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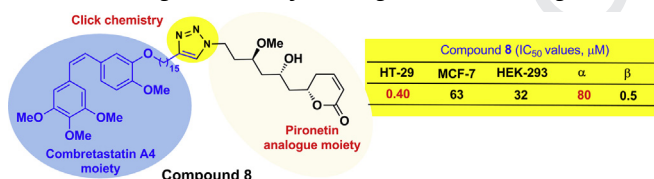
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Graphical Abstract

Design and synthesis of pironetin analogue/combretastatin A-4 hybrids containing a 1,2,3-triazole ring and evaluation of their cytotoxic activity

pp. 1–6

Concepción Vilanova, Sandra Torijano-Gutiérrez, Santiago Díaz-Oltra, Juan Murga*, Eva Falomir, Miguel Carda, J. Alberto Marco*



Highlights

- Hybrid molecules with combretastatin A-4 and pironetin-like moieties were prepared.
- Both moieties are connected by a variable spacer containing a 1,2,3-triazole ring.
- The molecules were tested for cytotoxicity on a normal and two tumoral cell lines.
- Some of the compounds show IC₅₀ values similar to those of combretastatin A-4.
- IC₅₀ values suggest that tubulin binding takes place through the combretastatin end.



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Original article

Design and synthesis of pironetin analogue/combretastatin A-4 hybrids containing a 1,2,3-triazole ring 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 connected through a spacer of variable length which includes a 1,2,3-triazole ring. 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 combretastatin A-4 and were less toxic than the latter compound for normal cells.

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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]. As a matter of fact, many 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, and the main component, 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 dynamic instability [5]. Any molecule which influences microtubule instability will also influence the cell division process, not only of normal cells but also of tumoral cells. Therefore, it is not surprising that tubulin-binding molecules (TBMs) constitute a very 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, the well-known 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].

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-

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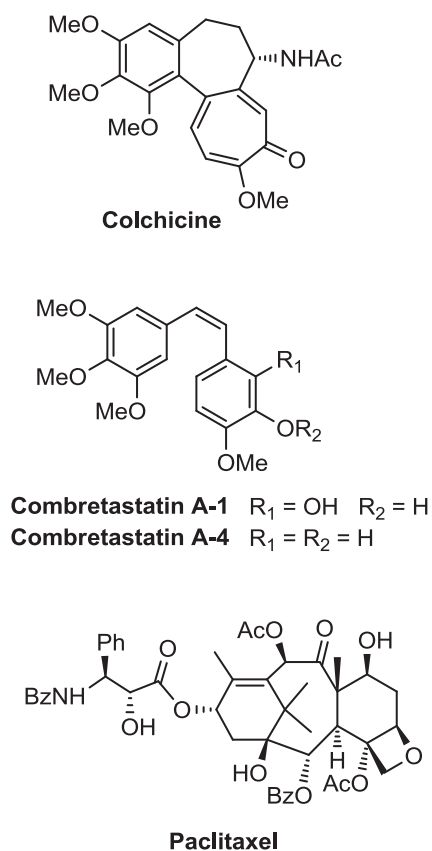


Fig. 1. Structures of some natural products reported to selectively bind to β -tubulin.

like hemisterlin 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].

Some structure–activity relationship (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 might also be relevant. It has been suggested that a nucleophilic residue, yet to be conclusively identified, of the α -tubulin chain adds in a Michael fashion to the conjugated double bond of pironetin [14], therefore forming a covalent bond with C-3 of the dihydropyrene ring [15].

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 [16], resistances have been reported to therapies with various types of anticancer agents [17]. 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 [18]. 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 Fig. 3. The elements that were maintained are the conjugated dihydropyrene 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].

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 observed for 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 either the α - or the β -tubulin subunits [19] 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 compounds with a hybrid structure [20] such as **1–8** (Fig. 4). These molecules display a moiety of combretastatin A-4 (itself numbered as **13** in Fig. 4) and another of the simplified pironetin type (**14**, *ent*-**14** and **15**), connected in turn by a spacer of variable length containing a 1,2,3-triazole ring generated by means of a “click-type reaction” [21]. Such heterocyclic rings are known to exhibit pharmacologically interesting

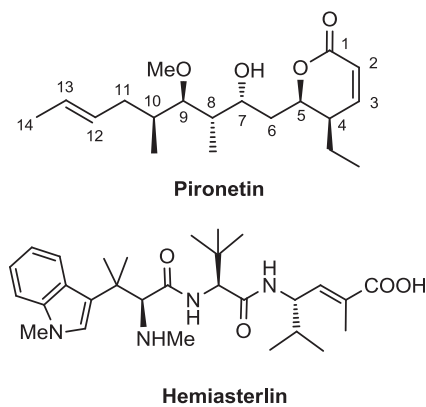


Fig. 2. Structure of two natural products reported to selectively bind to α -tubulin.

features that are beyond a mere connecting role [22] and, for example, may act as peptide isosters [21]. Some of the hybrid

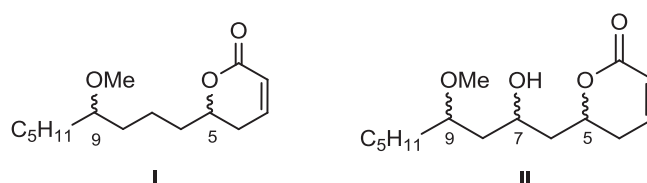


Fig. 3. General structures of simplified pironetin analogues of the first generation [5a].

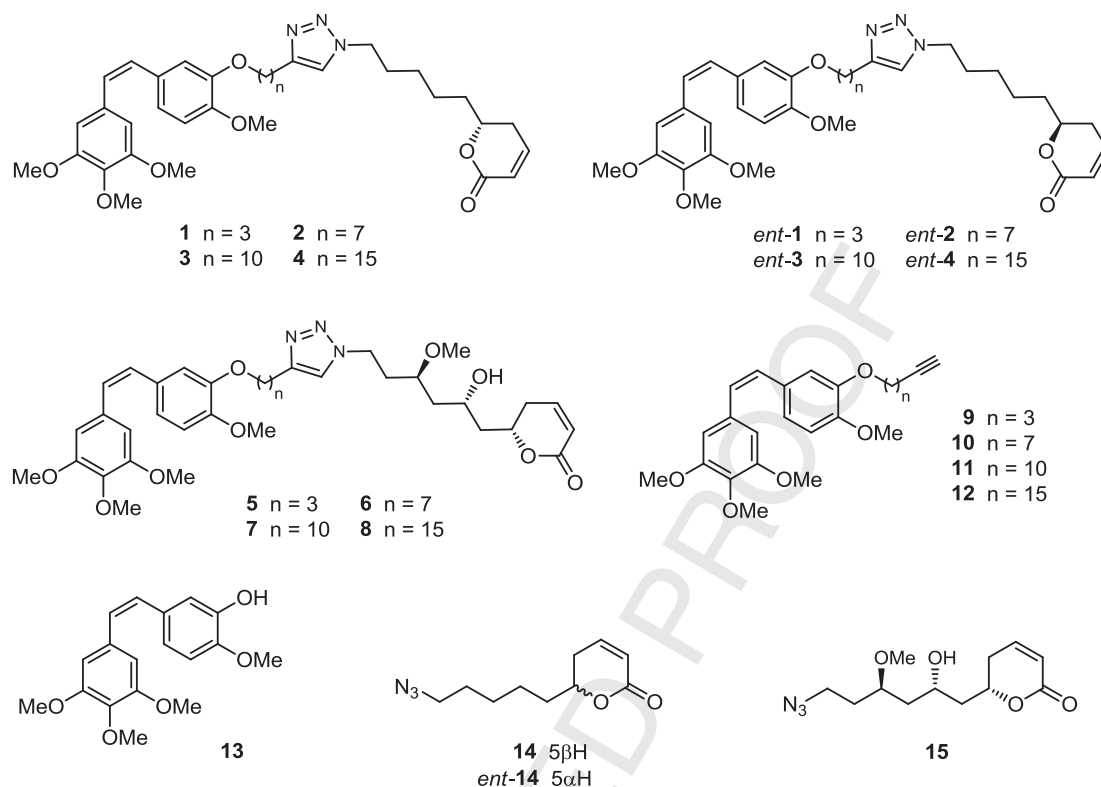


Fig. 4. Structures of the pironetin analogue/combretastatin A-4 hybrids used in this study (1–8) and of their synthetic precursors (9–15), including combretastatin A-4 (13).

molecules (1 to 4 and *ent*-1 to *ent*-4) have been prepared in both antipodal forms [23].

3. Synthetic work

Combretastatin A-4 (13) was prepared according to a literature procedure [24]. Its *O*-alkyl derivatives 9–12 were prepared as depicted in Scheme 1 by means of alkylation with ω -haloalkynes 16–19 (for experimental details, see the Supporting Information).

The synthesis of the pironetin fragments 14, *ent*-14 and 15 was performed as depicted in Scheme 2. Thus, the known pyrones 20, *ent*-20 and 22, prepared according to the general concept used in our previous papers [5a,b], were first tosylated to, respectively, 21, *ent*-21 and 23. Treatment of the latter compounds with sodium

azide in DMF furnished dihydropyrones 14, *ent*-14 and 24. Desilylation of the latter gave 15 [23].

Heating acetylenic compounds 9–12 with the aforementioned dihydropyrones under copper(I) catalysis gave rise to a 1,3-dipolar cycloaddition [21] with regioselective formation of the desired 1,2,3-triazoles 1 to 4, *ent*-1 to *ent*-4 and 5 to 8 (Scheme 3).

After the synthesis of the hybrid molecules was completed, the compounds were investigated in relation to their cytotoxic activity towards two types of tumoral cell lines and one normal cell line.

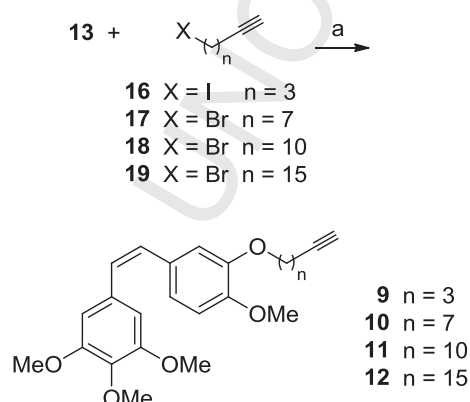
4. Biological work

4.1. Cellular effects of the compounds

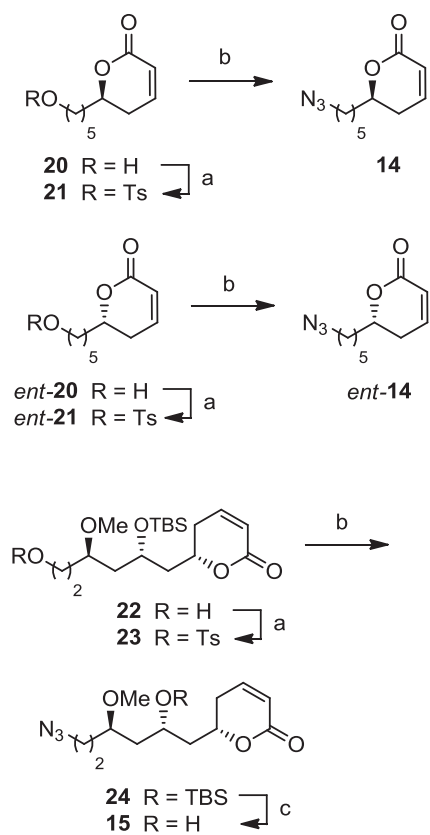
4.1.1. Cytotoxicity values

We carried out a measurement of the cytotoxicity of the synthetic combretastatin A-4 derivatives 1–4 and their respective enantiomers (*ent*-1 to *ent*-4), and 5–8. Cytotoxicity assays were performed as described in the Experimental Section using two types of tumoral cells, the human colon adenocarcinoma HT-29 and the breast adenocarcinoma MCF-7 cell lines. In addition, one normal cell line, the human embryonic kidney cell line (HEK-293) [25] was employed in the assays for comparison. Cytotoxicity values, expressed as the compound concentration ($\mu\text{mol/L}$) that causes 50% inhibition of cell growth (IC_{50}), are shown in Table 1.

The observed values are in the medium to low micromolar range. Compounds 2, 3, *ent*-1, *ent*-4, 6 and most particularly 8 showed the highest cytotoxicities for the HT-29 cell line, with IC_{50} values which were not very different of (in the case of 8 visibly lower than) those of combretastatin A-4 for this particular cell line. In the case of the MCF-7 cell line, the lowest IC_{50} value, not very different of that of combretastatin A-4, was shown by compound 6, followed by *ent*-3. A further aspect also worth mentioning is the



Scheme 1. Synthesis of *O*-alkylated combretastatin A-4 derivatives 9–12: (a) K_2CO_3 , DMF, 24 h, r.t. (yields: 9, 83%; 10, 82%; 11, 83%; 12, 88%). Acronyms and abbreviations: DMF, *N,N*-dimethylformamide.



Scheme 2. Synthesis of azides **14**, **ent-14** and **15**: (a) TsCl, Et₃N, DMAP, CH₂Cl₂, 3–4 h, r.t. (**21**: 83%; **ent-21**: 84%; **23**: 80%); (b) NaN₃, DMF, 3–4 h, r.t. (**14**: 85%; **ent-14**: 85%; **24**: 82%); (c) 48% aq HF, MeCN, 4 h, r.t. (95%). Acronyms and abbreviations: Ts, *p*-toluenesulfonyl; DMAP, 4-(*N,N*-dimethylamino)pyridine; TBS, *t*-butyldimethylsilyl.

fact that some of the synthetic compounds are more toxic for one or the other tumoral cell type than for normal cells, a desirable feature. This can be 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 safety margin of the compound in the corresponding cell line. Thus, combretastatin A-4 itself showed good values of both coefficients, most particularly in the case of the MCF-7 cell line. Pironetin, with IC₅₀ values in the low nanomolar range, showed similar values for the HT-29 and the MCF-7 cell lines. Among the compounds with an appreciable cytotoxicity (low IC₅₀ values), **ent-1**, **6** and **8** are worth mentioning as they exhibit good α values, with that of **8** being particularly high. Compound **6** also shows a good β value.

Some aspects related to the relation between the structures and the observed cytotoxicity deserve comment. In the case of two subsets of hybrid molecules [20] where there are differences between in the carbon chain length (e.g. **1–4** and **5–8**), differences in the degree of cytotoxicity are also observed but no obvious relation between both features are perceived. Thus, for compounds **1–4**, which display a very simplified pironetin moiety, the lowest IC₅₀ values are found for the two compounds that have the intermediate values of the spacer carbon chain length (**2**, $n = 7$ and **3**, $n = 10$). As for the compounds of the enantiomeric series (**ent-1** to **ent-4**), their cytotoxicities are not very different from those of **1–4**. However, for compounds **5–8**, which display a more complex pironetin part, the lowest IC₅₀ value is clearly that of the compound with the longest spacer carbon chain (**8**, $n = 15$). 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) or else at the combretastatin A-4 site (β -tubulin). The fact that the IC₅₀ values of the hybrid molecules are much more similar to those of combretastatin A-4 than to those of pironetin suggests that these compounds are possibly interacting with tubulin through the combretastatin A-4 site at β -tubulin. If this is true, one would conclude that the reversible non-covalent interaction at the β -tubulin site is kinetically faster than the covalent irreversible union at the α -tubulin site. More research in this direction will be necessary in order to definitively clarify this and other issues, such as the possible influence of the triazole fragment [26].

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. The two structural moieties have been connected through a spacer of variable length containing a 1,2,3-triazole ring, generated in turn by means of a 1,3-dipolar cycloaddition (“click reaction”). Some of these molecules have been synthesized in both enantiomeric forms. Their cytotoxic action (IC₅₀ values) towards a normal (HEK-293) and two tumoral (HT-29 and MCF-7) cell lines has then been measured. Several of the synthetic derivatives proved cytotoxic towards at least one of the two tumoral cell lines, with some of them showing cytotoxic activities of the same order as combretastatin A-4. For some compound/cell line combinations, most particularly **8** and the HT-29 cell line, the normal vs. tumoral cytotoxicity ratio was higher than in the case of the aforementioned natural product, that is, they proved comparatively much less cytotoxic towards normal cells (these compounds are highlighted in Table 1). This feature may potentially show pharmacological interest.

6. Experimental

6.1. Chemistry

6.1.1. 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

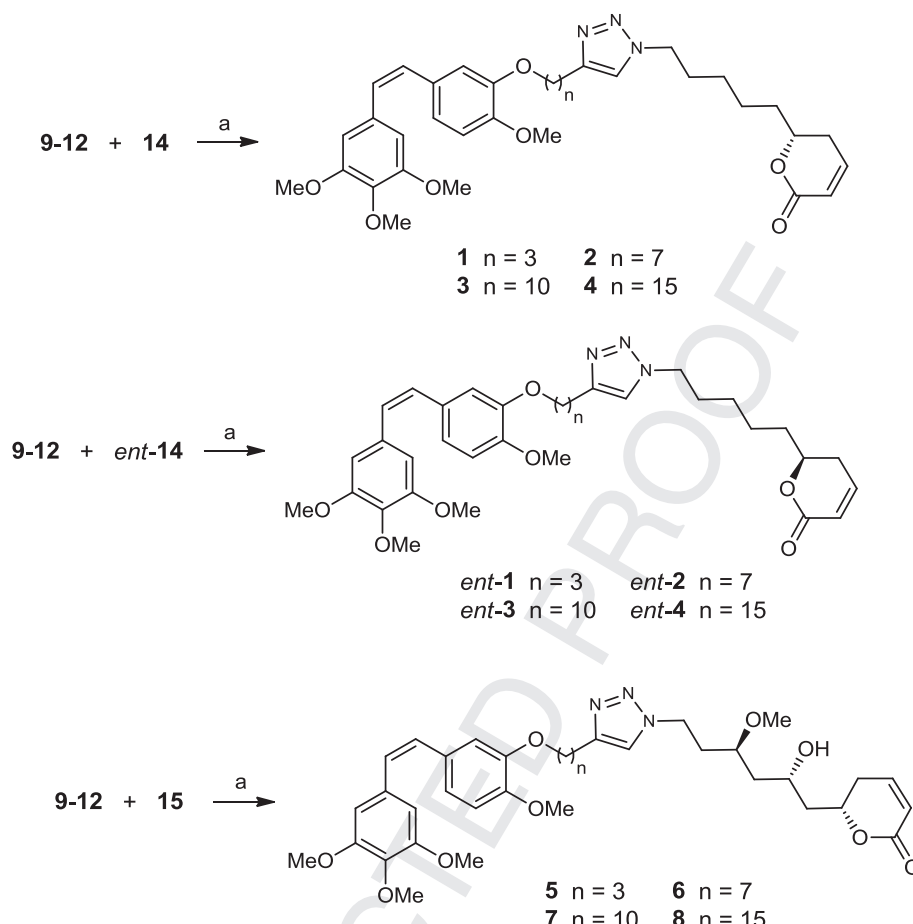
6.2.1. Materials and methods

6.2.1.1. 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 μ 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 μ g/mL) and amphoterycin (1.25 μ g/mL), supplemented with 10% FBS.

6.2.1.2. 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 [27].



Scheme 3. Synthesis of hybrid molecules **1** to **4**, *ent-1* to *ent-4*, and **5** to **8**: (a) THF/MeOH, r.t., then addition of Cu powder and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, r.t., 3–4 h (**1**: 46%; **2**: 51%; **3**: 51%; **4**: 52%; *ent-1*: 50%; *ent-2*: 55%; *ent-3*: 58%; *ent-4*: 52%; **5**: 55%; **6**: 58%; **7**: 53%; **8**: 55%).

Some 5×10^3 cells of HT-29, MCF-7 or HEK-293 cells in a total

Table 1
Cytotoxicity of pironetin analogue/combretastatin A-4 hybrids **1–8** and *ent-1* to *ent-4*.^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	0.0064 ± 0.0007	0.006 ± 0.002	0.017 ± 0.001	2.6	2.8
1	40 ± 2	40 ± 0.3	1.7 ± 0.3	0.04	<0.1
2	8 ± 1	39 ± 3	9.3 ± 0.2	1.2	<0.1
3	9 ± 1	29 ± 6	4.5 ± 0.3	0.5	0.2
4	22 ± 3	66 ± 4	16 ± 1	0.7	0.2
<i>ent-1</i>	9.3 ± 0.8	20 ± 5	34 ± 10	3.7	1.7
<i>ent-2</i>	11 ± 1	32 ± 4	40 ± 1	3.6	1.2
<i>ent-3</i>	17.5 ± 0.1	13 ± 3	13 ± 3	0.7	1
<i>ent-4</i>	8 ± 3	37 ± 3	2.6 ± 1.3	0.3	<0.1
5	24.0 ± 0.2	23 ± 6	30 ± 5	1.2	1.3
6	8 ± 1	7 ± 2	21 ± 4	2.6	3
7	17.3 ± 0.4	20 ± 3	40 ± 5	2.3	2
8	0.40 ± 0.05	63 ± 2	32 ± 10	80	0.5

^a IC_{50} values, which include those of the parent compounds combretastatin A-4 (CoA4) and pironetin, are expressed as the compound concentration ($\mu\text{mol/L}$ or μM) that causes 50% inhibition of cell growth. The values are the average (\pm s.d.) of three different measurements performed as described in the Material and Methods section.

^b $\alpha = \text{IC}_{50}(\text{HEK-293})/\text{IC}_{50}(\text{HT-29})$.

^c $\beta = \text{IC}_{50}(\text{HEK-293})/\text{IC}_{50}(\text{MCF-7})$. Values of α and β have been rounded off to a decimal figure. Compounds with both high cytotoxicity towards one tumoral cell line and comparatively low cytotoxicity towards the normal cell line have been highlighted in boldface italics.

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.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejmech.2014.09.053>.

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