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Original article

Substituted benzopyranobenzothiazinones. Synthesis and estrogenic activity on MCF-7 breast carcinoma cells

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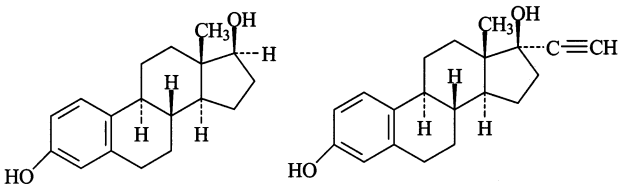
Abstract – In the search for new agents with estrogenic activity mediated by estrogen receptors (ER), six 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones **3a**–**f** were synthesized. These compounds were readily prepared by the addition of 2-aminothiophenol **2** to substituted 4-hydroxycoumarin derivatives **1a**–**e**. The estrogenic effect has been evaluated on the proliferation of MCF-7 breast adenocarcinoma cells and the specificity of described compounds was evaluated by the inhibition of their effect by ICI 182,780, an antiestrogenic compound. Among the compounds tested, 6,12-dihydro-3-methoxy-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one **3e** and 6,12-dihydro-3-hydroxy-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one **3f** exhibited an ER-dependent proliferation and a high binding affinity to ER, but a moderate capacity to activate the transcription of a reporter gene. Their pharmacological profiles are defined by their binding properties and their mechanism of action by computational modelling studies.

benzopyranobenzothiazinones / 2D-NMR / binding assay / estrogenic activity / SAR

1. Introduction

Even if estrogens have a well-established role in the growth of hormone-dependant tumours by an estrogen receptor (ER)-dependant mitogenic effect in cells containing ER [1], a member of the nuclear receptor superfamily [2], they exert numerous favourable activities in women. From a therapeutical point of view, 17β-estradiol (E2) and derivatives are well known not only as oral contraceptives, but also in hormone replacement therapy required in bone loss prevention or in the control of cardiovascular diseases (particulary atherosclerosis) in postmenopausal women [3]. The most widely used estrogens are 17β-estradiol [4] and the steroidal synthetic compound ethynylestradiol [5] (*figure 1*). Nevertheless, what distinguishes ER within the steroid receptor superfamily is its accommodation of a large variety of non-steroidal ligands.

Initially isolated as natural products [6], coumarins constitute a very relevant family of pharmacological active compounds. Actually, such derivatives have been developed as anticoagulant drugs [7], photosensitive drugs [8], potent and selective human dopamine



Estradiol (E2) Ethynylestradiol

Figure 1. Structure of 17β-estradiol and ethynylestradiol.

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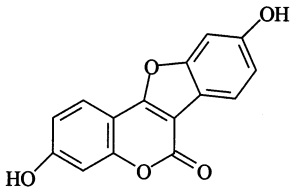
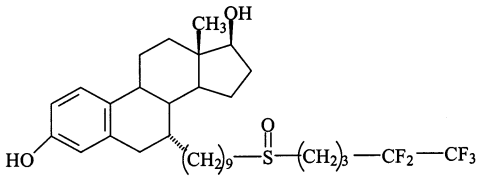


Coumestrol

Figure 2. Coumarinic estrogen coumestrol.



ICI 182,780 (faslodex)

Figure 3. Structure of ICI 182,780.

D4 antagonists [9], non-peptidic HIV protease inhibitors [10] or as antibiotic agents, such as novobiocin [11].

Among all the potential therapeutical applications revealed by coumarins, some estrogenic-like activities have been discovered, leading to the development of the natural potent non-steroidal estrogenic compound coumestrol [12] (*figure 2*).

The aim of our study was to synthesize a series of 2 or 3-substituted benzopyranobenzothiazin-6-ones and to test their estrogenic activity in vitro.

We describe the synthesis, the pharmacological profile and the design study of 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones **3a**–**f**. Their structure was elucidated by UV, FTIR, ^{1}H-NMR, ^{13}C-NMR, 2D-NMR (*J*, δ spectroscopy, correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC) and heteronuclear multiple quantum coherence (HMQC)) experiments and by EI-mass spectral data.

The proliferative activity of these compounds was tested on MCF-7 breast cancer cells and the involvement of ER was tested through the capacity of ICI 182,780 (*figure 3*), or faslodex, a pure antiestrogenic derivative [13, 14], to displace the tested molecules and to reverse their effect. We reported their ability to stimulate the proliferation of MCF-7 estrogen-sensitive mammary tumour cells, and for the most potent compounds **3e** and **3f**, their binding affinity, obtained by Scatchard analysis using a competitive binding assay with [^{3}H]-17β-estradiol as tracer. Finally, the ER-dependant proliferative activity of compounds **3e** and **3f** was determined, in transient transfection experiment, through the expression of a luciferase reporter gene driven by a consensus estrogen response element (ERE).

Subsequently, a structure–activity relationship study (SAR) was undertaken on described and evaluated compounds to rationalise the activity of this new class of non-steroidal estrogenic derivatives.

2. Chemistry

The synthesis of the quadricyclic 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one system **3a**–**e** was carried out according to the method of Tabacovic and co-workers [15].

The addition of 2-aminothiophenol **2** on the 2,4-chromandione anionic tautomeric form [16] of substituted 4-hydroxycoumarins **1a**–**e** was made in the oxidizing solvent DMSO [17]. This addition was accompanied by the elimination of one molecule of water and conducted to a non-isolable enaminone intermediate, bis(*o*-aminophenyl)disulfide (DAPDS) [18]. Subject to a nucleophilic attack at the 3-position of the coumarin, this enaminone led to the desired products **3a**–**e** by intracyclization. This heterocyclization was managed by the sulfur–sulfur bond scission, because of the very reactive 3-position of the substituted coumarins [19] (*figure 4*).

The expected compounds crystallised with DMSO when cooling to room temperature, and then were isolated by filtration under vacuum. Their structure was assigned from UV, FTIR, ^{1}H-NMR spectra, ^{13}C-NMR spectra, 2D-NMR experiments (*J*, δ experiment, COSY, HMQC, HMBC), EI-MS spectra and elemental analysis.

The synthesis of the hydroxylated derivative was unsuccessful with the classic methods using Lewis acid BBr_3 or the hard acid and soft nucleophile system EtSH–$AlCl_3$ [20]. Nevertheless, the use of the strong acid HI in acetic acid in the presence of acetic anhydride [21] successfully led to the desired product **3f** by deprotection of the methoxy group of compound **3e** (*figure 5*).

The UV spectra of the synthesized compounds exhibited three absorption maxima: a coumarinic chro-

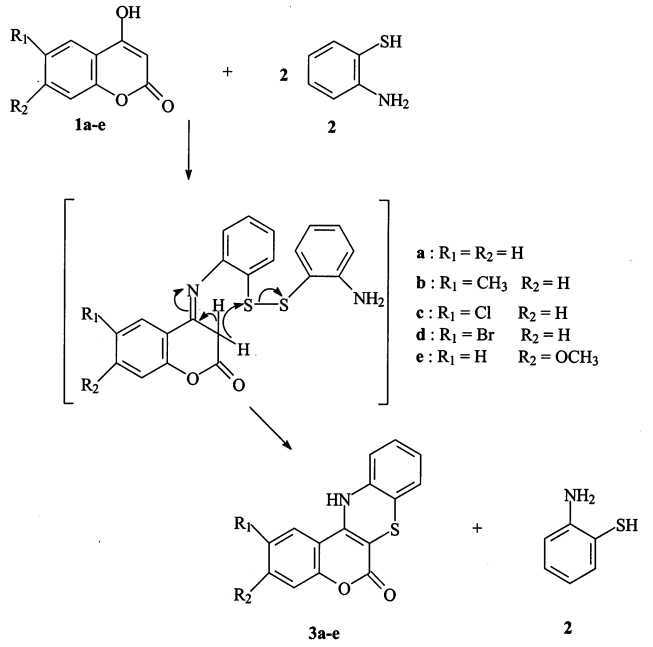


Figure 4. Synthesis of substituted benzopyranobenzothiazinones **3a**–**e**.

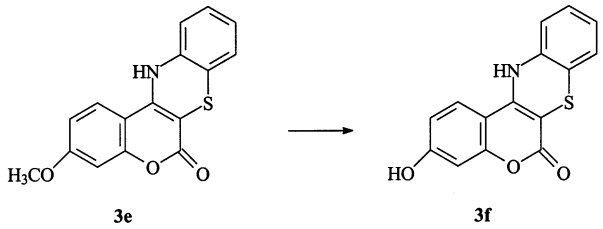


Figure 5. Synthesis of compound **3f**.

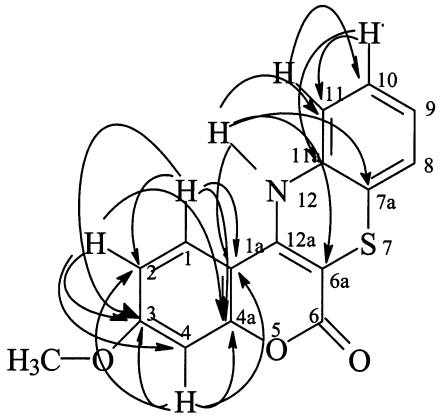


Figure 6. HMBC spectrum (H→C) of compound **7**.

mophore at 451 nm, a cinnamoyl chromophore at 315 nm and an aminophenyl chromophore at 290 nm [18].

The $^{1}H-^{1}H$ shift COSY, J, δ spectroscopy, HMQC and HMBC were successfully performed to establish the interfragment relationship, and to assign the proton and carbon signals of compound **3e** as shown in *figure 6.*

In the HMBC spectrum of **3e**, H-1 at δ 8.10 correlates with four aromatic carbon signals C-2 (δ 129), C-3 (δ 163), C-4a (δ 154) and C-1a (δ 119). H-2 at δ 7.02 is correlated with three aromatic carbon signals C-3 (δ 163), C-4 (δ 102) and C-4a (δ 154). Finally, H-4 at δ 6.98 is correlated with four aromatic carbon signals, precisely C-2 (δ 129), C-3 (δ 163), C-4a (δ 154) and C-1a (δ 119) of the coumarin ring. Moreover, H-12 at δ 9.00, the mobile proton of the amino group, and the four carbon signals C-11 (δ 118), C-1a (δ 119), C-7a (δ 107) and C-6a (δ 88) of the benzothiazine moiety, were correlated. Furthermore, H-11 at δ 6.98 and carbon signals C-11a (δ 138), C-10 (δ 126) were also correlated.

These results were supported by a ^{1}J correlation of H-1, H-2 and H-4 to, respectively, C-1 (δ 124), C-2 (δ 129) and C-4 (δ 102) in the HMQC spectrum.

3. Biological results and discussion

The biological profiles of these new non-steroidal molecules were evaluated by their capacity to induce the proliferation of MCF-7 breast carcinoma cells [22, 23], an ER-positive breast cancer cell line. The proliferative effect of these compounds was compared to the one observed with 17β-estradiol (*table I*) and the ER implication in cell proliferation was assessed with the pure and highly selective antiestrogen ICI 182,780 [24, 25].

The stimulation of MCF-7 cells with various concentrations of **3a**–**f** compounds was performed for 4 days (see Section 5) [26]. At the end of this period, an estimation of cell proliferation was performed using tetrazolium salt WST-1 (*figure 7*). The implication of estrogen receptors in the action of these compounds was appraised using ICI 182,780 at 1 μM. The results for compounds **3a**–**f** (*table I*) showed that **3a** induced a moderate (<20%) but significant increase ($P = 8.7 \times 10^{-5}$) of cell proliferation for the highest concentration tested (1 μM).

On the other hand, MCF-7 cells stimulation by 1 μM of compound **3e** induced a significant growth

Table I. Cell proliferation results for compounds 3a–f.

		Cell proliferation (% of control)	95% Confidence limit (%)	P [a]
17β-Estradiol	1 nM	49.2	20.2	<0.01
ICI 182,780	1 μM	−11.8	6.7	<0.01
E2+ICI	1 nM+1 μM	−8.9	1.1	<0.01
Compound **3a**	0.1 μM	−2.5	4.7	NS [b]
	1 μM	12.6	2.3	<0.01
	0.1 μM+ICI [c]	−3.2	6.8	NS
	1 μM+ICI	1.5	6.7	NS
Compound **3b**	1 μM	−1.6	1	<0.01
	10 μM	2	2.6	NS
	1 μM+ICI	−7.8	4.1	<0.01
	10 μM+ICI	−6.8	3.2	<0.01
Compound **3c**	1 μM	2.4	1	<0.01
	10 μM	1.5	3.4	NS
	1 μM+ICI	2.4	5.3	NS
	10 μM+ICI	−1.1	3.3	NS
Compound **3d**	1 μM	−13.9	4.8	<0.01
	10 μM	−2.7	9.4	NS
	1 μM+ICI	−25.9	4.3	<0.01
	10 μM+ICI	−15.2	4.5	<0.01
Compound **3e**	0.1 μM	40.3	9.9	<0.01
	1 μM	58.2	14	<0.01
	0.1 μM+ICI	16.3	9.7	<0.01
	1 μM+ICI	9.6	14.4	NS
Compound **3f**	1 μM	−5.6	5.1	NS
	10 μM	52.6	10.2	<0.01
	1 μM+ICI	−8.7	4.1	<0.01
	10 μM+ICI	−9.1	1.1	<0.01

[a] P value of t-test used to compare cell proliferation induced by the compounds with that induced in control cells.
[b] NS: not significant, i.e. $P>0.05$.
[c] ICI (ICI 182,780) concentration used is 1 μM.

response on MCF-7 cells of about 58.2±14% compared to the unstimulated cells ($P=2.0\times10^{-8}$) after 4 days of stimulation. The magnitude of the maximum effect induced by compound **3e** was found to be not different from the one observed in MCF-7 cells treated with 1 μM E2 (49.2±20%). The proliferative activity of compound **3e** was, as for the other compounds, almost completely inhibited with 1 μM ICI 182,780, demonstrating that such an effect was ER dependent.

Therefore, we focused on compound **3e** and we determined the concentration which induced the half-maximum effect, EC_{50} (*figure 8*). The stimulation of MCF-7 cells with compound **3e** from 10^{-11} to 10^{-5} M allowed one to find that the EC_{50} was obtained with a concentration of $2.4\pm1.6\times10^{-8}$ M ($n=4$).

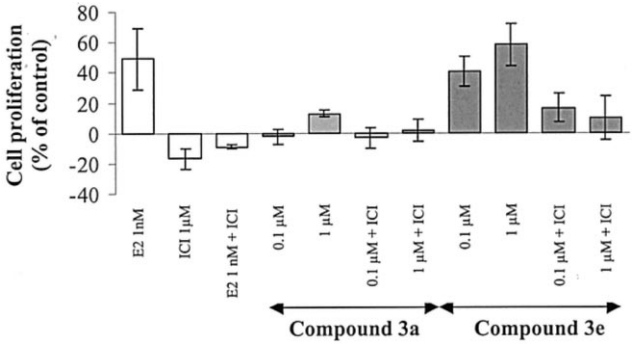


Figure 7. Effect of compounds **3a** and **3e** on MCF-7 cell proliferation.

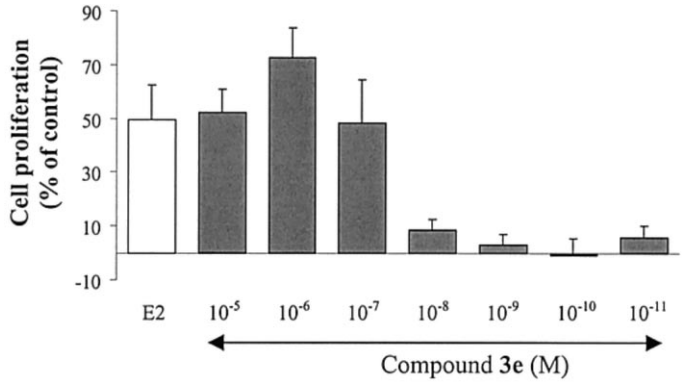


Figure 8. Concentration dependence of compound **3e** on MCF-7 cell proliferation.

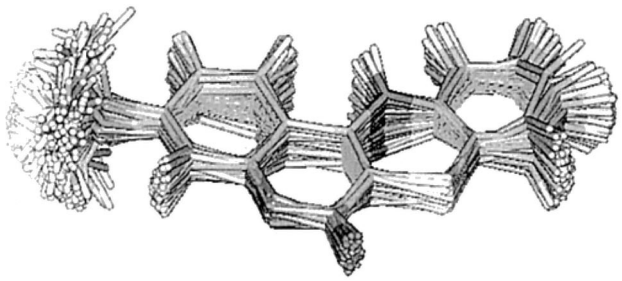


Figure 9. Molecular dynamic of compound **3e** at 300 K during 100 ps.

In an attempt to obtain further information about the relationships between **3e** and ER, its affinity for estrogen receptors as well as the activation of an ERE-containing promoter driving the luciferase gene were determined. Scatchard analyses showed that the number of estrogen receptors, evidenced with unlabelled diethylstilbestrol (DES), was 84 317±33 687 sites per cell and suggest that the compound **3e** was bound to only one class of estrogen receptor with a high affinity $K_d = 2.54 \pm 0.91$ nM, since the number of sites per cell was 25 264±13 655. The evaluation of the ER–**3e** complex capacity to bind an ERE sequence and to activate the luciferase reporter gene transcription was then performed and showed a moderate but significant capacity of 10^{-6} M of **3e** (1.34-fold, $P<0.01$) to activate the luciferase transcription compared with unstimulated cells treated with 10^{-8} M E2 (3.52-fold, $P<0.01$) as positive control.

Moreover, in order to explore the implication of the 3-methoxy group of compound **3e** in this estrogenic activity, we tested the free phenolic derivative **3f** in the same conditions. Stimulation on MCF-7 cells by 10 μM of compound **3f** induced a significant proliferative response (52.6±10.2%) compared to the unstimulated cells ($P = 5.0 \times 10^{-8}$) after 4 days of stimulation which is not different from **3e**. Activity of compound **3f** was, as for **3e**, almost completely inhibited with 1 μM ICI 182,780, demonstrating that such an effect was ER-dependent. The EC_{50} of compound **3f** was obtained with a concentration of $5.9 \pm 0.4.10^{-6}$ M ($n = 4$) (data not shown). Scatchard analyses showed that compound **3f** was bound to only one class of estrogen receptors with high affinity $K_d =$ 1.60±0.30 nM, and with a binding capacity of 39 677±14 537 sites per cell. These results show that **3f** has the same behaviour as **3e**. The evaluation of the ER–**3f** complex capacity to bind an ERE sequence and to activate the luciferase reporter gene transcription was then performed and showed a significant capacity of 10^{-5} M **3f** (2.64-fold, $P<0.01$) to increase the luciferase activity compared to unstimulated cells.

Since numerous ERα and ERβ variants are expressed in MCF-7 cells [27], we assume that **3e** and **3f** could bind one class of ER implicated in cell proliferation, but we cannot exclude the implication of another type of receptor in the observed effects of **3e** and **3f**. The different sensibility of MCF-7 cells to the proliferative effect of **3e** and **3f** derivatives could partly rely upon a slower degradation of **3e** in MCF-7 cells.

The estrogenic activity of compound **3e** mediated by estrogen receptors could be explained by a molecular modelling approach compared to E2. The molecules constructed into 3D molecular structures were minimised and the calculation performed until the maximum RMS derivative was less than 0.001 kcal $Å^{-1}$. The molecular dynamic study, carried out at 300 K during 100 ps, showed a very planar structure **3e**, with a maximum energy variation of 3 kcal mol^{-1} (*figure 9*). The planarity of this new substituted aromatic system does not appear to be an obstacle to a significant pharmacological activity, even if it is agreed that good ligands for the estrogen receptors need some degree of thickness in the central hydrophobic portion of the ligand binding domain (LBD).

Moreover, the volume of **3e** and **3f** occupies less total space than E2 (208.6 and 193.10 $Å^3$, respectively, versus 232.4 $Å^3$ for E2) in the 450 $Å^3$ binding pocket of the ER. Compared to E2, the excedentary volume

of **3e**, 52.06 Å^3, concerns the methyl group of the methoxy, the keto group and the angular aromatic D ring, whereas the excedentary volume of **3f**, 36.83 Å^3, concerns only the keto group and the angular aromatic D ring. Equally, when **3e** is compared to E2, the excedentary volume of E2 is 76.33 Å^3, and concerns the C ring and the cyclopentyl D ring of E2 (*figure 10*). These results concerning volumes of **3e** and **3f** compared to the volume of E2 are compatible with a binding of these derivatives in the pocket of estradiol in the ER. According to the studies of Duax et al. [28], the observation that the excedentary volume is principally localised in the D ring region is not an obstacle for the activity, since ER-LBD is flexible in this D-ring region [29, 30]. Moreover, the planarity induced by the keto group should have a better receptor match than the non-planar B ring [31].

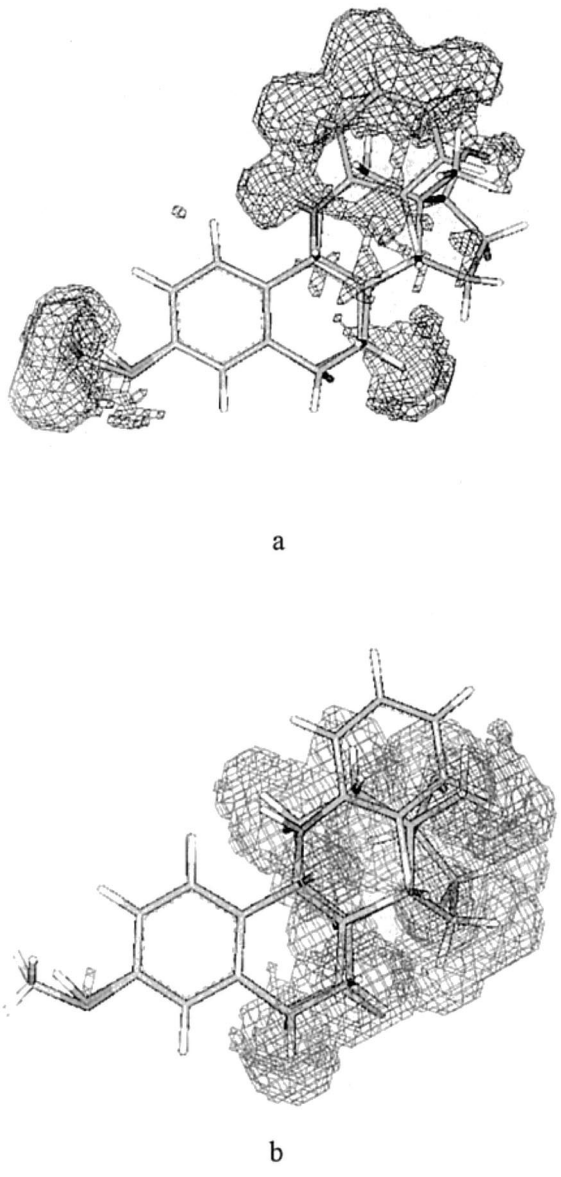


Figure 10. Excedentary volume of: (a) **3e** compared to E2; and (b) E2 compared to **3e**.

Furthermore, the charge density study of the benzopyranobenzothiazinone core clearly shows some analogies with E2. By molecular computational methods, we determined that the A ring of compound **3e**, compared to E2, had a similar charge density, even if the methoxy group, which might mimic the 3-hydroxyl group of **3f**, contributes to reducing the polarity of the 3-substituent. The calculation of the net atomic charge of the phenolic hydroxylic oxygen of **3f** is −0.38, suggesting that the phenol is a poor H-bond acceptor and is more H-bond donor as observed with the phenolic group of E2. Actually, this result has been already observed with E2; even if the phenol of E2 might be an H-bond donor and H-bond acceptor, there is evidence that the donor function is more important, with a net atomic charge of −0.253 [29].

Nevertheless, as we showed in the present study, the presence of a methoxy group is also implicated in the estrogenic activity as it has been already demonstrated with some non-steroidal estrogens such as TACE (chlorotrianisene), a triarylethylenic estrogen, or centchroman, a chroman derivative. Moreover, it is now admitted that the methyl group of the 3-methoxy substituent is removed by metabolism, resulting in free phenol. However, the polar substituent at the 3-position is indispensable since the loss of this substituent showed a significant loss from 20 to 120% of activity [32–34]. Actually, it is known that the apolar surface of the ligand interacts hydrophobically with the LBD of the estrogen receptor α, whereas the methoxy or the hydroxy group interacts with the His 524 (helix H11) and the Glu 419 (loop 6–7), a very conserved residue which plays a special role in polar ligand binding contacts, or with the Glu 353 (helix H3) of the LBD. Unfortunately, since the ligand orientation in the ligand binding cavity, limited to ERα, is still uncertain, it is difficult to come to a specific conclusion about the binding mode of the compound **3e** to the ER receptor [34].

4. Conclusions

In conclusion, our study provides evidence that non-steroidal compounds **3e** and **3f** act positively on MCF-7 cell proliferation and that this effect is mediated by estrogen receptors as evidenced by the inhibition with ICI 182,780. The binding studies of these compounds to ER as well as their capacity to induce the transcription of a reporter gene, driven by a consensus ERE sequence, allowed one to characterise the relationship between **3e**, **3f** and ER. Moreover, the volumes of **3e** and **3f** are totally compatible with the fact that such compounds may fit into the LBD of ER. This activity is easily assimilated to the methoxy group at the 3-position of **3e**, and to the free phenolic group of **3f**, which could mimic the phenolic group of E2, playing a fundamental role in the formation of the ligand–receptor complex. On the other hand, the non-substituted compounds and the two non-polar substituted compounds present a faint proliferative activity or none at all. Therefore, the effects may be useful in the treatment of pathologies in which estrogen-like properties play a fundamental role. Thus, compounds **3e** and **3f** could be new candidates that could contribute to the development of a large chemical library of related compounds by a combinatorial synthesis approach. Further studies are needed to elucidate with precision the type of receptor involved in the activity of **3e** and **3f** and their mechanism of action (binding mode).

5. Experimental protocols

5.1. Chemistry

All melting points (mp) were obtained with a Kofler Heizbank Reichert 18.43.21 and were uncorrected. Infrared spectra were recorded on a Shimadzu FTIR-8201PC spectrometer in potassium bromide pellets (ν in cm^{-1}). ^{1}H-NMR spectra, ^{13}C-NMR spectra and 2D-NMR experiments (J,δ experiment, COSY, HMQC, HMBC) were recorded on a Bruker AC 200 spectrometer. The samples were dissolved in DMSO-d_6 relative to tetramethylsilane as internal standard. All measurements were performed at 293 K. The chemical shift values are reported in parts per million (ppm, δ units) and spin–spin coupling J were exposed in Hz. The following abbreviations are used: singlet (s), doublet (d), triplet (t), multiplet (m). EI-MS was done on a Nermag R10-10H apparatus with a Coniphot detector and an ionizing voltage of 70 eV in the direct-inlet mode. UV spectra were recorded to a Shimadzu UV-160A spectrophotometer. Thin-layer chromatography (TLC) was carried out on an Alugram Sil G/UV_{254} plate with appropriate solvents. Microanalysis were carried out by the Service Central d'Analyses, Centre National de la Recherche Scientifique, Vernaison (France). Elemental analyses indicated by the symbols of the elements were within ±0.4% of the theoretical values for C, H and N. Yields were not optimised.

*5.1.1. General procedure for the condensation of 4-hydroxycoumarin derivatives **1a–e** with 2-aminothiophenol **2***

4-hydroxycoumarin derivatives **1a–e** (61.7 mmol) and 2-aminothiophenol **2** (11.56 g, 92.5 mmol) were added to 20 mL of DMSO and heated to 150°C for 10 h. On cooling at room temperature, the products crystallised and are filtered under vacuum.

*5.1.1.1. 6,12-Dihydro-1-benzopyrano[3,4-b][1,4]-benzothiazin-6-one (**3a**)*

Prepared by coupling 4-hydroxycoumarin **1a** with 2-aminothiophenol **2**. Red amorphous powder. (60%), mp>300°C. IR (KBr) (cm^{-1}): 3335, 3010, 1668, 1618, 1015, 737; ^{1}H-NMR (DMSO-d_6) δ: 6.90–7.10 (4H, m, Ar-H), 7.50 (2H, m, Ar-H), 7.65 (1H, dd, $^4J = 6.5$ Hz, $^3J = 7.5$ Hz, H-2), 8.20 (1H, m, $^3J = 7.5$ Hz, H-1), 9.00 (1H, s, NH). MS m/z: 267 [M^+]; Anal. $C_{15}H_9NO_2S$ (C,H,N).

*5.1.1.2. 6,12-Dihydro-2-methyl-1-benzopyrano-[3,4-b][1,4]benzothiazin-6-one (**3b**)*

Prepared by coupling 4-hydroxy-2-methylcoumarin **1b** with 2-aminothiophenol **2**. Pale red amorphous powder. (40%), mp>300°C. IR (KBr) (cm^{-1}): 3348, 3010, 2950, 1655, 1038, 739; ^{1}H-NMR (DMSO-d_6) δ: 2.20 (3H, s, CH_3), 6.80–7.10 (4H, m, Ar-H), 7.25 (1H, d, $^3J = 8$ Hz, H-4), 7.35 (1H, d, $^3J = 8$ Hz, H-3), 7.90 (1H, s, H-1), 8.9 (1H, s, NH). MS m/z: 281 [M^+]; Anal. $C_{16}H_{11}NO_2S$ (C,H,N).

*5.1.1.3. 2-Chloro-6,12-dihydro-1-benzopyrano-[3,4-b][1,4]benzothiazin-6-one (**3c**)*

Prepared by coupling 6-chloro-4-hydroxycoumarin **1c** with 2-aminothiophenol **2**. Orange amorphous powder. (50%), mp = 230°C. IR (KBr) (cm^{-1}): 3323, 3065, 1672, 1616, 1030, 745; ^{1}H-NMR (DMSO-d_6) δ: 6.80–7.00

(4H, m, Ar-H), 7.36 (1H, s, $^3J = 8.0$ Hz, H-4), 7.65 (1H, dd, $^4J = 4.0$ Hz, $^3J = 8.0$ Hz, H-3), 8.25 (1H, d, $^4J = 8.0$ Hz, H-1), 8.95 (1H, s, NH). MS $m/z = 301$ [M^+]; Anal. $C_{15}H_8NO_2SCl$ (C,H,N).

5.1.1.4. 2-Bromo-6,12-dihydro-1-benzopyrano-[3,4-b][1,4]benzothiazin-6-one (3d)

Prepared by coupling 6-bromo-4-hydroxycoumarin **1d** with 2-aminothiophenol **2**. White amorphous powder. (36%), mp = 290°C. IR (KBr) (cm^{-1}): 3323, 3006, 1672, 1618, 1028, 752; ^{1}H-NMR (DMSO-d_6) δ: 6.50–6.80 (4H, m, Ar-H), 7.00 (1H, d, $^3J = 10$ Hz, H-4), 7.20 (1H, d, $^3J = 10$ Hz, H-3), 7.95 (1H, d, H-1), 10.8 (1H, s, NH). MS $m/z = 347$ [$M^+ + 1$]; Anal. $C_{15}H_8NO_2SBr$ (C,H,N).

5.1.1.5. 6,12-Dihydro-3-methoxy-1-benzopyrano-[3,4-b][1,4]benzothiazin-6-one (3e)

Prepared by coupling 4-hydroxy-7-methoxycoumarin **1e** with 2-aminothiophenol **2**. Pink amorphous powder. (60%), mp = 230. IR (KBr) (cm^{-1}): 3335, 3010, 2934, 1670, 1616, 1022, 750; ^{1}H-NMR (DMSO-d_6) δ: 3.85 (3H, s, OCH_3), 6.85 (2H, m, Ar-H), 6.98 (1H, d, $J = 2.5$ Hz, H-4), 6.95 (2H, m, Ar-H), 7.02 (1H, dd, $^4J = 2.5$ Hz, $^3J = 9.1$ Hz, H-2), 8.10 (1H, d, $^3J = 9.1$ Hz, H-1), 9.01 (1H, s, NH). ^{13}C-NMR (DMSO-d_6): δ 56.8 (OCH_3), 88.3 (C-6a), 101.9 (C-4), 106.5 (C-7a), 113.0 (C-9), 117.7 (C-11), 118.7 (C-1a), 124.0 (C-1), 126.0 (C-10), 127.4 (C-8), 128.5 (C-2), 137.6 (C-11a), 147.5 (C-12a), 154.1 (C-4a), 158.0 (C-6), 163.1 (C-3). MS $m/z = 297$ [M^+]; Anal. $C_{16}H_{11}NO_3S$ (C,H,N).

5.1.1.6. 6,12-Dihydro-3-hydroxy-1-benzopyrano-[3,4-b][1,4]benzothiazin-6-one (3f)

Prepared by refluxing during 1 h 1 mmol of **3e** with 10 mL of HI in a mixture of 5 mL of acetic anhydride and 5 mL of acetic acid. On cooling at room temperature, the product precipitates and is then filtered under vacuum. Ochre amorphous powder. (35%), mp>300°C. IR (KBr) (cm^{-1}): 3415, 3246, 3010, 1664, 1616, 1050, 763; ^{1}H-NMR (DMSO-d_6) δ: 5.10 (1H, s, NH), 6.60 (3H, m, Ar-H), 6.90 (1H, d, $J = 7.2$ Hz, H-4), 7.30 (2H, m, Ar-H), 7.70 (1H, d, $^3J = 8.6$ Hz, H-1), 10.70 (1H, s, OH). MS $m/z = 282$ [$M^+ - 1$]; Anal. $C_{15}H_9NO_3S$ (C,H,N).

5.2. Pharmacological methods

5.2.1. Cell line and culture

MCF-7 cells were purchased from ATCC (Biovalley, Conches, France) and maintained in DMEM medium containing phenol red (Sigma, Saint Quentin, Fallavier, France) and supplemented with 15 mM HEPES, 2 mM glutamine, 1% antibiotic cocktail (streptomycin 10 mg mL^{-1}, penicillin 10 000 U mL^{-1}, amphotericin B 25 μg mL^{-1}) and 10% fetal bovine serum (FBS). MCF-7 cells were tested for the absence of mycoplasma before starting the experiments. For the experiments, cells were trypsinised and seeded in 96-well plates (Falcon, Elvetec, Venissieux, France) with 5000 cells per well for 4 days in a phenol red-free DMEM medium supplemented with 15 mM HEPES, 2 mM glutamine, 1% antibiotic cocktail and 10% desteroided-FBS (dextran-charcoal-treated FBS).

All compounds (**3a**–**f**) were made soluble in DMSO. For experiments, the DMSO solution was diluted by a factor 1000 into the stimulation medium [35]. Stimulations were performed with the same medium supplemented with various concentrations of compounds in eight wells for each condition. Positive controls were performed with 10^{-9} M 17β-estradiol and results of proliferation were compared to those obtained with cells incubated with the medium alone. The implication of estrogen receptors in the action of these compounds was tested by the inhibition of their effect on cell proliferation with a pure antiestrogenic compound, ICI 182,780, at 10^{-6} M.

5.2.2. Cell proliferation

The measurement of cell proliferation was then performed by a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to formazan by mitochondrial dehydrogenase in viable cells after 4 days of stimulation. Assays were performed according to the manufacturer's protocol (cell proliferation reagent WST-1, Roche, Meylan, France).

5.2.3. Binding studies in MCF-7 cells

The determination of the **3e** and **3f** compounds binding affinity to the estrogen receptor was performed according to the method described by Taylor et al. [36]. MCF-7 cells (5×10^4 cells) were grown for 5 days in 24-well plates in phenol red-free DMEM supplemented with 10% charcoal-treated FBS, 2 mM glutamine and 15 mM HEPES. Thereafter, as confluence was reached, cells were incubated with 6 nM [^{3}H]-estradiol (specific activity: 84.1 Ci $mmol^{-1}$, NEN) and various concentrations of **3e** and **3f** compounds (0–10^{-6} M) for 1 h at 37°C in phenol red-free DMEM supplemented with 0.1% bovine serum albumin (BSA). Medium was then

discarded and cells incubated for 30 min at room temperature (RT) with a phosphate buffer pH 7.4 (sodium phosphate 5 mM, sucrose 0.25 M, glycerol 10%, BSA 0.5%). MCF-7 cells were then rinsed twice with the same cold phosphate buffer and incubated with 1 mL absolute ethanol for 30 min at RT. Ethanol fractions, containing the bound fraction of estradiol, were then transferred to counting vials and placed in a β-counter (Beckman LS 6000 IC). Scatchard analysis was then performed to determine the dissociation constants (K_d) and the number of binding sites (N), using a non-linear adjustment according to the Marquart's method processed on SAS software (SAS Institute, Cary, NC). Binding studies were performed in triplicate in three independent experiments.

5.2.4. Transient transfection experiments

Transient transfection of the ERE-wt-tk-Luc and luciferase activity quantification were performed to evaluate the implication of the estrogen receptors in the action of compounds **3e** and **3f**. MCF-7 cells were transiently transfected with a plasmid containing a consensus ERE triggering the luciferase gene expression. Transfections were achieved with the following plasmids: pUC 18 vector containing a consensus ERE linked to the firefly luciferase reporter gene and the eukaryotic expression vector pSGΔ2 containing NLS LacZ from pMMuLV NLS LacZ [37] which was used as a positive control for monitoring transfection efficiency. MCF-7 cells, grown to a 30–40% confluence, were transiently transfected using Lipofectine® Reagent (Life Technologies, Cergy-Pontoise, France) for 12 h with 1 μg of each plasmid and 4 μg Lipofectine®, in 1 mL phenol red-free DMEM, supplemented with 0.1% charcoal-treated FBS and 2 mM glutamine. Cells were washed twice with 2 mL phenol red-free DMEM and were subsequently stimulated for 5 h with various concentrations of **3e** and **3f**, and with 10^{-8} M 17β-estradiol as positive control, in phenol red-free DMEM supplemented with 15 mM Hepes, 2 mM glutamine, 10% charcoal-treated FBS and 1% antibiotic cocktail. Transfected MCF-7 cells were then lysed with Reporter lysis buffer (Promega, Charbonnières, France) and luciferase and β-galactosidase activities were measured. β-Galactosidase activity was assayed in 40 μg cellular extracts, in 1 mL buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1 mM $MgSO_4$, 10 mM KCl, 50 mM β-mercaptoethanol) and 200 μL ONPG (4 mg mL^{-1} in buffer Z). Samples were incubated at 37°C until a yellow colouring appeared. Luciferase activity was determined using luciferase detection Kit (Promega). Results are expressed as ratio of the luciferase activity (normalised with β-galactosidase activity) between stimulated over unstimulated cells. Transient transfection studies were performed in quadruplicate in two independent experiments.

5.3. Computational methods

5.3.1. Molecular modelling

Modelling studies were performed on a Silicon Graphics Iris Indigo 2 running the INSIGHT II software package (Biosym/MSI., version 95.0). The structure of estradiol and the compound **3e** were constructed from standard bond lengths and bond angles using the SKETCH option from the Builder module. The 2D sketches of estradiol and compound **3e** were fully optimised in vacuo and converted into 3D molecular structures. After construction, molecules were minimised using the Steepest Descents method and CFF91 forcefield supported by the Discover program. Calculations were performed until the maximum RMS derivative was less than 0.001 kcal $Å^{-1}$. The molecular dynamics at 300 K during 100 ps was done with the Discover program and the volumes were calculated using the Search/Compare module.

6. Statistics

Results were expressed as mean ± 95% confidence limits. EC_{50} values were determined from the linear portion of concentration/proliferation curves by regression analysis, and expressed as a mean ± SE. Statistical analysis was performed using Student's test (t-test) and probability values (P) below 0.05 were considered significant. Before performing the t-test, the data were tested using an F test, for their variance homogeneities.

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