Pharmacological Profile of 6,12-Dihydro-3-methoxy-1-benzopyrano[3,4-b] [1,4]benzothiazin-6-one, a Novel Human Estrogen Receptor Agonist

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Pharmacological studies were carried out to characterize further the endocrinological profile and the binding mode to the estrogen receptor (ER) of 6,12-dihydro-3-methoxy-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one (1). Binding experiments were conducted with highly purified recombinant human estrogen receptors hERα and β. Potent estrogenic activity of compound 1 was assessed by testing its ability to down-regulate ERs and to enhance estrogen receptor element (ERE)-dependent transcription. The latest step of our work dealt with the synthesis of the 9-fluorinated derivative 15 for ionic microscopy experiments to determine the intracellular localization of compound 1. Although failed to compete with [3H]E2 for binding to both ER isoforms, evidence was reported that it interacted with hERα in MCF-7 cells (ER down-regulation/ERE-dependent luciferase induction). Hence, an appropriate conformation of the hormone binding domain, most probably conferred by co-regulators of ER, is required for the onset of an activity of the compound 1. Estrogenic activity was weak but on the order of magnitude of that of coumestrol (slightly weaker). The synthesis of the 9-methoxyalted derivative 16 and its pharmacological evaluation led us to propose a binding mode of 1 on hERα. Compound 1 appears to interact with ERα mainly through interactions of its 3-methoxy substituent with the residue His-524 of the hormone binding domain.

Key words benzopyrano[3,4-b][1,4]benzothiazin-6-ones; coumestrol; estrogenicity; MVLN; binding mode; ionic microscopy

The intranuclear estrogen receptor (ER),1,2) of which three isoforms (α, β, and γ) have been identified, plays a crucial role in several processes such as the control of vertebrate reproduction or the development of secondary sexual characters. It has important functions in many non reproductive tissues such as bone,3) the cardiovascular system,4—7) or the central nervous system (CNS).8) Estrogens, which bind to ERs, are used in the treatment of primary or secondary ovarian deficiencies, in the maintenance of contraception and the prevention of diseases associated with menopause. More recently, Mook-Jung et al.9) have reported that E2, the endogenous estrogen in women, presents a significant benefit in the treatment of some cognitive deficiencies such as in Alzheimer disease. The growing interest in estrogen replacement therapy in women, particular for decreasing osteoporosis and cardiovascular diseases such as coronary atherosclerosis, has led us to synthesize estrogenic coumarins that could be candidates for drug development.10)

The role of estrogens is now increasingly well established, and considerable advances in the understanding of their molecular mechanism of action have been achieved with recombinant human estrogen receptors α (hERα) and β (hERβ).11—13) Hormone-bound receptors form an active dimer that mediates biological response in the nucleus by interacting with specific short nucleotide sequences of DNA called estrogen response elements (EREs) located in the promoter region of target genes. Numerous co-activators or corepressors are involved in the associated activation processes of the transcriptional machinery, leading to the biological cellular response (Fig. 1).14—17)

Xenoestrogens are synthetic compounds that encompass the simple phenolic structure of steroidal estrogens. Nonsteroidal estrogens are being increasingly developed and span a remarkable range of acyclic structures with a great variety of heterocycles,18,19) This is the case of many natural derivatives with a coumarinic, flavone, or flavene core structure.20) Among these natural phytoestrogens, coumestanes with a coumarinic structure are mainly represented by one derivative, coumestrol.21,22)

In our work to develop new active xenoestrogens, we have recently reported the 6,12-dihydro-3-methoxy-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one (1) that appeared to compete with E2 for ER binding on MCF-7 breast cancer cells.10) The present work further analyses the estrogenic activity of this compound on MVLN cells (ERE-regulated luciferase expression cells) in comparison with E2, coumestrol, and the pure antiestrogen RU 58668.23) Studies conducted with highly purified hERα and β failed to show any binding

Fig. 1. General E2-Induced Transcriptional Mechanism in ER+ MCF-7 Cells
E2, 17β-estradiol; R, estrogen receptor; hsp, heat-shock protein; R-E2, complex estradiol-estrogen receptor; (R-E2)2, dimer of the complex estradiol-estrogen receptor; CoA, coactivator(s); ERE, estrogen response element.

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affinity for 1, 6,12-dihydro-9-fluoro-3-methoxy-1-benzopyran-4[3,4-b][1,4]benzothiazin-6-one (15), and 6,12-dihydro-9-methoxy-1-benzopyran-4[3,4-b][1,4]benzopyran-6-one (16). On the contrary, the incubation of MCF-7 cells with 1 led to a decrease in their capacity to concentrate \([1^3\text{H}]\text{E}_2\) (down-regulation), confirming their ability to interact with ERs in living cells. MVLN cells (MCF-7 cells stably transfected with a pVit-tk-Luc reporter plasmid) revealed a significant ERE-dependent transcriptional activity of 1 and 15 similar to that of coumestrol. Additional investigations using ion microscopy and concerning the subcellular localization of the analogous compound 15 suggested an intranuclear localization. Such results confirm our preliminary results, suggesting the involvement of ERs in the biological activity of 1. A binding mode for 1 within the human estrogen receptor \(\alpha\)-ligand binding domain (hER\(\alpha\)-LBD) is proposed.

### MATERIALS AND METHODS

**Compounds Utilized** Estradiol and tritium-labeled estradiol \([1^3\text{H}]-17\beta\text{-E}_2\) were purchased from Sigma (Bornem, Belgium) and Amersham Pharmacia (Roosendaal, the Netherlands), respectively. Coumestrol was purchased from Fluka (Bornem, Belgium) and RU 58668 was obtained from Roussel Uclaf (Romainville, France). Starting materials for the synthesis of compounds 15 and 16 were purchased from Acros Organics (Noisy-le-Grand, France) for 4-hydroxy-7-methoxy coumarin (2) and from Aldrich Chemical Company (Sigma Aldrich, Saint-Quentin Fallavier, France) for 4-hydroxy coumarin (3), 4-fluoroaniline (4), 4-methoxyaniline (5), and ammonium thiocyanate (6). Compound 1 was obtained according to the procedure described by Tabakovic et al.\(^{26}\) Its spectral and physicochemical characteristics have been previously reported.\(^{10}\) Melting points were determined on a Kofler Heizbank Reichert 18.43.21 and were uncorrected. IR spectra were recorded on a Shimadzu FTIR-8201PC spectrometer in potassium bromide pellets (\(v \text{ in cm}^{-1}\)). NMR spectra in DMSO-\(d_6\) were recorded on a Bruker AC 300 spectrometer. Chemical shifts \(\delta\) are given in ppm and coupling constants \(J\) are expressed in Hz. The following abbreviations are used: s, singlet; d, doublet; t, triplet; q, quartet; and m, multiplet. MS spectra were recorded on a Nermag R10-10H apparatus by electron impact (EI).

### 4-Fluorophenylthiourea (9) and 4-Methoxyphenylthiourea (10)

Compounds 9 and 10 were prepared following the synthetic route illustrated in Chart 2:\(^{27,28}\) 4-fluoroaniline hydrochloride (7) or 0.01 mol of 4-methoxyaniline hydrochloride (8), respectively, were obtained by adding slowly concentrated hydrochloric acid on the appropriate aniline dissolved in \(\text{CH}_2\text{Cl}_2\). To 0.01 mol of the substituted aniline hydrochlorides was added 0.01 mol of ammonium thiocyanate (6) in 100 mL of water. The mixture was refluxed for 4 h. On cooling at room temperature, the precipitate was filtered under vacuum, rinsed 4 times with cold water, and dried to give, respectively, 4-fluorophenylthiourea (9) or 4-methoxyphenylthiourea (10).

9: Gray powder, yield: 45%, \(C_7H_7FN_2S\); mp 173 °C. IR (KBr) cm\(^{-1}\): 3442—3374, 3008, 1627, 1604—1409, 1118. \(1^\text{H}-\text{NMR}\) (DMSO-\(d_6\)) \(\delta\): 7.15 (d, 2H, \(J=8.97\)), 7.40 (d, 2H, \(J=8.97\), \(J=5.16\)), 9.65 (s, 1H). EI-MS \(m/z\) 170 (M\(^+\)).

10: Gray powder, yield: 64%, \(C_8H_{10}N_2OS\); mp 262 °C. IR (KBr) cm\(^{-1}\): 3398—3276, 3028, 2962, 1622, 1618—1417. \(1^\text{H}-\text{NMR}\) (DMSO-\(d_6\)) \(\delta\): 7.31 (s, 3H), 6.88 (d, 2H, \(J=8.72\)), 7.21 (d, 2H, \(J=8.72\)), 9.45 (s, 1H). EI-MS \(m/z\) 182 (M\(^+\)).

### 2-Amino-6-fluorobenzothiazole (11) and 2-Amino-6-methoxybenzothiazole (12)

10 \(\mu\)mol of 9 or 10 were added to 50 mL of distilled chloroform at 5 °C and 0.01 mol of bromine was added dropwise. The mixture was allowed to stand at room temperature for 1 h and was refluxed for 1 h. After cooling at room temperature, the mixture was treated with an aqueous solution of sulfurous acid and filtered. Concentrated ammonium hydroxide was added to the filtrate, and the crude precipitate corresponding to 2-amino-6-fluorobenzothiazole (11) or 2-amino-6-methoxybenzothiazole (12), respectively, was filtered and dried under vacuum.

11: White powder, yield: 42%, \(C_7H_7FN_2S\); mp 185 °C. IR (KBr) cm\(^{-1}\): 3446—3372, 3014, 1627, 1606—1409, 1116. \(1^\text{H}-\text{NMR}\) (DMSO-\(d_6\)) \(\delta\): 7.30 (d, 1H, \(J=8.76\)), 7.46 (s, 2H), 7.47 (s, 1H), 7.58 (dd, 1H, \(J=8.76\), \(J=2.14\)). EI-MS \(m/z\) 168 (M\(^+\)).

12: Gray powder, yield: 35%, \(C_8H_9N_2OS\); mp 149 °C. IR (KBr) cm\(^{-1}\): 3390—3294, 3105, 2947, 1643, 1604—1434. \(1^\text{H}-\text{NMR}\) (DMSO-\(d_6\)) \(\delta\): 3.71 (s, 3H), 6.77 (dd, 1H, \(J=8.55\), \(J=2.63\)), 7.21 (d, 1H, \(J=8.33\)), 7.22 (s, 2H), 7.28 (d, 1H, \(J=2.63\)). EI-MS \(m/z\) 180 (M\(^+\)).

### 2-Amino-5-fluorothiophenol (13) and 2-Amino-5-methoxythiophenol (14)

Compounds 11 or 12 were refluxed in a 12 : 1 solution of acuous sodium hydroxide for 48 h. After standing at room temperature, the mixture was filtered in glacial acetic acid and yellow crystals appeared, corresponding to the unstable 2-amino-5-fluorothiophenol (13) \(^{29—32}\) Compound 13 was obtained as a yellow oil and used later without further purification.

13: Yellow needles, yield: 51%, \(C_7H_8N_2S\); mp 106 °C. IR (KBr) cm\(^{-1}\): 3452—3361, 3001, 2952, 2400, 1622—1490. \(1^\text{H}-\text{NMR}\) (DMSO-\(d_6\)) \(\delta\): 3.65 (s, 3H), 4.10 (s, 1H), 6.69 (s, 1H), 6.72 (s, 2H), 6.81 (d, 1H, \(J=3.09\)), 6.84 (d, 1H, \(J=3.09\)). EI-MS \(m/z\) 155 (M\(^+\)).

14: Yellow needles, yield: 51%, \(C_7H_9NOS\); mp 106 °C. IR (KBr) cm\(^{-1}\): 3452—3361, 3001, 2952, 2400, 1622—1490. \(1^\text{H}-\text{NMR}\) (DMSO-\(d_6\)) \(\delta\): 3.65 (s, 3H), 4.10 (s, 1H), 6.69 (s, 1H), 6.72 (s, 2H), 6.81 (d, 1H, \(J=3.09\)), 6.84 (d, 1H, \(J=3.09\)). EI-MS \(m/z\) 155 (M\(^+\)).
b][1,4]benzopyran-6-one (15), 6,12-dihydro-9-methoxy-1-benzopyrano[3,4-b][1,4]benzopyran-6-one (16) Aminothiophenols 13 and 14 were added to 4-hydroxy-7-methoxy-coumarin and 4-hydroxycoumarin, respectively, in 20 ml of DMSO and heated to 150 °C for 10 h. On cooling at room temperature, the products crystallized and were filtered under vacuum.

15: Brown powder, yield: 25%, C16H10FNO3S, mp 260 °C. IR (KBr) cm⁻¹: 3327, 3087, 2920, 1660, 1618, 1560—1438, 1109. ¹H-NMR (DMSO-d₆) δ: 3.85 (s, 3H), 6.80 (d, 1H, \( J = 8.27 \)), 6.84 (d, 1H, \( J = 8.48 \)), 6.94 (s, 1H), 6.98 (d, 1H, \( J = 8.27 \)), 7.17 (d, 1H, \( J = 8.48 \)), 8.25 (d, 1H, \( J = 9.12 \)), 9.00 (s, 1H). EI-MS \( m/z \) 315 (M⁺).

16: Orange powder, yield: 21%, C16H11NO3S, mp 300 °C. IR (KBr) cm⁻¹: 3400, 3066, 2850, 1649, 1627, 1600—1452. ¹H-NMR (DMSO-d₆) δ: 3.65 (s, 3H), 6.55 (d, 1H, \( J = 2.64 \)), 6.61 (dd, 1H, \( J = 2.85, J = 8.77 \)), 6.92 (d, 1H, \( J = 8.55 \)), 7.37 (m, 2H), 7.60 (t, 1H, \( J = 8.55 \)), 8.08 (d, 1H, \( J = 8.33 \)), 8.95 (s, 1H). EI-MS m/z 297 (M⁺).

Receptor Binding Studies Highly purified recombinants hERα or β were purchased from Calbiochem, Eurobiochem, Bruges, Belgium. These commercial preparations were diluted 300 times in a bovine serum albumin (BSA) solution (1 mg/ml) to be subsequently adsorbed onto hydroxyapatite (HAP) according to the procedure of Maaroufi and Leclercq (1994). After removal of unbound material by centrifugation, the HAP suspensions were incubated overnight at 0—4 °C with \( [³H]E₂ \) in the presence or the absence of increasing amounts of E₂ (control) or the investigated compound (from 10⁻⁶ M to 10⁻¹⁰ M). Suspensions were centrifuged and the corresponding pellets were washed. Radioactivity adsorbed onto the HAP pellets was extracted with ethanol and measured by liquid scintillation counting.

Influence of Compounds on ER Level The influence of compounds on the ER level was evaluated according to the method reported by Gyling and Leclercq. MCF-7 cells were incubated overnight with 1 at 10⁻⁶ M. Control cells were maintained in culture in the absence of the compound. After removal of the medium, treated and untreated cells were incubated for 1 h with increasing amounts of \( [³H]E₂ \) (5.10⁻¹¹ to 10⁻⁹ M) with or without an excess of unlabeled E₂ (10⁻⁶ M, nonspecific binding). After extraction with ethanol, incorporated \( [³H]E₂ \) was measured by liquid scintillation and the data analyzed by Scatchard plot analysis after correction for nonspecific binding (³H]E₂—(³H] + excess E₂)). \( [³H]E₂ \) binding capacity (abscissa) provides an estimation of the receptor level.

ERE-Dependent Luciferase Expression Compounds 1, 15, and 16 were solubilized in DMSO and diluted 1000 times in Dulbecco’s modified Eagle medium (DMEM, Gibco, Ghent, Belgium). E₂, coumestrol, and RU 58668 were solubilized in ethanol and diluted in DMEM. MVLN cells (MCF-7 cells stably transfected with a pVit-tk-Luc reporter plasmid) were cultured for 3 to 4 d in 95-mm Falcon dishes (plating density 80000 cells/dish) in fetal calf serum depleted of endogenous steroid by dextran-coated charcoal (DCC) treatment. Compounds 1, 15, 16, coumestrol (from 10⁻⁶ M to 10⁻¹⁰ M), E₂ 0.1 nM, and RU 58668 10⁻⁷ M were added to the medium and cultured until firefly luciferase was detected (24 h). For that purpose, the medium was removed and the cells were washed twice with a phosphate–buffered saline (PBS) solution. A minimal volume (250 µl) of a 5-fold diluted lysis solution (E153A Promega, Leiden, the Netherlands) was then added to the dishes and the latter maintained under mild agitation for 20 min to extract luciferase. Lysed cells were subsequently detached with a scraper (Costar 3010) and centrifuged for 5 s at 12000×g to clarify their extracts. Twenty microliters of each extract were finally mixed at room temperature with 100 µl of a luciferase–reactant medium (Promega E151A/E152A), prepared according to

Chart 2. General Synthesis of Compounds 15 and 16

![Chart 2](image)
the manufacturer’s protocol. Induced light was then measured with a Berthold luminometer (Luma LB 9507) and induction of the ERE-dependent biological response was expressed in arbitrary units with regard to the light measured with a blank (relative light unit, RLU). To compare RLUs, the protein content of each extract was measured using the Coomassie method (PIERCE, Erembodegem, Belgium).

The statistical significance of differences among values was assessed by Student’s t-test. Differences vs. control were considered significant at \( p < 0.01 \). It should be noted that \( p < 0.01 \) was reached for all the pharmacological data reported in this work.

**Ion Microscopy** The monolayer cell culture was washed in PBS 10 ml and cells were dissociated in 0.02% trypsin–EDTA medium. MCF-7 cells were cultured on thin ultrapure gold plates with a surface of 1 cm\(^2\) and thickness of 0.05 cm (ENGELHARD-CLAL, Noisy-le-Sec, France). MCF-7 cells were purchased from the ATCC (American Type Culture Collection, ref. HTB22, Biovalley, Conches, France) and maintained in DMEM containing phenol red (Sigma) and supplemented with 15 nM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), 2 mM glutamine, and 1% of an antibiotic cocktail (streptomycin 10 mg/ml, penicillin 10000 U/ml, amphotericin B 25 \( \mu \)g/ml). Cell incubation (1.5 h) was carried out by adding a solution of compound 15 at 10\(^{-5}\) M to the DMEM medium. Compound 15 was made soluble in DMSO and was diluted by a factor of 1000 in the DMEM culture medium. Unbound ligands were removed with 0.01 M cacodylate buffer (pH 7.4) and the thin gold plates were fixed with 1.2% glutaraldehyde for 30 min at 4°C. The plates were washed with 0.1 M cacodylate buffer and with deionized and distilled water to eliminate buffer salts. Residual water was then eliminated with filter paper. Plates were then stored at 37°C for 48 h to dry the preparation before analysis. Ion microscopy, based on selected ion monitoring mass spectrometry (SIM mode) allowed us to distinguish CN \((M=26)\) and F \((M=19)\), providing separate images reflecting the distribution of incorporated fluor. Analysis was carried out at high mass resolution \((M/\Delta M)\) close to 3000 to avoid interference by polyatomic ions. The histologic photographs were obtained with an exposure of 5 s for \( M=26 \), which allowed detection of an ionic current of 8.1 \( \times 10^{-14} \) A. On the other hand, exposure of 4 min for \( M=19 \) was necessary to detect an ionic current of 2.0 \( \times 10^{-16} \) A.

**RESULTS AND DISCUSSION**

The loss of \([\text{H}]\text{E2}\) binding detected in living MCF-7 cells may be relevant to a competitive mechanism involving hER\(\alpha\) or hER\(\beta\) as reported in our previous publication.\(^{10}\) Binding assays were performed with highly purified hERs to determine whether this occurred. In contrast to E2 and 4-OH-Tam, 1 failed to compete with \([\text{H}]\text{E2}\) for binding to both ER isoforms (Figs. 2 and 3). Discrepancies between binding data established on ERs in living cells (whole-cell assay) and purified hERs (hER\(\alpha\) and hER\(\beta\)) have been recently reported by Kim and Katzenellenbogen.\(^{36}\) According to those authors, if this difference in binding was due to a nonspecific association, we should have observed a higher binding affinity for the pure receptors hER\(\alpha\) and/or hER\(\beta\). Since we observed the opposite tendency (higher binding affinity for ERs in

![Fig. 2. Affinity of Compound 1 on hER\(\alpha\) Compared to E2 and 4-Hydroxytamoxifen (4-OH-Tam)](image)

![Fig. 3. Affinity of Compound 1 on ER\(\beta\) Compared to E2 and 4-Hydroxytamoxifen (4-OH-Tam)](image)

MCDF-7 cells, Kd=2.5±0.91 nM), we can reject the possibility of such a nonspecific binding phenomenon. Thus the discrepancies appear to favor a mechanism in which coactivators could assume a suitable configuration of ERs to optimize the affinity of compound 1 for the receptors.\(^{37}\) Down-regulation of ERs in the whole-cell assay, i.e., ligand induced degradation leading to its progressive elimination,\(^{38-41}\) may be also considered. Preincubation (16 h) of MCF-7 cells with compound 1 \(10^{-6}\) M led to a significant decrease \((t= \text{ca. } 30\%)\) in their capacity to incorporate \([\text{H}]\text{E2}\) specifically as shown in the Scatchard plot analysis of the binding data in Fig. 4 (Kd=4.5 \( \times 10^{-11}\) M for compound 1 vs. Kd=5.1 \( \times 10^{-11}\) M for the control). Note that E2 at \(10^{-9}\) M always induces an almost total elimination of the capacity of the cells to incorporate \([\text{H}]\text{E2}\) under identical conditions.\(^{34}\)

The assessment of luciferase induction in MVLN cells confirms this view of an intracellular association of 1 with ER. We found 53% enhancement for compound 1 at \(10^{-6}\) M (control, 100%; E2, 178% at \(10^{-10}\) M; RU 58668, 34% at \(10^{-7}\) M), suggesting an estrogenic effect (Fig. 5).\(^{42}\) We compared the transcriptional activity of 1 with coumestrol at concentrations ranging from \(10^{-10}\) to \(10^{-6}\) M. As reference standards, E2 \(10^{-10}\) M and the pure antiestrogen RU 58668
$10^{-7} \text{M}$ were used. Whereas coumestrol provoked an expected increase in luciferase (estrogenicity) with a maximum of activity at $10^{-6} \text{M}$ (287%), compound 1 produced a weaker increase (only 188%) at the same concentration suggesting a lower efficiency. In agreement with this result, coumestrol $10^{-7} \text{M}$ exhibited such significant luciferase induction (Fig. 6) whereas compound 1 (Fig. 5a) failed to exhibit a significant increase in luciferase induction (for comparison, note the activity of E2 and RU 58668, Fig. 7). A comparison of the luciferase induction profiles of E2, coumestrol, and 1 revealed a markedly lower potency of the latter two compounds (optimal luciferase increase was always reached at $10^{-10} \text{M}$ E2). The decrease in the level of luciferase always seen with RU 58668 may be relevant to an inhibition of transcription induced by growth factors from either the serum or released by the cells (autocrine regulation).

In another experiment, we tested the ability of RU 58668 to inhibit the luciferase induced by coumestrol and compound 1. Figure 5b clearly shows inhibition of the luciferase activity induced by compound 1 when RU 58668 was added to the medium at $10^{-7} \text{M}$, which produced optimal inhibition. These results confirm that the estrogenic activity induced by compound 1 is mediated by ERs.

Since the transcriptional pointed to an estrogenic mechanism mediated by ERs, we determined the in situ localization of compound 1 in MCF-7 cells using ionic microscopy, a very sensitive imaging method to explore the localization of the intracellular targets of drugs. To carry out this experiment, we synthesized a fluor-labeled derivative of 1, i.e., compound 15. The advantage of such a labeled derivative is that fluor is not usually present in cells and therefore its detection in MCF-7 cells reflects the intracellular distribution of compound 15. In a preliminary phase of this experiment, we verified that compound 15 has estrogenic activity in
MVLN cells similar to that of compound 1. A slightly weaker transcriptional activity with a maximal efficiency at $10^{-6}$ M was seen, as shown in Fig. 8a. RU 58668 again inhibited the activity of compound 15, confirming the involvement of ER (Fig. 8b).

Analytical microscopy of MCF-7 cells after 1.5 h of stimulation by fluorinated derivative 15 revealed an image at the mass of 19 corresponding to fluor 19F. Figure 9 shows the intranuclear distribution of compound 15 at the mass of 19. The marked intranuclear distribution of fluor supports the concept of a direct binding of compound 15 to nuclear ERs.

Finally, the potential binding mode of compound 1 on ERs was investigated. This study was inspired by a recent article from Pike et al.43 dealing with the ligand binding mode of nonsteroidal ligands within the hERα-LBD and hERβ-LBD. Two types of contacts bonding to specific amino acids of the LBD are required for E2: the first concerns the 3-phenolic group (A-ring) and the second the 17β-hydroxyl group (D-ring). In the hERα-LBD, the phenolic group binds to the residue Glu-353 (helix H3) of the hERα-LBD while the 17β-hydroxyl binds to the δ nitrogen of the His-524 (helix H11) and the δ oxygen of Glu-419 (loop 6—7) (Fig. 10a). The donor and acceptor characteristics of the phenol at position 3 and of the hydroxyl at position 17β, respectively, are involved in these interactions. Even if the donor and acceptor characters of the 17β-hydroxyl are required for contacts with His-524 and Glu-419, the interaction with His-524 (donor
character) is the most significant in terms of energy.\(^4\) Moreover, it is now well established that this 17β-hydroxyl interaction with the His-524 region of the hERα-LBD is more important than that of the 3-phenolic group with the Glu-353 region, as reported by Wurtz et al.\(^4\) For this reason, some methoxylated ligands exhibit good binding affinity for ERs. This is the case for TACE (or chlortriainisen), centchroman,\(^46\) and apparently compound \(^1\) (i.e., interaction of the oxygen of its methoxyl group with the His-524, donor interaction). Hence, the outcome of these considerations is that the methoxyl group of compound \(^1\) would not interact with Glu-353, and should interact exclusively with the His-524 (but not with Glu-419) of hERα-LBD. Nevertheless, it is important to take into account the enzymatic metabolism conducting in vivo to the hydroxylated drugs. In this regard, we found that the 3-hydroxylated derivative of \(^1\), i.e., 6,12-dihydro-3-methoxy-1-benzopyran-3,4,6-[1,4]benzothiazin-6-one \(^1\) did not enhance the activity or the binding affinity for ERs.\(^10\) When the estrogenic effects of compounds \(^1\) and \(^16\) were compared, the results were unequivocal since they revealed no transcriptional activity of compound \(^16\) (113% at 10\(^{-6}\)M), whereas compound \(^1\) exhibited a significant estrogenic effect (188% at 10\(^{-6}\)M). These observations confirm that the methoxy group at position 3 of compound \(^1\) favors an estrogenic effect, whereas its presence at position 9 is unfavorable. It is therefore highly probable that the 3-methoxy group of its methoxyl group with the His-524, donor interaction).\(^3\)

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