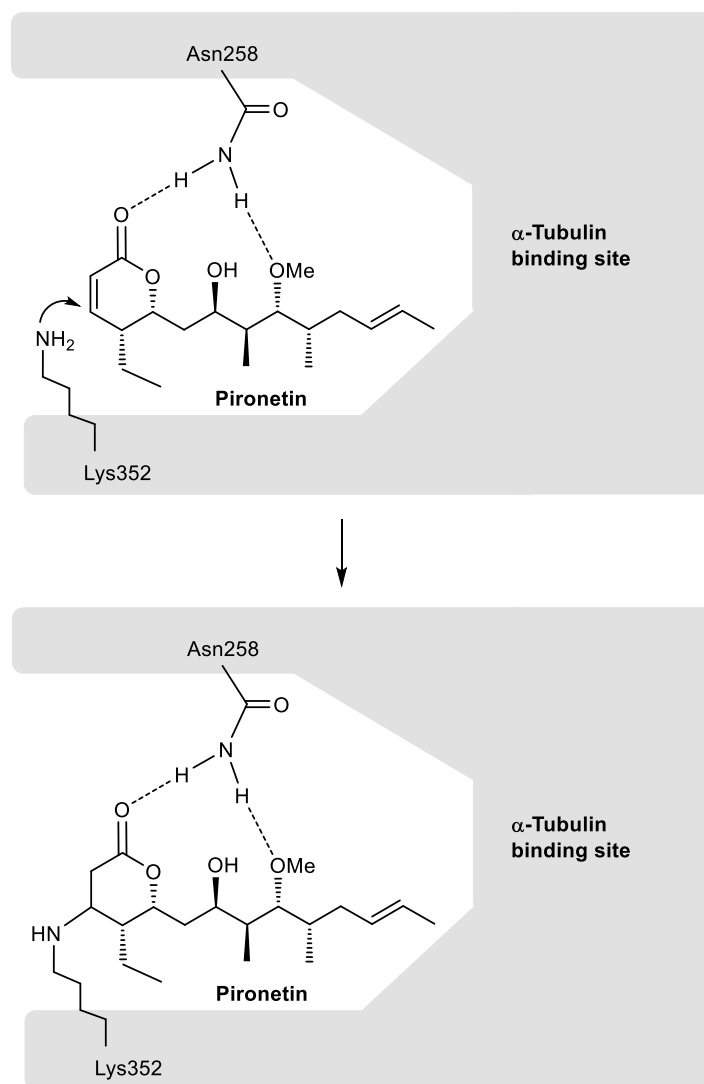


Figure 3. Schematic model of the covalent union of pironetin to its binding site at the α -tubulin surface.

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

[15], resistances have been reported to therapies with various types of cytotoxic agents [16]. The investigation of new compounds with such biological properties therefore constitutes an important goal in chemistry and pharmacology.

2. Concept and design of hybrid tubulin-binding ligands

As a member of the up to now small group of TBMs that bind to α -tubulin, pironetin constitutes a pharmacologically interesting target. Not surprisingly, an appreciable number of total syntheses of this natural compound has appeared in the literature [17]. In order to develop SAR studies based upon the pironetin framework, we designed two years ago [5a] a simplified model structure where all elements that had not yet proven to be essential for the biological activity were removed. The target structures **I/II** are schematically shown in Figure 4. The elements that were maintained are the conjugated dihydropyrone ring and the side chain with the methoxy group at C-9. The hydroxyl group at C-7 was removed in some substrates (**I**) and retained in others (**II**), in order to see its influence on the activity. All alkyl pendants (methyl groups at C-8 and C-10, ethyl at C-4) and the isolated C12–C13 double bond were removed. The configurations of the two/three remaining stereocentres were then varied in a systematic way. Thus, all four possible stereoisomers with general constitution **I**, with no hydroxyl group at C-7, were prepared. Likewise, all eight stereoisomers exhibiting general structure **II**, with a hydroxyl group at C-7, were synthesized [5a].

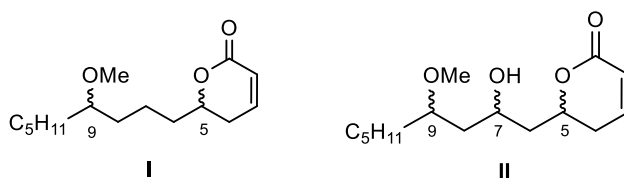


Figure 4. General structures of simplified pironetin analogues of the first generation (ref. 5a).

The cytotoxic activity of these analogues and their interactions with tubulin were subsequently investigated. It was found on one hand that analogues **I/II** were cytotoxic in the low micromolar range, about three orders of magnitude less active than the parent molecule. On the other hand, we also found that they behave in the same way as pironetin, share the mechanism of action of the natural compound and compete for the same binding site in α -tubulin. As the parent compound, they also lead to disruption of the microtubule network [5a].

With the aim at extending our project, our idea was to prepare cytotoxic TBMs with a dual ability to bind to both α - and β -tubulin and exert a microtubule-destabilizing effect. Since these properties are specifically exhibited by pironetin (binds to α -tubulin) and combretastatin A-4 (binds to β -tubulin),

respectively, we decided to prepare hybrid molecules such as **1-8** (Fig. 5) containing a moiety of combretastatin A-4 (itself numbered as **13** in Fig. 5) and another of the simplified pironetin type (**14**, *ent*-**14** and **15**), connected in turn by an ester spacer of variable length. Some of them have been prepared in both antipodal forms (**1** to **4** and *ent*-**1** to *ent*-**4**) [18].

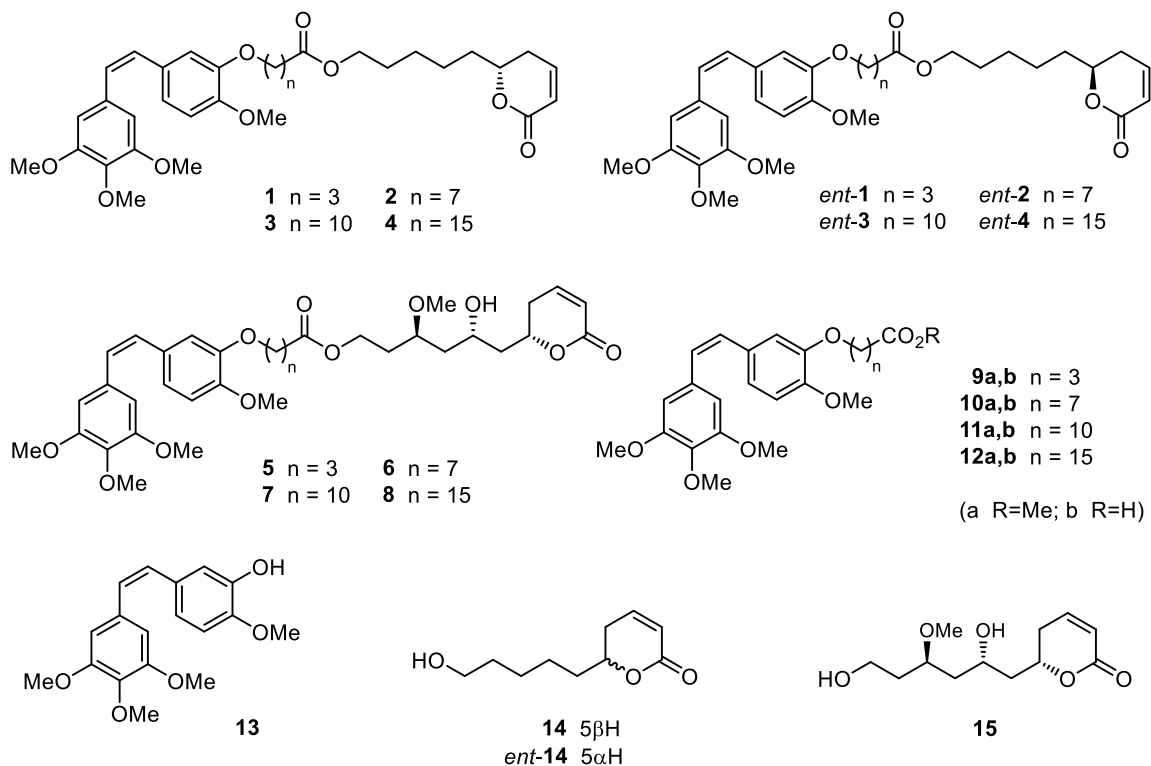
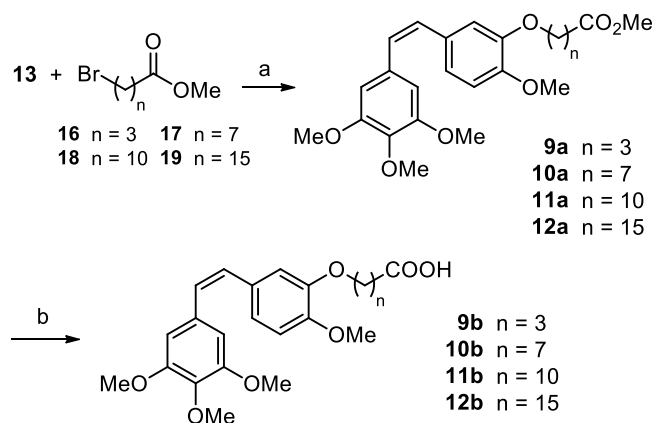


Figure 5. Structures of the pironetin analogue/combretastatin A-4 hybrids used in this study (**1-8**) and of their synthetic precursors (**9-15**), including combretastatin A-4 (**13**).

3. Synthetic work

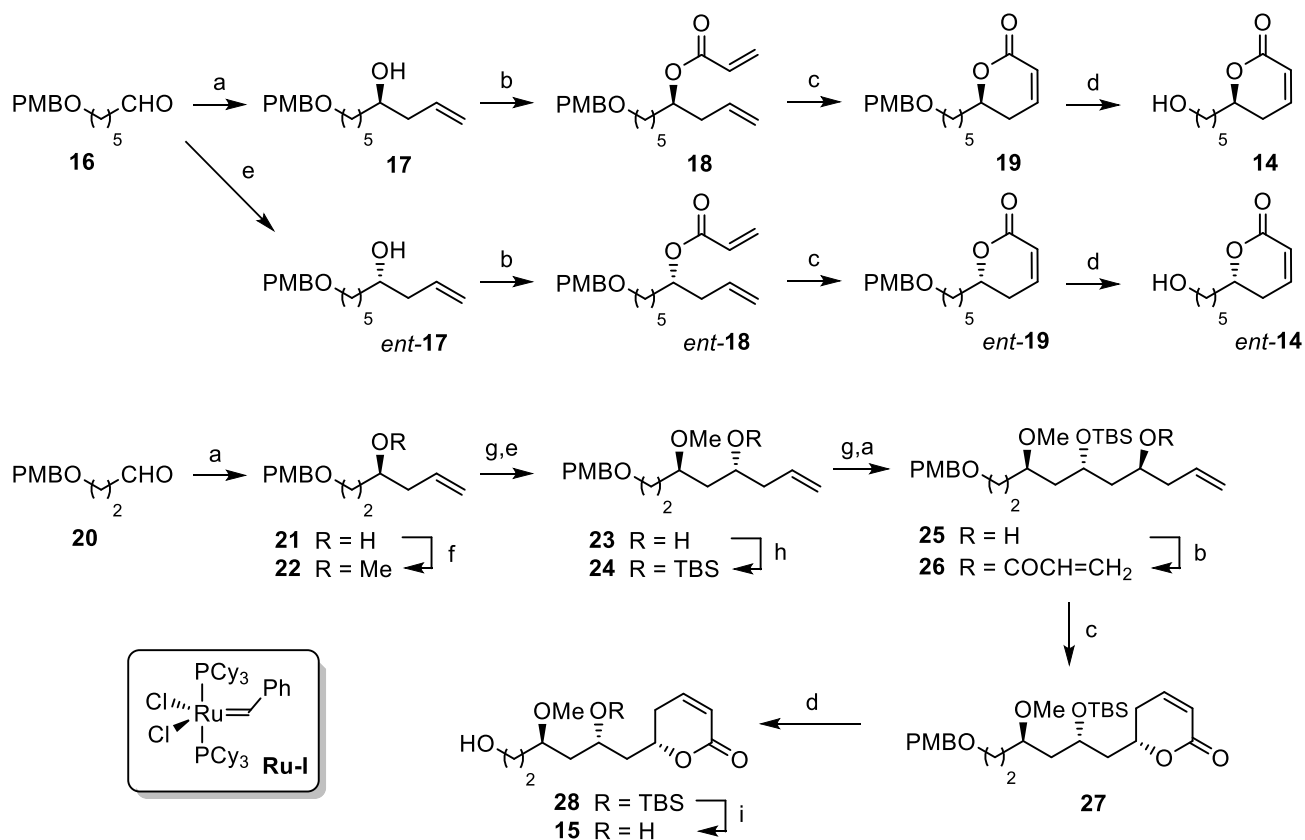
Combretastatin A-4 (**13**) was prepared according to a literature procedure [19]. Its *O*-alkyl derivatives **9a-12a** were prepared by means of *O*-alkylation with commercially available ω -bromoesters **16-19** (Scheme 1). Saponification of esters **9a-12a** gave the corresponding acids **9b-12b**.



Scheme 1. Synthesis of combretastatin A-4 derivatives **9a-12a** and **9b-12b**: (a) K_2CO_3 , DMF, RT, 24 h (**9a**, 91%; **10a**, 91%; **11a**, 98%; **12a**, 90%); (b) aq NaOH, MeOH, RT, 18 h (**9b**, 90%; **10b**, 78%; **11b**, 72%; **12b**, 75%). Acronyms and abbreviations: DMF, *N,N*-dimethylformamide.

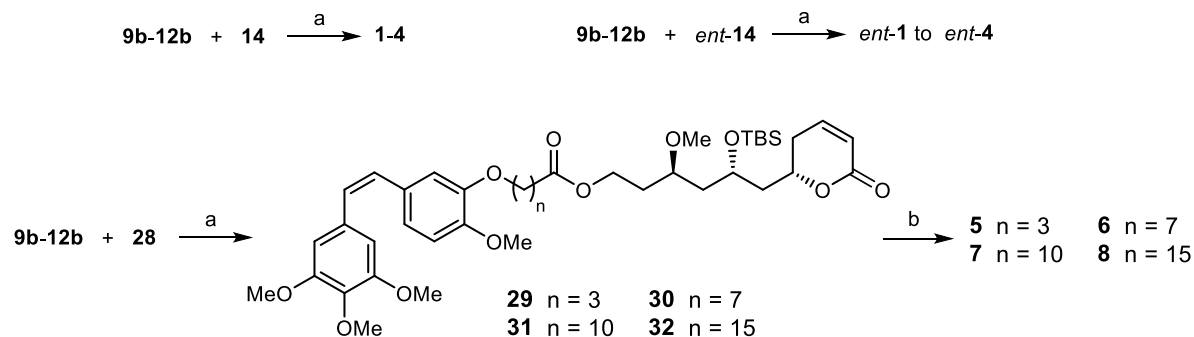
The synthesis of the pironetin fragments **14**, *ent*-**14** and **15** depicted in Scheme 2, was performed according to the general concept used in our previous papers [5a,b]. Thus, Brown's asymmetric allylation [20] of the known aldehyde **16** [21] afforded homoallyl alcohol **17**. The required chiral allyborane was prepared through reaction of allylmagnesium bromide with the commercially available (–)-diisopinocampheylboron chloride, (–)-Ipc₂BCl. Reaction of **17** with acryloyl chloride at low temperature gave acrylate **18**, which was subjected to ring-closing metathesis (RCM) [22] in the presence of Grubbs first-generation catalyst **Ru-I**. This furnished dihydropyrone **19**, deprotection of which provided **14** in good yield. Dihydropyrone *ent*-**14** was prepared in the same way except that (+)-Ipc₂BCl was the chiral boron reagent used.

Dihydropyrone **15** was not connected as such with the combretastatin A-4 fragment but as its *O*-silyl derivative **28** (Scheme 2), which was also prepared according to the previous methodology. Brown's asymmetric allylation of the known aldehyde **20** [23] furnished alcohol **21**, which was then converted into methyl ether **22**. The latter was then sequentially subjected to a two-step oxidative cleavage of the olefinic bond, followed by Brown's asymmetric allylation to yield **23**. Silylation of the hydroxyl group of **23** and repetition of the previous sequence gave alcohol **25**, which was esterified with acryloyl chloride. The resulting acrylate **26** was subjected to ring-closing metathesis to **27**, which was then oxidatively deprotected [24] to **28**. Desilylation of **28** gave **15**, which was used in the biological evaluations.



Scheme 2. Synthesis of pironetin fragments **14**, *ent*-**14** and **15**. (a) (–)-Ipc₂BCl, allylMgBr, Et₂O, –78 °C, 1 h, then addition of the aldehyde, 1 h, –78 °C (**17**: 76%, e.r. 96:4; **21**: 90%, e.r. 94:6; **25**, 70% overall yield from **24**, d.r. 76:24); (b) CH₂=CHCOCl, CH₂Cl₂, *i*Pr₂NEt, –78 °C, 30 min (**18**: 94%; *ent*-**18**: 90%; **26**, 98%); (c) 10% cat. **Ru-I**, CH₂Cl₂, Δ, 2 h (**19**: 81%; *ent*-**19**: 80%; **27**: 83%); (d) DDQ, wet CH₂Cl₂, 30 min. (**14**: 69%; *ent*-**14**: 67%; **28**: 84%); (e) (+)-Ipc₂BCl, allylMgBr, Et₂O, –78 °C, 1 h, then addition of the aldehyde, 1 h, –78 °C (*ent*-**17**: 89%, e.r. 96:4; **23**: 90% overall yield from **22**, d.r. 86:14); (f) NaH, THF, 0 °C, then MeI, RT, overnight (88%); (g) 1) OsO₄, NMO, aq *t*BuOH, THF, RT, overnight. 2) Pb(OAc)₄, CH₂Cl₂, 0 °C; (h) TBSOTf, 2,6-lut, 0 °C, 1 h (80%); i) PPTS (cat.), MeOH, Δ, overnight (85%). Acronyms and abbreviations: Ipc, isopinocampheyl; DDQ, 2,3-dichloro-5,6-dicyano-*p*-benzoquinone; TBS, *tert*-butyldimethylsilyl; NMO, *N*-methylmorpholine *N*-oxide; 2,6-lut, 2,6-lutidine; PPTS, pyridinium *p*-toluenesulfonate.

Dihydropyrones **1-8**, including *ent*-**1** to *ent*-**4**, were prepared by esterification of acids **9b-12b** with dihydropyrones **14**, *ent*-**14** and **28** by means of the Yamaguchi method [25] (Scheme 3). In the case of compounds **5-8**, an additional desilylation step was required.



Scheme 3. Synthesis of hybrid molecules **1-4**, *ent-1* to *ent-4*, **5-8**. (a) **9b-12b**, 2,4,6-trichlorobenzoyl chloride, Et₃N, RT, then addition of the alcohol component, RT, 2 h (**1**: 34%; **2**: 47%; **3**: 37%; **4**: 35%; *ent-1*: 42%; *ent-2*: 55%; *ent-3*: 37%; *ent-4*: 49%; **29**: 25%; **30**: 40%; **31**: 44%; **32**: 21%); (b) PPTS (cat.), MeOH, Δ, overnight (**5**: 85%; **6**: 95%; **7**: 80%; **8**: 85%).

After the synthesis of the pironetin-combretastatin A-4 hybrids was completed, the compounds were investigated in relation to their cytotoxic activity towards two types of tumoral lines and one normal cell line.

4. Biological work

4.1. Cellular effects of the compounds. Cytotoxicity values.

We carried out a measurement of the cytotoxicity of the synthetic combretastatin A-4 derivatives **1-4** and their enantiomers, **5-8**, **9a-12a** and **9b-12b**. Pironetin analogues **14**, *ent-14* and **15** were also tested. Cytotoxicity assays were performed as described in the Experimental Section 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 [26]. Cytotoxicity values, expressed as the compound concentration (μmol/L) that causes 50% inhibition of cell growth (IC₅₀), are shown in Table 1. The observed values are in most cases in the low to medium micromolar range. Compounds *ent-1*, **3**, **7**, **10a**, **11a**, **12a** and **12b** showed the highest cytotoxicities for the HT-29 cell line, with IC₅₀ values which were not very different (in some cases even lower) of those of combretastatin A-4 and pironetin for this particular cell line. In the case of the MCF-7 cell lines, the lowest IC₅₀ values were shown by compounds **7**, **9b**, **10a**, **11a**, **11b**, **12a**, **12b** and **15**. But a further aspect which is also worth mentioning is the fact that some of the synthetic compounds are much more toxic for tumoral cells than for normal ones, an obviously desirable feature. This can better appreciated with the α and β coefficients, obtained by dividing the IC₅₀ values of the normal cell line (HEK-293) by those of one or the other tumoral cell line (see footnote in the Table). The higher value of either the α or the β coefficient, the higher the

therapeutic safety margin of the compound in the corresponding cell line. Thus, combretastatin A-4 itself shows good values of both coefficients, most particularly in the case of the MCF-7 cell line. Pironetin shows a similarly good value for the HT-29 cell line (α), but much less so for the MCF-7 cell line. Among the compounds which show a high cytotoxicity (low IC_{50} values), *ent*-**1**, **7**, **11a**, **12a** and **12b** are particularly worth mentioning as they exhibit high both α and β values. Compound **3** shows a good selectivity only in the case of the HT-29 line ($\alpha > 30$) whereas compounds **11b** and **15** show a good selectivity in the specific case of the MCF-7 line ($\beta > 60$).

Some aspects related to the relation between the structures and the observed cytotoxicity deserve comment. In the case of two subsets of hybrid molecules [27] where there are differences between in the carbon chain length (e.g. **1-4** and **5-8**), the cytotoxicity reaches a maximum for compounds having a ten-carbon chain in the combretastatin A-4 segment (**3**, **7**). The cytotoxicities of the compounds of the enantiomeric series (*ent*-**1** to *ent*-**4**) are not too different from those of **1-4**, even though the maximum cytotoxicity is found here for *ent*-**1**, the compound with the shorter carbon chain. It is difficult to propose now an explanation for this fact, as we do not yet know whether these compounds are interacting with tubulin at the pironetin site (α -tubulin), at the combretastatin A-4 site (β -tubulin) or at both sites simultaneously. Research in this direction is being currently performed.

We have also tested the synthetic intermediates used for the preparation of the aforementioned hybrid molecules. As regards combretastatin A-4 derivatives **9-12a/b**, a dependence of the cytotoxicity from the carbon chain length is also observed (Table 1). For the methyl esters (**9a-12a**), a marked increase in the cytotoxicity is observed in going from the compound with the three-carbon chain (**9a**) to those having longer chains (**10a-12a**), with the IC_{50} values of the three latter being very similar (with both tumoral cell lines) and comparable with those of combretastatin A-4 itself. As for the free acids (**9b-12b**), the cytotoxicities vary in a rather erratic way and no relation is perceived between IC_{50} values and the chain length. Furthermore, marked differences also observed in some cases between the IC_{50} values for the two tumoral cell lines.

The cytotoxicities of the three pironetin analogues **14**, *ent*-**14** and **15** were also measured. Compound **14** and its enantiomer, which display a single stereocentre, represent the most simplified model possible for pironetin. From the results presented in Table 1, it is evident that **14** and *ent*-**14** show a very low cytotoxicity, much below of that observed for the hybrid molecules (**1** to **3**, and *ent*-**1** to *ent*-**3**) bearing these fragments (exceptions to this are **4** and *ent*-**4**, which show very high IC_{50} values, perhaps because of solubility problems). Even if conclusions must be still provisory, this seems to point to **1-3** and their enantiomers owing their cytotoxicity to interactions with tubulin through the combretastatin A-4 end. As regards compound **15**, it has three stereocentres and corresponds more closely to the pironetin models we have been investigating in recent years [5]. Table 1 shows that its cytotoxicity for the two tumoral

cells is indeed much higher than those of **14/ent-14**, most particularly in the case of the MCF-7 cell line. From the hybrid molecules **5-8** having this structural fragment, the highest cytotoxicity is shown by **7**, the molecule that displays the ten-carbon chain, as commented above. It is also worth noting that the IC₅₀ value of **7** with the HT-29 cell line is much lower than the IC₅₀ values of both **11b** and **15**, its two precursor components. This suggests that a hydrolysis of **7** into **11b** and **15** is not taking place within the cell.

Table 1. Cytotoxicity of pironetin analogue/combretastatin A-4 hybrids **1-8** and *ent-1* to *ent-4*, combretastatin A-4 derivatives **9a-12a** and **9b-12b**, and pyrones **14**, *ent-14* and **15**.^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
Pironetin	8 ± 2	18 ± 3	46 ± 6	5.7	2.5
1	12 ± 2	52 ± 10	39 ± 4	3.2	0.7
2	32 ± 5	> 300	80 ± 14	2.5	< 0.3
3	10 ± 1	> 300	> 300	> 30	–
4	107 ± 5	> 300	> 300	> 2.8	–
<i>ent-1</i>	3.5 ± 0.9	17 ± 2	114 ± 5	32	6.7
<i>ent-2</i>	19 ± 6	> 300	> 300	> 15	–
<i>ent-3</i>	38 ± 3	69 ± 7	59 ± 10	1.6	0.8
<i>ent-4</i>	64 ± 7	> 300	72 ± 3	1.1	< 0.3
5	15.4 ± 0.5	31 ± 1	22 ± 2	1.4	0.7
6	44 ± 5	> 300	> 300	> 6.8	–
7	2.9 ± 0.2	5.6 ± 0.1	22 ± 2	7.6	3.9
8	50.8 ± 0.6	21 ± 4	81 ± 10	1.6	3.8
9a	60.1 ± 0.5	10.4 ± 0.5	121 ± 5	2	12
9b	50 ± 2	2.9 ± 0.2	67 ± 2	1.4	23
10a	3.5 ± 0.3	9.6 ± 0.2	9.6 ± 0.3	2.7	1
10b	16 ± 2	17 ± 4	> 300	> 19	> 18
11a	8 ± 1	3 ± 0.6	> 300	> 37	> 100
11b	103 ± 6	5 ± 0.9	> 300	> 30	> 60
12a	5 ± 1	9.6 ± 0.2	> 300	> 60	> 30
12b	1.9 ± 0.3	4.4 ± 0.8	39 ± 5	20	8.8
14	109 ± 20	> 300	206 ± 15	1.9	< 0.7
<i>ent-14</i>	81 ± 12	109 ± 8	119 ± 16	1.5	1.1
15	25.4 ± 0.2	2.5 ± 0.4	> 300	> 12	> 120

^aIC₅₀ values, which include those of the parent compounds combretastatin A-4 (CoA4) and pironetin, are expressed as the compound concentration (μmol/L or μM) that causes 50% inhibition of cell growth. The values are the average (± s.d.) of three different measurements performed as described in the Material and Methods section. ^b α = IC₅₀ (HEK-293) / IC₅₀ (HT-29).

$\alpha\beta = IC_{50} \text{ (HEK-293)} / IC_{50} \text{ (MCF-7)}$. Values of α and β have been rounded off to a decimal figure. Compounds with both high cytotoxicity towards one or the two tumoral cell lines and low cytotoxicity towards the normal cell line have been highlighted.

5. Summary and conclusions

We have prepared a set of synthetic hybrid molecules containing a combretastatin A-4 moiety and a fragment structurally related to the natural product pironetin. Some of these molecules have been synthesized in both enantiomeric forms. Their cytotoxic action (IC_{50} values) towards a normal (HEK-293) and two tumoral (HT-29 and MCF-7) cell lines has then been measured. While most of the synthetic derivatives proved cytotoxic towards at least one of the two tumoral cell lines, some of them showed cytotoxic activities of the same order as the natural compounds pironetin and combretastatin A-4. More interesting was the fact that, for some of the compounds, the normal vs. tumoral cytotoxicity ratio was markedly higher than in the case of the two aforementioned natural products, that is, they proved comparatively less cytotoxic towards normal cells (these compounds are highlighted in Table 1). This may possibly endow these compounds with pharmacological interest.

6. Experimental

6.1. Chemistry. General procedures

The general reaction conditions and the physical and spectral data of all synthetic intermediates and final compounds are described in detail in the Supporting Information. The samples of compounds used for the biological studies were purified to > 95% by means of preparative HPLC.

6.2. Biological studies. Materials and methods

6.2.1. Reagents and cell culture

Cell culture media were purchased from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was a product of Harlan-Seralab (Belton, U.K.). 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 Thermo Scientific™ BioLite. All tested compounds were dissolved in DMSO at a concentration of 10 $\mu\text{g/mL}$ 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 $\mu\text{g/mL}$) and amphoterycin (1.25 $\mu\text{g/mL}$), supplemented with 10% FBS.

6.2.2. Cytotoxicity assays

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 [28]. 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.

Acknowledgments

Financial support has been granted by the Spanish Government (Ministerio de Economía y Competitividad, projects CTQ2008-02800 and CTQ2011-27560), by the Conselleria d'Empresa, Universitat i Ciència de la Generalitat Valenciana (projects PROMETEO/2013/027 and ACOMP09/113) and by the Universitat Jaume I (projects P1-1B-2008-14 and PI-1B-2011-37). S. T.-G. thanks the Generalitat Valenciana for a predoctoral fellowship of the Santiago Grisolia program. C.V. thanks the Ministerio de Economía y Competitividad for a predoctoral fellowship of the FPI program. J. A. M. thanks the COST Action CM0804 for aid in establishing cooperations with other research groups.

Supplementary Information

Description of general features and reaction conditions. Spectral data and graphical NMR spectra of all new compounds.

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Graphical Abstracts

Design and Synthesis of Pironetin Analogue-Combretastatin A-4 Hybrids and Evaluation of Their Cytotoxic Activity

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