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Novel benzopsoralen analogues: Synthesis, biological activity and molecular docking studies

Carla S. Francisco [a], Lígia R. Rodrigues [b], Nuno M.F.S.A. Cerqueira [c], Ana M.F. Oliveira-Campos [a], Ana P. Esteves [a, *]

[a] Chemistry Centre, School of Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
[b] CEB - Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
[c] REQUIMTE, Faculty of Sciences, University of Porto, Rua do Campo Alegre s/n, 4169-007 Porto, Portugal

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ABSTRACT

New benzopsoralen analogues were synthesized and their inhibitory effect on the growth of tumour-tumour cell lines (MDA MB231 and TCC-SUP) was evaluated. The *in vitro* antitumour activity of the new benzopsoralen analogues was discussed in terms of structure–activity relationship. Molecular docking studies with human-CYP2A6 enzymes were also carried out with the synthesized compounds to evaluate the potential of these molecules to interact with the haem group of the enzymes. The results demonstrated that the compounds that are able to interact with the iron ion of the haem cofactor and at the same time with active site Asn297 are those that have better anti-proliferative activity.

1. Introduction

Psoralens are naturally occurring furocoumarins present in many plant families such as *Apicaceae* and *Umbelliferae* [1]. Furocoumarins extracted from plants are commonly used as food additives, in phytomedicine and as cosmetics [2]. Additionally, Psoralen and 8-methoxypsoralen (8-MOP, named Xanthotoxin) are used for the treatment of Psoriasis in combination with UVA irradiation (PUVA therapy) [3]. It is important to notice that these compounds have also been reported to have an anti-cancer effect independently of its photoactivation [4,5]. It has been reported that Bergapten (5-methoxypsoralen) enhances p53 gene expression and induces apoptosis in human breast cancer cells [6].

Psoralens have been developed as pharmaceuticals for a wide range of disorders (vitiligo, psoriasis, skin cancers) that require cell division inhibitors [7] and are known to interrupt drug metabolism due to their ability to competitive and/or mechanistically inhibit a variety of human cytochrome P450 enzymes, including CYP1A2, CYP2A6 and CYP3A4/5 [8,9]. Xanthotoxin, Bergapten and Psoralen have been reported to suicide-inactivate the human CYP2A6 (enzyme involved in the coumarin metabolism) [10,11] and they contribute to the programmed cancer cell death and increased sensitivity to chemotherapy [10,11].

Some psoralens, e.g. Bergapten, have been demonstrated to exert an anti-cancer effect against different cancers, independently of its photoactivation [6]. For instance, Lee et al. [12] reported the chemopreventive role of Bergapten in a human hepatocellular carcinoma. The authors propose that there are at least three modes of suppressive effects shown by Bergapten, namely killing the cells directly; inducing apoptosis by arresting cells at the G2/M phase in the cell cycle; and inducing apoptosis through an independent pathway with cell cycle arrest at a given exposure time. It is also pertinent to mention that structurally related Bergapten exerts its anticarcinogenic properties by a cytotoxic effect, inducing apoptosis and inhibiting cell proliferation. In the current work, no mechanistic studies have been included, therefore only considerations regarding cytotoxicity and a potential effect on cell proliferation can be advanced.

The human CYP2A6 protein is involved in the metabolism of coumarins [13] and is known to be overexpressed in several cancer cells [14,15], including breast and bladder cancers. Therefore, it is an adequate model for molecular docking studies of the compounds herein synthesized. Previously, we reported that these compounds interact very closely with the iron ion of the haem group of the enzyme [4,5]. Ye and Zhang [16] demonstrated that the depletion of iron (haem deficiency) caused the apoptosis of HeLa cells, which

* Corresponding author.
E-mail address: aesteves@quimica.uminho.pt (A.P. Esteves).

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involved the release of cytochrome c and the activation of caspase 3 (involved in the programmed cell death). It appears that haem deficiency inhibits cell growth by selectively interfering with the progression of the S phase of the cell cycle. Indeed, the haem altered metabolism has been associated with numerous diseases, including cancer.

Earlier in our group Psoralen derivatives were synthesized and their anti-proliferative effects on different human tumour cell lines were demonstrated [4,5]. The molecular docking results of these compounds with the CYP2A6 enzyme showed that, in general, the compounds carrying few bulky groups attached to the coumarin moiety adopted a conformation that allows the carbonyl group of the coumarin moiety to interact very closely with the iron ion of the haem cluster. Interestingly, these compounds were found to have a better ability to inhibit the proliferation of the three cell lines studied (MDA MB231, HeLa, TCC-SUP).

In the current work, two angular benzopsoralen analogues derived from 4-hydroxydibenzothiophene **1** were synthesized (compounds **2** and **3**, Scheme 1) and their biological activities were evaluated. Other two linear benzopsoralen analogues derived from 2-hydroxydibenzofuran and 2-hydroxycarbazole (compounds **4** and **5**, Scheme 2) were also synthesized and evaluated. One compound devoid of a coumarin moiety, (*E*)-ethyl 3-(3-hydroxy-9*H*-carbazol-9-yl)acrylate (**10**) (Scheme 3), was also obtained and tested.

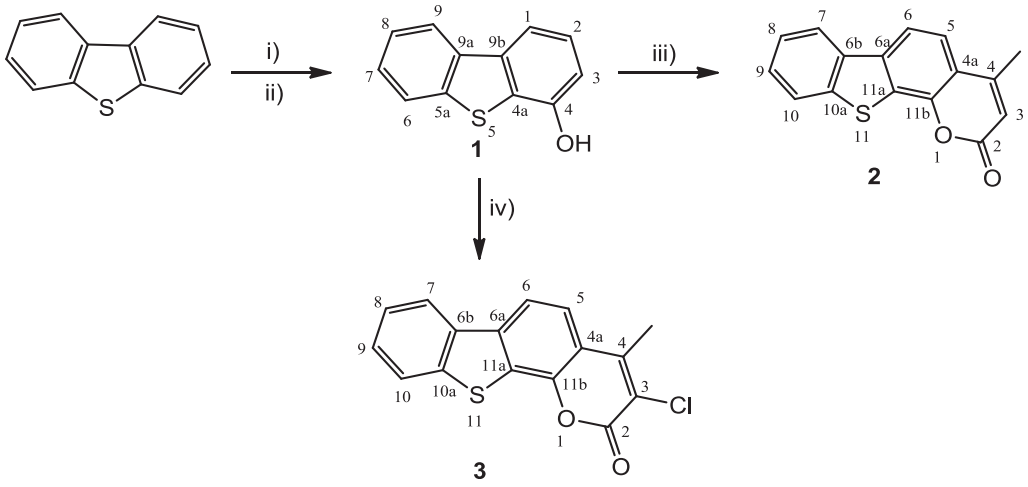
2. Results and discussion

2.1. Chemistry

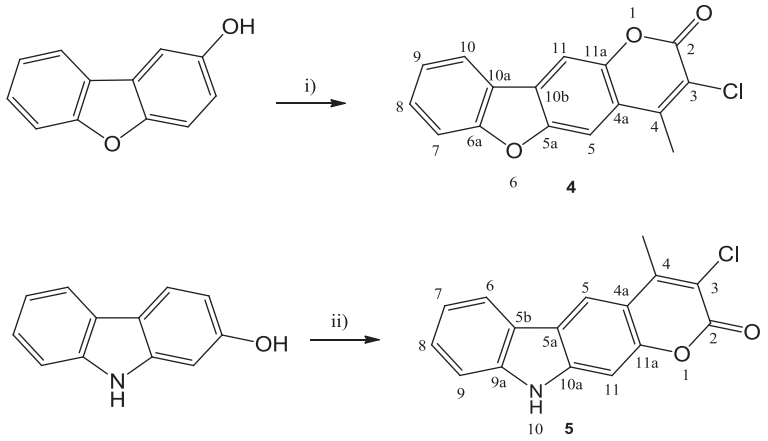
Benzopsoralen derivatives with sulphur nucleus were obtained by Pechmann reaction using 4-hydroxydibenzothiophene **1** as the precursor. Compound **1** was prepared by the reaction of dibenzothiophene (commercial) with TMEDA, $B(OBu)_3$ and *n*-BuLi in the presence of 30% H_2O_2, in dry diethyl ether [17]. Although a good resolution of the multiplicity of signals was not achieved, in the ^1H NMR spectrum it was possible to identify the signal corresponding to the hydroxyl group at δ 9.95 ppm and also the other seven aromatic proton signals (δ 7.02–8.28 ppm). By Pechmann reaction of 4-hydroxydibenzothiophene **1** with ethyl acetoacetate and ethyl 2-chloroacetoacetate in the presence of concentrated H_2SO_4 compounds **2** and **3** were obtained in 21 and 53% yields, respectively (Scheme 1). The NMR data for compound **2** are consistent with the proposed structure, namely the presence of two doublets at 8.05 and 7.68 ppm, J = 8.4 Hz belonging to protons H-5 and H-6, respectively. The methyl group appears as a doublet at 2.56 ppm due to the long range coupling with H-3 and therefore the signal of H-3 is as an apparent doublet 6.37 ppm. For compound **3** ^1H NMR spectrum showed the signals of protons H-5 and H-6 as doublets at 8.08 (J = 8.4 Hz) and 7.69 ppm (J = 8.7 Hz), respectively, a singlet at 2.70 for the methyl group and absence of H-3 signal, that confirms the formation of the product.

The benzopsoralen analogues **4** and **5** were also prepared under Pechmann conditions (Scheme 2). The compound **4** was obtained, in 14% yield, from 2-hydroxydibenzofuran and ethyl 2-chloroacetoacetate. Its formation was confirmed by analysis of the ^1H NMR spectrum that showed the presence of singlets at 7.89 (H-11), 7.79 (H-5) and 2.70 (CH_3) ppm. The reaction of 2-hydroxycarbazole with ethyl 2-chloroacetoacetate afforded compound **5** in 16% yield. By analysis of the ^1H NMR spectrum the presence of two singlets at 8.59 and 7.38 ppm for the protons H-5 and H-11, respectively, and a singlet at 2.67 (CH_3) was observed.

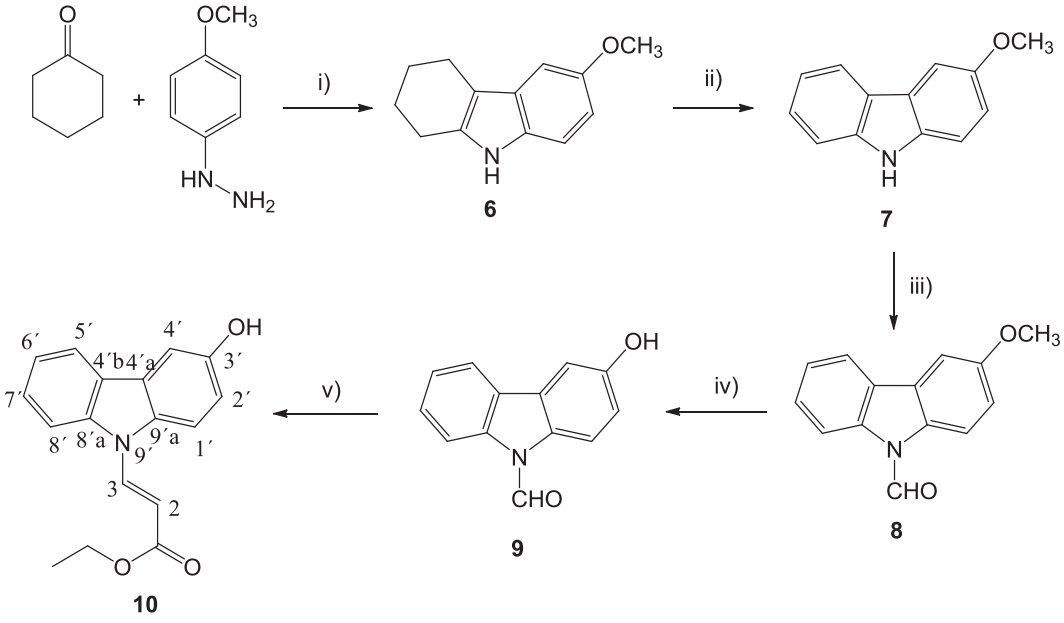
Compound **6** was prepared by reaction of cyclohexanone with 4-methoxyphenyl hydrazine according to the method described by Rogers and Corson [18], in a yield of 71% (Scheme 3). ^1H NMR spectrum showed a singlet at δ 7.56 corresponding to –NH and a doublet at 6.93 ppm J = 2.4 Hz corresponding to H-4 and the expected signals for the aliphatic protons. Dehydrogenation reaction of compound **6** in *p*-cymene/water and in the presence of 10% Pd/C, under reflux, afforded the carbazole **7** in 53% yield (Scheme 3). The introduction of the –CHO group at 4-position of carbazole **7** was attempted under the conditions of Vilsmeier-Haack formylation ($POCl_3$ in dry DMF) and a solid was obtained in 76% yield. However, after analysis of the ^1H NMR spectrum the absence of the signal corresponding to the –NH and the presence of a singlet at 9.80 ppm, led to the conclusion that *N*-formylation had occurred with formation of compound **8** (Scheme 3). Demethylation of **8** with a 1 M solution of BBr_3 in CH_2Cl_2 under nitrogen atmosphere gave compound **9**, in 48% yield after purification by column chromatography (Scheme 3). In the proton NMR spectrum the disappearance of the OCH_3 signal was observed together with the presence of a singlet at δ 9.60 ppm (OH group). The alkene **10** was prepared by Wittig reaction between **9** and $Ph_3PCHCOOEt$ (in *N*,*N*-diethylaniline, 15 h, reflux) (Scheme 3). The proton NMR spectrum of the product showed two doublets at 8.54 (H-3) and 6.32 (H-2)



Scheme 1. Reagents and conditions: i) TMEDA, *n*-BuLi, rt → reflux, 1 h, dry ether; ii) $B(OBu)_3$, 30% H_2O_2, reflux, 90 min; iii) ethyl acetoacetate, conc. H_2SO_4, 2 h, rt; iv) ethyl 2-chloroacetoacetate, conc. H_2SO_4, 8 h, rt.



Scheme 2. Reagents and conditions: i) ethyl 2-chloroacetoacetate, 80% H_2SO_4, 4 days, rt; ii) ethyl 2-chloroacetoacetate, 80% H_2SO_4, 48 h, rt.



Scheme 3. Reagents and conditions: i) acetic acid, reflux, 4.5 h; ii) *p*-cymene, H_2O, 10% Pd/C, reflux, 48 h; iii) dry DMF, dry CH_2Cl_2, $POCl_3$, reflux, 3 h; iv) dry CH_2Cl_2, BBr_3 in CH_2Cl_2, N_2 atmosphere, 24 h, rt; v) *N*,*N*′-diethylaniline, carbethoxymethylenetriphenylphosphorane, reflux, 15 h.

ppm both with $J = 14$ Hz, typical of a *trans* alkene, confirming the formation of the product **10**.

2.2. Anti-proliferative effect on human cancer cell lines

The ability of the compounds **2**–**5** and **10** to inhibit the *in vitro* growth of MDA MB231 and TCC-SUP cell lines was evaluated. The results, given in concentrations that were able to cause 50% of cell growth inhibition (GI_{50}), are summarized in Table 1.

Table 1
Anti-proliferative effect of the studied compounds on human cancer cell lines.

Compound	Inhibition of cancer cell lines GI_{50} (μM)	
	MDA MB231	TCC-SUP
2	0.049 ± 0.002	0.253 ± 0.022
3	0.460 ± 0.038	0.311 ± 0.031
4	0.082 ± 0.003	0.243 ± 0.027
5	0.421 ± 0.031	0.149 ± 0.013
10	0.198 ± 0.017	0.025 ± 0.008

A high anti-proliferative activity was observed for all the compounds even at the minimum concentration tested (0.5 μM). Compound **2** was found to be the most active against MDA MB231, while for TCC-SUP cells the compound **10** showed a higher anti-proliferative effect. Furthermore, the compound **4** showed better activity for the MDA MB231 cell line than compounds **3** and **5**, although for the TCC-SUP cell line it gave values on the same range as the compounds **3** and **5**. The results herein obtained are in agreement with our previous studies using benzopsoralen analogues [4,5].

As previously mentioned, it is expected that a compound that interacts with the haem group of CYP2A6 will have an effect on the cell proliferation and apoptosis, which can be the mechanism by which psoralens exerted the observed anti-proliferative activity. Some differences could be observed between the two different cell lines (Table 1), namely in what regards the most active compound. These differences could be due to distinct CYP2A6 expression

levels, presence of polymorphisms in the CYP2A6 gene, but also different responsiveness to hormones (e.g. oestrogen) [19–22].

2.3. Molecular docking

Given that psoralens have been shown to be potent inhibitors of several enzymes from the cytochrome P450 superfamily, in the current work molecular docking methodologies were used to predict the binding pose between each of these ligands and the enzyme CYP2A6. The enzyme CYP2A6 was chosen since it is one of the 57 CYP isoenzymes found in humans [23] and is involved in the metabolism of several pharmaceuticals, carcinogens, and a number of coumarin-type alkaloids [24,25].

The co-crystallized X-ray structure of Xanthotoxin in CYP2A6 (PDB code 1Z11) reveals that the substrate fits very well in the narrow binding site. The active site is formed by a cluster of phenylalanine residues (Phe107, Phe111, Phe108, Phe209 and Phe480) that line the "roof" of the active site and by the presence of a single polar residue, Asn297 (Fig. 1). It is suggested that Asn297 influences substrate orientation, and metabolism, and therefore might be critical for substrate recognition and binding. Indeed, mutations of this residue often lead to dramatic changes in the kinetics of the reaction that is catalysed by this enzyme.

The molecular docking results show that all the studied compounds bind in the same region of the protein nearby the haem cofactor (Fig. 1). Similarly to what was found in our previous molecular docking studies [4,5], the C=O group of compounds **2** and **5** binds very near to the iron ion of the haem cofactor (2.5 Å and 3.3 Å). The pyrrole and the thiophene rings point towards the amino group of Asn297 (3.2 Å and 5.4 Å). Compounds **3** and **2** are very similar; the only difference between them is the presence of a chlorine atom bound to position 3 of the coumarin moiety. Interestingly, the sulphur atom of thiophene ring of compound **3** no longer interacts with the amino group of Asn297, neither the C=O group interacts directly with the iron ion of the haem cofactor. The methyl group and the chlorine atom interact now with the iron ion (2.2 Å) and the thiophene ring is pointing towards the opposite direction of Asn297. A similar trend is observed in compound **4**. The chlorine atom linked to position 3 of the coumarin moiety causes the methyl group to be close to the iron ion of the cofactor (3.0 Å) instead of the C=O group. In this case the benzofuran is still in close contact with Asn297 (2.8 Å). This is possible only because the new orientation of the molecule did not preclude this sort of interaction, something that was not observed in compound **3**. Product **10** has a different scaffold from all the other compounds. However, the bulky ester group attached to the carbazole ring interacts very closely to the iron ion of the haem cofactor (2.5 Å). The hydroxyl group that is attached to the carbazole moiety points towards Asn297 (3.4 Å). This type of binding pose is in line with compounds **2** and **5**.

Based on the measured anti-proliferative activity data and the best scored solutions obtained from the molecular docking protocol, it can be concluded that the inhibitory activity of the compounds is dependent on their ability to interact with the iron ion of the cofactor and with Asn297. This result goes in line with our previous results and is observed with compound **2** and **10** [4,5]. Compound **5** has a similar binding pose to these compounds but it has poor anti-proliferative activity. The molecular docking results suggest that this might be related with the poor interaction that this compound has with Asn297 which causes the binding pose to be less specific.

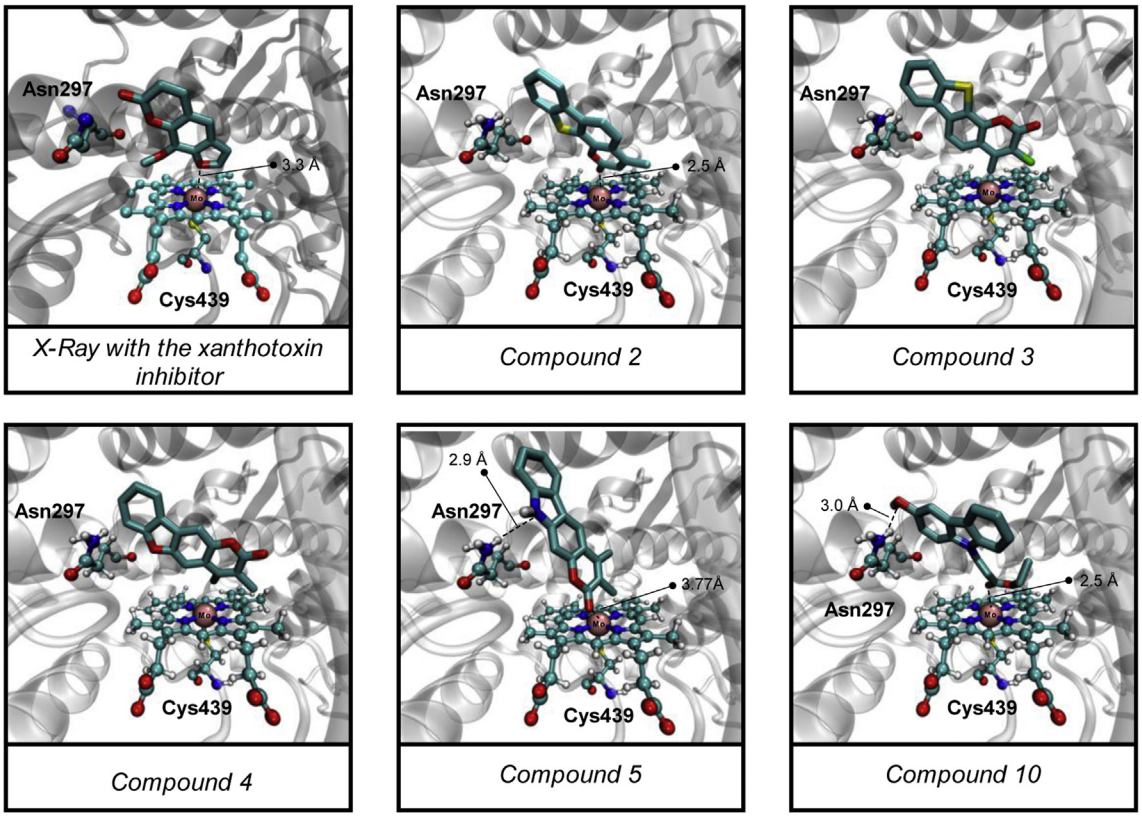


Fig. 1. Molecular docking results for compounds **2**–**5** and **10**. The substrates are represented in bonds, the haem, Asn297 and Cys439 in ball and stick, and the protein in cartoon. The X-Ray structure containing the Xanthotoxin inhibitor has the PDB code 1Z11.

Compound **4** also shows an interesting anti-proliferative activity and similar to compounds **2** and **10**. This compound forms a hydrogen bond with Asn297 through the oxygen atom of the benzofuran ring, and interacts with the iron ion of the haem cofactor through the methyl group and the chlorine atom. This sort of interaction is different to what is observed in compound **2** and **10** but based on the anti-proliferative activity data, endorses a similar inhibitory effect. Compound **3** has a similar binding pose to compound **4**, but shows poor anti-proliferative activity. The molecular docking results suggest that the reason behind this trend is related with the lack of the interaction of this compound with Asn297 that might induce a less specific binding pose.

3. Conclusions

Four new Benzopsoralen analogues were synthesized and their biological activities were tested. All of them significantly inhibited the proliferation of two human tumour cell lines, which we proposed to be mainly linked with the inhibition of CYP2A6.

The molecular docking results revealed that all the compounds interact with the ferryl haem of CYP2A6 and this might be one of the main causes that preclude the observed tumour cell proliferation. Comparing the anti-proliferative activity results of each compound and the best scored solutions obtained from the molecular docking protocol, it may be concluded that the compounds that are able to interact with the iron ion of the haem cofactor and at the same time with the active site Asn297 are those that have better anti-proliferative activity. Comparing our previous results with those herein obtained, it becomes evident that the compounds that do not interact with Asn297 have systematically lower anti-proliferative activities. This may be related with the lack of specificity in the binding pose of those compounds in the active site of CYP2A6 that precludes an efficient inhibitory activity.

4. Experimental

4.1. Chemistry

Melting points were determined on a *Gallenkamp* melting point apparatus and are uncorrected. ^{1}H NMR (300 MHz) and ^{13}C NMR (75.4 MHz) spectra were recorded on a *Varian Unity Plus* Spectrometer at 298 K or on a *Bruker Avance III 400* spectrometer (400 MHz for ^{1}H and 100.6 MHz for ^{13}C). Chemical shifts are reported in ppm relative to solvent peak or TMS; coupling constants *J* are given in Hz. Double resonance, HMQC (heteronuclear multiple quantum coherence) and HMBC (heteronuclear multiple bond correlation) experiments were carried out for complete assignment of ^{1}H and ^{13}C signals in the NMR spectra. High-resolution mass spectra (ESI-TOF) were obtained on a *Bruker FTMS APEXIII* spectrometer. Elemental analyses were performed on a *Leco CHNS-932* instrument. TLC was carried out on plates coated with silica gel 60 F_{254}. Column chromatography was performed on silica gel (70–230 or 230–400 mesh). Light petroleum refers to the fraction boiling in the range 40–60 °C.

4.1.1. *Dibenzo*[b,d]*thiophen-4-ol* (***1***)

To a solution of dibenzothiophene (2.50 g, 13.7 mmol) and TMEDA (2.5 mL, 16.7 mmol) in dry diethyl ether (35 mL), under stirring and nitrogen atmosphere, 2.5 M *n*-BuLi in hexane (5.9 mL, 14.8 mmol) was added dropwise at room temperature. The reaction mixture was refluxed for 1 h. Then it was cooled to 0 °C and tributylborate (4.0 mL, 14.8 mmol) was added, maintaining 0 °C for 40 min and at room temperature for 1 h. After cooling to 0 °C, 30% H_2O_2 (5.0 mL) was added dropwise under vigorous stirring and the mixture refluxed for 90 min. After cooling, 5 M HCl (5.0 mL) was added and the layers were separated. The organic layer was washed with a cold 10% solution of ammonium and iron(II) sulphate (2 × 50 mL) and then extracted with 2 M NaOH solution (2 × 100 mL), dried ($MgSO_4$), filtered and evaporated to dryness to give a white solid which was identified as dibenzothiophene. The aqueous phase was acidified with 5 M HCl until precipitation of a beige solid which was separated by filtration and dried and identified as the dibenzo[*b*,*d*]thiophen-4-ol (**1**). Yield: 0.860 g (0.21 mmol, 31%). mp 146–147 °C (Lit. mp 157–159 °C) [26]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.28 (d, 1H, J = 9.2 Hz, H-9), 8.00 (d, 1H, J = 8.8 Hz, H-1), 7.84 (d, 1H, J = 8.0 Hz, H-6), 7.52 (m, 2H, H-7 and H-8), 7.38 (t, 1H, J = 7.6 Hz, H-2), 7.03 (d, 1H, J = 8.0 Hz, H-3) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ 153.06 (C-4), 140.38 (C-9a), 138.38 (C-5a and C-4a), 136.91 (C-9b), 127.64 (C-7 or C-8), 126.68 (C-2), 125.25 (C-7 or C-8), 123.78 (C-1), 122.81 (C-9), 114.01 (C-6), 112,13 (C-3) ppm.

4.1.2. *4-Methyl-2*H-*benzo[4,5]thieno[3,2-*h*]chromen-2-one* (***2***)

A mixture of dibenzo[*b*,*d*]thiophen-4-ol **1** (0.200 g, 1.0 mmol), ethyl acetoacetate (0.25 mL, 2.0 mmol) and chilled concentrated H_2SO_4 (1.5 mL) was stirred at room temperature for 2 h. After cooling the mixture was poured over crushed ice and the beige solid formed was separated by filtration and dried to give compound **2**. Yield: 0.055 g (0.21 mmol, 21%). mp 204–207 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.21 (dd, 1H, J = 6.9 and 2.4 Hz, H-7), 8.05 (d, 1H, J = 8.4 Hz, H-5), 7.95 (dd, 1H, J = 6.6 and 2.4 Hz, H-10), 7.68 (d, 1H, J = 8.4 Hz, H-6), 7.57–7.52 (m, 2H, H-8 and H-9), 6.37 (app d, 1H, J = 1.2 Hz, H-3), 2.56 (d, 3H, J = 1.2 Hz, CH_3) ppm. ^{13}C NMR (100.6 MHz, $CDCl_3$): δ 160.09 (C=O), 153.12 (C-4), 149.22 (C-6a), 140.91 (C-6b), 139.26 (C-11b), 134.83 (C-10a and C-11a), 127.91 (C-8), 127.01 (C-4a), 124.90 (C-9), 123.26 (C-10), 122.32 (C-7), 120.81 (C-6), 117.22 (C-5), 114.41 (C-3), 19.19 (CH_3) ppm. HRMS (ESI-TOF) calcd for $C_{16}H_{10}O_2S$ 266.04015; found 267.04757 (MH^+).

4.1.3. *3-Chloro-4-methyl-2*H-*benzo[4,5]thieno[3,2-*h*]chromen-2-one* (***3***)

A solution of ethyl 2-chloroacetoacetate (0.28 mL, 2.0 mmol) and dibenzo[*b*,*d*]thiophen-4-ol **1** (0.200 g, 1.0 mmol) was slowly added to chilled concentrated H_2SO_4 (1.5 mL) and stirred at room temperature for 8 h. The mixture was poured over crushed ice and the beige solid formed was separated by filtration and dried. Yield: 0.16 g (0.53 mmol, 53%). mp 239–245 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.21 (dd, 1H, J = 6.6 and 2.4 Hz, H-7), 8.08 (d, 1H, J = 8.4 Hz, H-5), 7.96 (dd, 1H, J = 6.6 and 1.8 Hz, H-10), 7.69 (d, 1H, J = 8.7 Hz, H-6), 7.60–7.51 (m, 2H, H-8 and H-9), 2.70 (s, 3H, CH_3) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ 155.02 (C=O), 148.80 (C-4), 146.17 (C-11b), 139.35 (C-6a), 138.46 (C-4a), 134.12 (C-11a), 127.94 (C-8 or C-9), 125.04 (C-8 or C-9), 124.96 (C-10a), 123.04 (C-10), 122.63 (C-7), 122.08 (C-6), 118.85 (C-3), 117.91 (C-5), 116.70 (C-6b), 16.09 (CH_3) ppm. HRMS (ESI-TOF) calcd for $C_{16}H_9O_2SCl$ 300.00118; found 301.00924 (MH^+).

4.1.4. *3-Chloro-4-methyl-2*H-*benzofuro[2,3-g]chromen-2-one* (***4***)

To a mixture of 2-hydroxydibenzofuran (1.02 g, 5.43 mmol) and ethyl 2-chloroacetoacetate (1.14 mL, 8.19 mmol), 80% H_2SO_4 (2.50 mL) was added and the mixture was stirred at room temperature for 4 days. The mixture was poured onto ice, the precipitated brown solid was filtered and recrystallized from hot ethanol, affording a beige solid that was separated by filtration and dried. Yield: 0.217 g (0.16 mmol, 14%). mp 261–262 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.02 (d, 1H, J = 7.2 Hz, H-10), 7.89 (s, 1H, H-11), 7.79 (s, 1H, H-5), 7.65–7.53 (m, 2H, H-7 and H-8), 7.42 (dt, 1H, J = 1.8 and 6.9 Hz, H-9), 2.70 (s, 3H, CH_3) ppm. ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 157.88 (C=O and C-6a), 152.72 (C-5a), 147.58 (C-4 and C-11a), 129.34 (C-8), 127.73 (C-10b), 123.41 (C-9), 123.04 (C-10a), 121.73 (C-

10), 120.91 (C-3), 118.86 (C-4a), 112.03 (C-7), 108.25 (C-11), 106.56 (C-5), 16.66 (CH_3) ppm. HRMS (ESI-TOF) calcd for $C_{16}H_9ClO_3$ 284.02402; found 285.0250 (MH^+).

*4.1.5. 3-Chloro-4-methylpyrano[2,3-b]carbazole-2(10H)-one (**5**)*

To a mixture of 2-hydroxycarbazole (1.0 g, 5.43 mmol) and ethyl 2-chloroacetoacetate (1.14 mL, 8.19 mmol), 80% H_2SO_4 (2.50 mL) was added and the mixture was stirred at room temperature for 48 h. The mixture was poured onto ice, the brown precipitate was separated by filtration, washed with cold water and recrystallized from EtOAc/light petroleum. The yellow solid formed was separated by filtration and dried. Yield: 0.254 g (0.90 mmol, 16%). mp 247–250 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.66 (s, 1H, NH), 8.59 (s, 1H, H-5), 8.23 (d, 1H, J = 7.8 Hz, H-6), 7.50 (dd, 1H, J = 8.1 and 1.2 Hz, H-9), 7.43 (dt, 1H, J = 1.2 e 7.7 Hz, H-8), 7.38 (s, 1H, H-11), 7.23 (dt, 1H, J = 1.2 and 7.5 Hz, H-7), 2.67 (s, 3H, CH_3) ppm. ^{13}C NMR (75.4 MHz, DMSO-d_6): δ 156.66 (C=O), 150.00 (C-10a or C-11a), 149.81 (C-10a or C-11a), 141.81 (C-5a), 140.95 (C-9a), 122.08 (C-5b), 126.46 (C-8), 120.75 (C-4), 120.70 (C-6), 119.64 (C-7), 117.68 (C-5), 115.19 (C-3), 112.07 (C-4a), 111.26 (C-9), 97.10 (C-11), 16.48 (CH_3) ppm. HRMS (ESI-TOF) calcd for $C_{16}H_{10}ClNO_2$ 283.0400; found 283.0405.

*4.1.6. 6-Methoxy-2,3,4,9-tetrahydro-1H-carbazole (**6**)*

To a solution of cyclohexanone (0.30 mL, 2.86 mmol) in glacial acetic acid (1 mL), methyl 4-methoxyphenyl hydrazine (0.501 g, 2.87 mmol) was added over 1 h. The mixture was refluxed for 3.5 h and left stirring until reaching room temperature. It was then cooled in ice for 20 min and then 75% (v/v) aqueous MeOH (4 mL) was added. The solid formed was filtered and washed with 12 mL of the same aqueous methanol to give a beige solid. Yield: 0.409 g (2.03 mmol, 71%). mp 93–95 °C. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.56 (s br, 1H, NH), 7.18 (d, 1H, J = 9.0 Hz, H-1), 6.93 (d, 1H, J = 2.4 Hz, H-4), 6.77 (dd, 1H, J = 8.7 and 2.4 Hz, H-2), 3.86 (s, 3H, OCH_3), 2.72–2.68 (m, 4H, H-6 and H-7), 1.90–1.89 (m, 4H, H-5 and H-8) ppm. ^{13}C NMR (75.4 MHz, $CDCl_3$): δ 153.84 (C-3), 153.06 (C-8a), 130.68 (C-9a), 128.17 (C-4a), 110.90 (C-1), 110.50 (C-2), 110.02 (C-4b), 100.23 (C-4), 55.95 (OCH_3), 23.33 (C-6 or C-7), 23.18 (C-5 and C-8), 20.94 (C-6 or C-7) ppm. Anal. calcd for $C_{13}H_{15}NO$: C, 77.91; H, 7.223; N, 6.983%; found: C, 77.58; H, 7.51; N, 6.96%.

*4.1.7. 3-Methoxy-9H-carbazole (**7**)*

To a solution of compound **6** (0.409 g, 2.03 mmol) in *p*-cymene and H_2O (5 mL, 4:1) 10% Pd/C (0.206 g) was added and the mixture heated under reflux for 48 h. After cooling it was filtered over Celite and washed with boiling EtOAc. The filtrate was evaporated to dryness, and the product was obtained as an oil. Yield: 0.210 g (1.07 mmol, 53%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.04 (d, 1H, J = 8.1 Hz, H-5), 7.57 (d, 1H, J = 2.4 Hz, H-4), 7.41–7.40 (m, 2H, H-7 and H-8), 7.34 (d, 1H, J = 8.7 Hz, H-1), 7.24–7.20 (m, 1H, H-6), 7.07 (dd, 1H, J = 9.0 and 2.4 Hz, H-2), 3.94 (s, 3H, OCH_3) ppm. NH was not observed.

*4.1.8. 3-Methoxy-9H-carbazole-9-carbaldehyde (**8**)*

To a solution of dry DMF (0.10 mL, 1.30 mmol) in dry CH_2Cl_2 (5 mL) cooled to 0 °C, $POCl_3$ (0.07 mL, 0.75 mmol) was slowly added and the mixture was kept under stirring for 30 min. Then a solution of compound **7** (0.100 g, 0.51 mmol) in dry CH_2Cl_2 (10 mL) was added and the reaction mixture was heated under reflux for 3 h. After cooling, water (10 mL) was added and the phases were separated. The organic phase was sequentially washed with 10% HCl (5 mL) and saturated $NaHCO_3$ (2 × 5 mL), dried ($MgSO_4$), filtered and evaporated to dryness. The product **8** was obtained as a solid, m.p. 67–70 °C. Yield: 0.087 g (0.39 mmol, 76%). ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.81 (s, 1H, CHO), 8.34 (d, 1H, J = 8.4 Hz, H-1), 8.17 (d, 1H, J = 8.4 Hz, H-5), 8.08 (d, 1H, J = 7.2 Hz, H-8), 7.75 (d, 1H, J = 2.4 Hz, H-4), 7.51 (t, 1H, J = 7.6 Hz, H-6), 7.42 (t, 1H, J = 7.6 Hz, H-7), 7.11 (dd, 1H, J = 8.8 e 2.8 Hz, H-2), 3.87 (s, 3H, OCH_3) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ 159.31 (CHO), 156.70 (C-3), 138.15 (C-8a), 130.74 (C-9a), 127.24 (C-7), 125.07 (C-4b), 123.91 (C-6), 120.87 (C-5), 116.75 (C-1), 115.16 (C-2), 112.23 (C-4a), 111.41 (C-8), 104.07 (C-4), 55.64 (OCH_3) ppm.

*4.1.9. 3-Hydroxy-9H-carbazole-9-carbaldehyde (**9**)*

To a cold solution of compound **8** (0.069 g, 0.31 mmol) in dry CH_2Cl_2 (5 mL) under nitrogen atmosphere a 1 M solution of BBr_3 in CH_2Cl_2 (0.60 mL, 0.60 mmol) was slowly added. After reaching room temperature the mixture was kept under stirring for 24 h and then it was poured onto ice and extracted with CH_2Cl_2 (3 × 10 mL). The organic phase was dried ($MgSO_4$), filtered and evaporated to dryness. The residue obtained was purified by preparative layer chromatography (eluent: diethyl ether/light petroleum, 1:1). The compound **9** was obtained as a solid. Yield: 0.031 g (0.14 mmol, 48%). mp 149–150 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 9.79 (s, 1H, CHO), 9.60 (sl, 1H, OH), 8.25 (d, 1H, J = 8.4 Hz, H-1), 8.06 (d, 2H, J = 8.4 Hz, H-5 and H-8), 7.49 (t, 1H, J = 7.6 Hz, H-7), 7.46 (d, 1H, J = 2.4 Hz, H-4), 7.39 (t, 1H, J = 7.6 Hz, H-6), 6.94 (dd, 1H, J = 8.8 and 2.0 Hz, H-2) ppm. ^{13}C NMR (100.6 MHz, DMSO-d_6): δ 159.08 (CHO), 154.70 (C-3), 138.15 (C-8a), 129.84 (C-9a), 127.09 (C-7), 125.06 (C-4b), 123.85 (C-6), 120.69 (C-5), 116.76 (C-1), 115.64 (C-2), 112.05 (C-4a), 111.33 (C-8), 105.73 (C-4) ppm.

*4.1.10. (E)-Ethyl 3-(3-hydroxy-9H-carbazol-9-yl)acrylate (**10**)*

To a solution of compound **9** (0.070 g, 0.33 mmol) in *N*,*N*′-diethylaniline (2.0 mL) carbethoxymethylenetriphenylphosphorane (0.160 g, 0.46 mmol) was added and the mixture was refluxed for 15 h. After cooling, water was added (10 mL) and extracted with CH_2Cl_2 (4 × 5 mL). The organic phase was washed with 5% HCl (5 × 5 mL), dried ($MgSO_4$), filtered and evaporated to dryness. The residue obtained was purified by preparative thin layer chromatography (eluent: diethyl ether/light petroleum, 7:3) and a yellow solid was obtained. Yield: 0.021 g (0.09 mmol, 28%). m.p. > 200 °C (dec.). ^{1}H NMR (400 MHz, acetone-d_6): δ 8.54 (d, 1H, J = 14.4 Hz, H-3), 8.57 (s, 1H, OH), 8.10 (dd, 1H, J = 8.0 and 0.8 Hz, H-5′or H-8′), 7.90 (d, 1H, J = 8.4 Hz, H-5′or H-8′), 7.81 (d, 1H, J = 8.8 Hz, H-1′), 7.61 (d, 1H, J = 2.4 Hz, H-4′), 7.58 (td, 1H, J = 8.0 and 1.2 Hz, H-7′), 7.39 (td, 1H, J = 8.0 and 0.8 Hz, H-6′), 7.15 (dd, 1H, J = 2.4 and 8.8 Hz, H-2′), 6.32 (d, 1H, J = 14.0 Hz, H-2), 4.28 (q, 2H, J = 7.2 Hz, CH_2), 1.35 (t, 3H, J = 7.2 Hz, CH_3) ppm. ^{13}C NMR (100.6 MHz, acetone-d_6): 168.18 (C=O), 154.66 (C-3′), 140.64 (C-8′a), 137.65 (C-3), 133.61 (C-9′a), 127.98 (C-7′), 127.64 (C-4′a), 126.14 (C-4′b), 123.31 (C-6′), 121.27 (C-5′or C-8′), 116.49 (C-2′), 113.97 (C-1′), 112.50 (C-5′ or C-8′), 106.72 (C-4′), 100.90 (C-2), 60.50 (CH_2), 14.74 (CH_3) ppm.

4.2. Tumour cell growth assay

The compounds were evaluated for their anti-proliferative effect on human cancer cell lines MDA MB231 (breast adenocarcinoma) and TCC-SUP (bladder transitional cell carcinoma). The MDA MB231 epithelial cell line was established from a pleural effusion obtained from a 51-year-old female patient with breast cancer. The TCC-SUP cell line was established from a tumour specimen resected from the urinary bladder transitional cell carcinoma (undifferentiated, grade IV) of a 67-year-old woman. All the cell lines were kindly provided by IPATIMUP (Portugal). The cells were maintained in an incubator with a 5% CO_2 atmosphere and at 37 °C. The culture medium used was the Dulbecco's modified Eagle medium (DMEM) (GIBCO®, Invitrogen, Barcelona, Spain) supplemented with Foetal Bovine Serum (FBS) (GIBCO®, Invitrogen, Barcelona, Spain) (10% for

MDA MB231 and 15% for TCC-SUP) and 1% of penicillin/streptomycin (Invitrogen, Barcelona, Spain).

4.2.1. Cell viability

Cells were exposed to five concentrations of compounds starting from a maximum concentration of 75 μM. Compounds, prepared in dimethyl sulfoxide (DMSO), were freshly diluted with cell culture medium just prior the assays. Final concentrations of DMSO were less than 1% not interfering with cell growth. The cell viability was determined using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) method. A commercial kit was used according to the manufacturer instructions (Promega, PROM G35800001, Lisbon, Portugal). In these experiments, 100 μL of cell suspension was added to each well of a 96-well plate. Additionally, control wells were included consisting of DMEM medium and the compounds prepared in DMSO at the concentrations under study. When a cell concentration of 1×10^4 cells/mL was obtained, adequate volumes of the compounds solutions were added to the wells and incubated for 48 h. Afterwards, 20 mL of the CellTitter 96 Aqueous One Solution Cell Proliferation Assay reagent (MTS) was added to each well and left in the incubator (37 °C, 5% CO_2) for 2 h after which the cell viability was quantified by recording the absorbance at 490 nm. For each test compound and for each cell line a dose–response curve was generated and the growth inhibition of 50% (GI_{50}) of cell population was determined (GI_{50} corresponds to the concentration of compound that inhibits 50% of the cell growth). The results are expressed as percentage of viable cells compared to the control and represent an average of 3 independent cultures with 4 wells per concentration in each experiment.

4.3. Molecular docking

All the compounds were studied using the molecular docking software AutoDock [27] and the vsLab plug-in Ref. [28]. The structure of the receptor was built from the PDB structure 1Z10 containing the Human Microsomal P450 2A6. The ligands were built with GaussView, protonated at physiological pH and optimized with gaussian09 (HF/6-31G(d)). In the docking process the Lamarckian genetic algorithm (LGA) was used. The number of generations, energy evaluations, and docking runs were set to 370,000, 1,500,000, and 50, respectively. The types of atomic charges were taken as Kollman-all-atom for the receptor and Gasteiger for the compounds. The final solutions were retrieved from the molecular docking process according to the criteria of interacting energy.

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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.2014.09.066.

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