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RESEARCH ARTICLE

N-Arylbenzamides: extremely simple scaffolds for the development of novel estrogen receptor agonists

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Abstract
The research of estrogen receptor (ER) ligands has benefited in the last decade from the implementation of combinatorial chemistry. The general pharmacophore has been identified and subsequently a multitude of compounds have been synthesized. Surprisingly, up to now simple amides have not been taken into consideration. Here we show that amides resulting from the condensation of hydroxybenzoic acids with aminophenols result in compounds retaining the pharmacophore structure of an ER ligand with a clear estrogenic activity.

Keywords: Estrogen, receptor, amides, scaffold, agonist

Introduction

The regulation of the reproductive systems and of many functions in liver, bones, central nervous system and cardiovascular systems are some examples of the effects of estrogens, endocrine molecules exemplified by 17β-estradiol (Figure 1)[1] The latter binds to the estrogen receptor (ER), known to be present in two isoforms (ER-α and ER-β), leading to a conformational change that allows the ER to act as transcription factor, giving rise to gene expression changes and modulatory functions[2].

ERs are known to be able to recognize non-steroidogenic substances. Indeed, the promiscuity of the ER has led to the development of molecules with estrogenic and therapeutical potential[3]. In the past decades, the tremendous effort made to characterize the interaction of the ER with its ligands and the activation cascade[4] has led to a detailed knowledge of the receptor-ligand interactions and the pharmacophore is actually known[5,6].

The pharmacophore ligand model (Figure 1) summarizes the key structural features deducted from 17β-estradiol: (1) H-bonding ability of the phenolic ring mimicking the 3-OH, (2) H-bond donor mimicking the 17β-OH, (3) O-O distance between 3 and 17β-OH, (4) precise steric hydrophobic centers mimicking steric 7α and 11β-substituents, (5) hydrophobicity and (6) a ring structure[6]. Indeed, such understanding has led the pharmaceutical industry to develop a number of estrogen agonists and antagonists to modulate the hormonal activity in the different body districts and health conditions[7].

In the last decades, the search for compounds endowed with higher affinity and selectivity (eventually with respect to the ER subtype) has led to the preparation or isolation of different estradiol analogs, the best known families being diarylethanes (e.g. hexestrol[8,9]), di/triarylethenes (e.g. diethylstilbestrol[10,11], tamoxifen[12,13]) and phytoestrogens[14,15] (flavones, flavanones, isoflavones, etc.). In addition to these, a large number of synthetic estradiol analogs have been built over the pharmacophore model[16]. The main variations are observed in the central region, where different substructures have been employed to bear two phenolic ring with the OH-OH distance predicted by the established model. To this purpose, several analogs with central spacing structures such as five-membered heterocyclic rings[17,18] have been synthesized and tested. Additional derivatives involve fusion of one of the phenolic ring with carbo-[19,20] and

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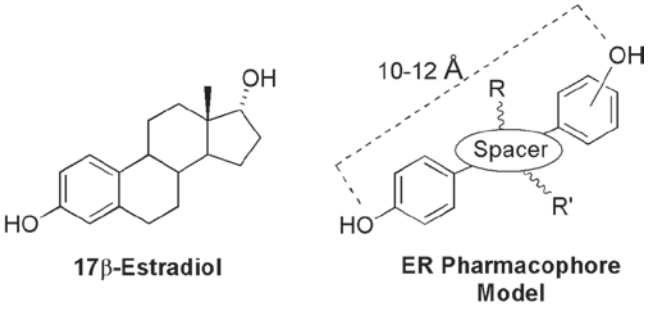

Figure 1. 17-β estradiol and a simplified model of the ER pharmacophore.

hetero-cycles[21], five-membered rings being a common choice[22–24] and leading to well known leads such as raloxifen[25]. Quite surprisingly, simpler spacing substructures have been scarcely employed[26,27] and we were therefore prompted to explore if simpler functional groups could be engaged in the preparation of efficient estrogen analogs. In particular, we were interested in the use of a simple carboxylic amide as the central section of novel potential ER ligands. The amide group can be readily assembled from carboxylic acids and amines precursors, each of them bearing the required structural features; its size is such that the phenolic rings may be positioned with a correct OH-OH distance. The restricted rotation around the C-N bond imparts an overall molecular rigidity while maintaining a residual conformational mobility. The nitrogen atom may be eventually decorated with substituents in order to fine-tuning the lipophilicity of the molecule. Finally, the easy formation of the amide functional group is compatible with solid-phase and combinatorial synthetic methods, allowing eventual wide-range screening to be performed with limited synthetic efforts.

In this work, we report the preparation of a small library of eight polyhydroxy-*N*-arylbenzamides (Figure 2), and preliminary tests on their activity towards ERs.

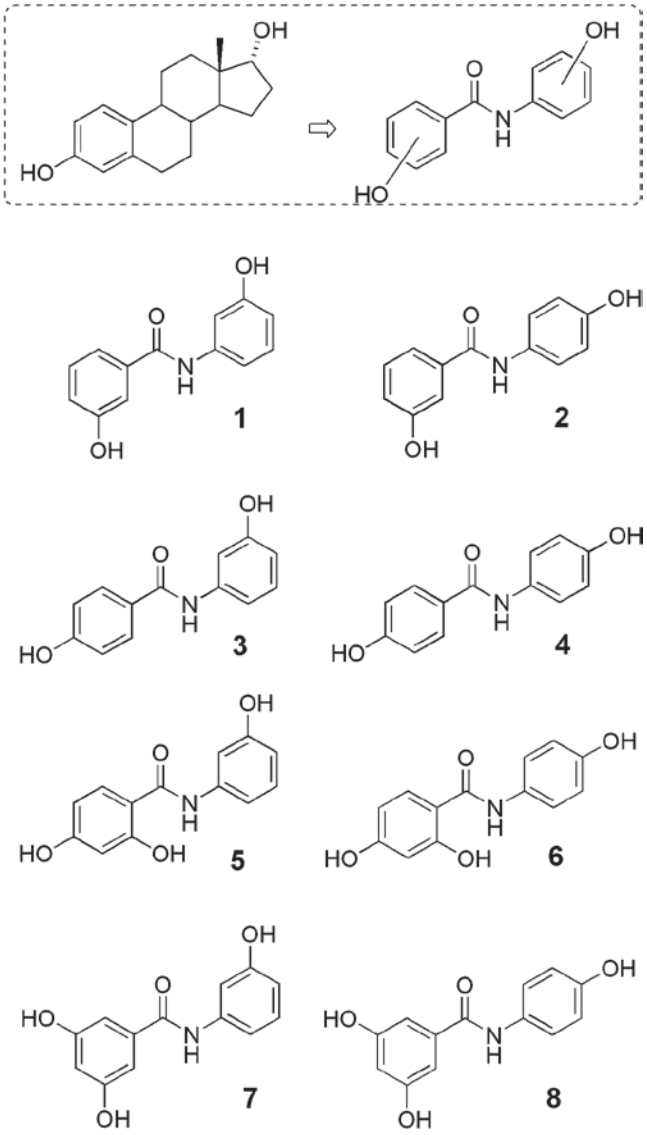

Figure 2. *N*-arylbenzamides library.

Material and methods

General procedure for the synthesis of compounds (1–8)

The aminophenol (1.0 mmol), the hydroxybenzoic acid (1.0 mmol) and EDC (*N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride, 1.2 mmol) were dissolved in acetone (5 mL). The mixture was stirred and refluxed 12 h under N_2 atmosphere. The solvent was evaporated and the residual solid was dissolved in ethyl acetate and washed three times with 1 M aq HCl, and 10% aq Na_2CO_3. The organic phase was dried over Na_2SO_4 and evaporated in vacuum to give the desired polyhydroxyamide.

3-Hydroxy-N-(3-hydroxyphenyl)benzamide (1)

^{1}H NMR (300 MHz, CD_3OD) δ 7.34 (dt, J = 7.5, 1.5 Hz, 1H), 7.31–7.27 (m, 3H), 7.14 (t, J = 8.0 Hz, 1H), 7.06 (ddd, J = 8.2, 1.2, 1.2 Hz, 1H), 6.97 (ddd, J = 7.8, 2.5, 1.4 Hz, 1H), 6.58 (ddd, J = 8.0, 2.5, 1.2 Hz, 1H) ppm; ^{13}C NMR (75.4 MHz, CD_3OD) δ 167.7, 157.5, 139.6, 136.5, 129.4, 129.2, 118.5, 118.1, 114.2, 112.1, 113.3, 108.1 ppm; MS(EI): 230.2 ($M{\cdot}H^+$); m.p. 187.5–188.7°C.

3-Hydroxy-N-(4-hydroxyphenyl)benzamide (2)

^{1}H NMR (300 MHz, CD_3OD) δ 7.42 (d, J = 8.8 Hz, 2H), 7.37-7.28 (m, 3H), 6.87 (ddd J = 7.8, 2.4, 1.4 Hz, 1H), 6.78 (d, J = 8.8, Hz, 2H) ppm; ^{13}C NMR (75.4 MHz, CD_3OD) δ 167.5, 157.6, 154.4, 136.4, 130.2, 129.3, 123.1, 118.4, 118.0, 114.9, 114.1 ppm; MS(EI): 230.1 ($M{\cdot}H^+$); m.p. 208.0–209.2°C.

4-Hydroxy-N-(3-hydroxyphenyl)benzamide (3)

^{1}H NMR (300 MHz, CD_3OD) δ 7.80 (d, J = 8.9 Hz, 2H), 7.26 (t, J = 2.3 Hz 1H), 7.13 (t, J = 8.0 Hz, 1H), 7.05 (ddd, J = 8.1, 2.3, 1.1 Hz, 1H), 6.86 (d, J = 8.6 Hz, 2H), 6.56 (ddd, J = 8.1, 2.3, 1.1 Hz, 1H) ppm; ^{13}C NMR (75.4 MHz, CD_3OD) δ 167.4, 161.0, 157.5, 139.8, 131.4, 129.4, 129.1, 125.7, 114.8, 112.2, 111.1, 108.2 ppm; MS(EI): 230.1 ($M{\cdot}H^+$); m.p. 200.8–201.6°C.

4-Hydroxy-N-(4-hydroxyphenyl)benzamide (4)

^{1}H NMR (300 MHz, CD_3OD) δ 7.79 (d, J = 8.6, Hz, 2H), 7.40 (d, J = 8.9 Hz 2H), 6.85 (d, J = 8.9 Hz, 2H), 6.77 (d, J = 9.2 Hz, 2H) ppm; ^{13}C NMR (75.4 MHz, CD_3OD) δ 168.6, 162.4, 155.6, 131.7, 130.5, 126.7, 124.5, 116.2 ppm; MS(EI): 230.2 ($M{\cdot}H^+$); m.p. 198–199.5°C.

2,4-Dihydroxy-N-(3-hydroxyphenyl)benzamide (5)

^{1}H NMR (300 MHz, CD_3OD) δ 7.80 (d, J = 8.9 Hz, 1H), 7.23 (t, J = 2.1 Hz, 1H), 7.14 (t, J = 8.0 Hz, 1H),7.06 (dd, J = 8.7, 2.3 Hz, 1H) 7.00 (bd, J = 8.0 Hz, 1H), 6.57 (ddd, J = 8.0, 2.4, 0.9 Hz, 1H) 6.33 (d, J = 2.4 Hz, 1H) ppm; ^{13}C NMR (75.4 MHz, CD_3OD) δ 167.9, 162.8, 161.7, 157.6, 139.1, 129.9, 129.2, 112.3, 111.2, 108.3, 108.2, 107.4, 102.6 ppm; MS(EI): 246.1 ($M{\cdot}H^+$); m.p. 214.9–215.8°C.

2,4-Dihydroxy-N-(4-hydroxyphenyl)benzamide (6)

^{1}H NMR (300 MHz, CD_3OD) δ 7.76 (d, J = 8.9 Hz, 1H), 7.36 (d, J = 8.6 Hz, 2H), 6.77 (d, J = 8.3 Hz, 2H), 6.39-6.29 (m, 2H) ppm; ^{13}C NMR (75.4 MHz, CD_3OD) δ 168.2, 162.3, 162.0, 154.5, 129.6, 129.5, 123.6, 115.0, 107.9, 107.3, 102.7 ppm; MS(EI): 246.2 ($M{\cdot}H^+$); m.p. 201.4–201.8°C.

3,5-Dihydroxy-N-(3-hydroxyphenyl)benzamide (7)

^{1}H NMR (300 MHz, CD_3OD) δ 7.26 (t, J = 2.2 Hz, 1H), 7.21 (t, J = 8.0 Hz, 1H), 7.05 (m, 1H), 6.78 (d, J = 2.2 Hz, 2H) 6.58 (ddd, J = 7.8, 2.4, 1.0 Hz, 1H), 6.44 (t, J = 2.3, Hz, 1H) ppm; ^{13}C NMR (75.4 MHz, CD_3OD) δ 169.2, 159.9, 158.8, 140.9, 138.6, 130.4, 113.4, 112.5, 110.0, 107.0, 106.7 ppm; MS(EI): 246.1 ($M{\cdot}H^+$); m.p. 220.5–221.1°C.

3,5-Dihydroxy-N-(3-hydroxyphenyl)benzamide (8)

^{1}H NMR (300 MHz, CD_3OD) δ 7.41 (d, J = 8.8 Hz, 2H), 6.78-6.74 (m, 3H), 6.44 (t, J = 2.1 Hz, 1H) ppm; ^{13}C NMR (75.4 MHz, CD_3OD) δ 169.0, 158.8, 154.6, 137.4, 130.5, 124.4, 115.2, 105.2, 105.7 ppm; MS(EI): 246.2 ($M{\cdot}H^+$); m.p. 229.7–230.8°C.

Biological methods

Plasmids

Estrogen responsive element-luciferase (ERE-Luc), kindly provided by Dr. J-M. Renoir, is a reporter for estrogen driven transcriptional activity, where the Firefly luciferase gene expression is under the control of the ERE. Cyto Megalo Virus (CMV)-hERα present the constitutive expression of the human ER α drove by the strong promoter of CMV, it allows an high expression of the human ER α. The pGL4.73 Renilla Luciferase plasmid from Promega (Milan, Italy) presents an elevated expression of the Renilla luciferase gene and served as transfection normalizer.

Cell culture, transfection and luciferase assay

HEK293 cell line was cultured in RPMI-1640, 10% fetal calf serum (Invitrogen, USA), Penicilin/streptomicyn 1% (Sigma Aldrich, Germany). At the day of transfection, 10000 cells were seed in a 96 well plate in 50 µL of OPTI-MEM (Invitrogen, USA) Penicilin/streptomicyn 1% (Sigma Aldrich, Germany) and 1% DCC stripped medium (Invitrogen, USA). After 6 h, cells were transfected by 0.2 µL of Lipofectamine (Invitrogen, USA) mixed with 33 ng of reporter plasmid, ERE-Luc, 33 ng of CMV-ERa and 33 ng pGL4.73 Renilla luciferase (Promega, as a normalizer of transfection). Sixteen hours after transfection, cells were treated by the compounds for 24 h. All compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, Germany). DMSO treatment served as negative control.

Thereafter, luciferase assay was performed by Dual-Glo luciferase assay (Promega, USA), following the producer indication. The sample-value of firefly luciferase was devided by the relative renilla luciferase value to normalize the transfection level.

Statistic was performed according to the *t*-student test.

Results and discussion

Dihydroxy-*N*-phenylbenzamides **1**–**4** are the simplest members of the library and are derived from all the possible combinations of 3- and 4-hydroxybenzoic acids with 3- and 4-aminophenols. The aminophenols were retained even in compounds **5**–**8**, while including an additional hydroxyl group in the benzoic acid counterpart. Trihydroxyamides **5**–**6** originate from 2,4-dihydroxybenzoic acid, seeking the formation of an intramolecular hydrogen bond between the additional 2-OH group and the amide group with a potential gain in the conformational rigidity. Entries **7**–**8** embody two OH groups exchangeable through rotation of their aryl ring around the Ar-C=O single bond, thereby doubling the hydrogen bond ability of this aryl ring.

Transcriptional activity of estrogen is a straightforward method to study estrogenic potential of a compound[28]. To evaluate the possibility that these compounds exhibit an estrogenic activity we transfected HEK293 cells with three plasmids: Estrogen responsive element-luciferase (to monitor estrogenic activity), CMV-hERα (to provide an high expression of ERα in the cell line[29])and Renilla luciferase pGL4.73 (Promega, as a normalizer of transfection). Cells were treated with Phenol Red-free medium and stripped bovine serum for 24 h at the reported concentration, and then luciferase signal was measured by Dual-Glo luciferase assay (Promega) and reported in Figure 3. Cells that are not transfected with CMV-hERα, did not produce any firely luciferase signal (data not shown).

A preliminary test of estrogen activity was conducted for all compound exposing the cells after transfection by the reporter plasmid to 100 nM of drug, only compounds **3**, **4**, **6** and **7** showed a statistical significance ($p < 0.05$). Consequently, a dose/effect curve was performed for **3**, **4**, **6** and **7** (Figure 3). Compounds **3** and **4** resulted the most active with an estrogenic activity at 100 nM of 219% and 335%, respectively compared to the control. Compound **3** showed the most interesting agonistic activity, maintaining a statistically significant agonistic activity up to 10 nM.

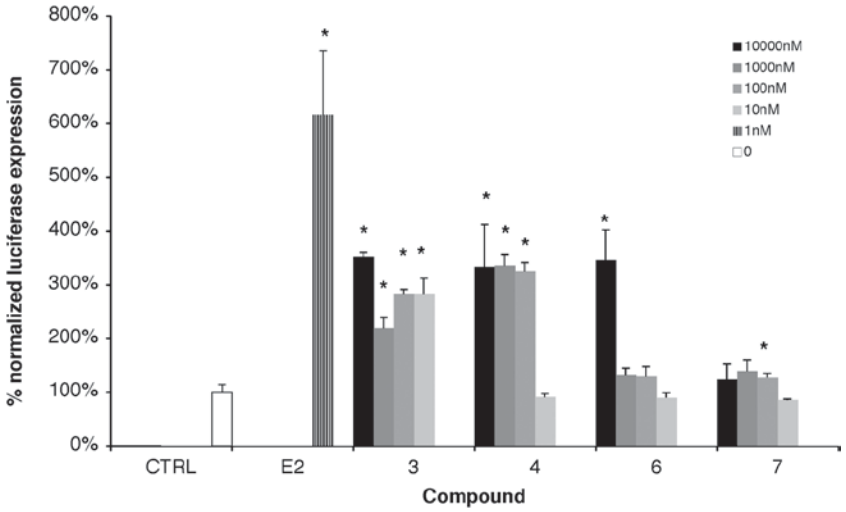

Figure 3. Normalized firefly luciferase level reporting the estrogenic activity of the compounds after 24 h exposure, compared to EtOH-treated control. E2 (17-β estradiol) served as positive control for the assay.

These data allow extrapolating some indications on the structure-activity relationships. Amides **3** and **4** are characterized by the presence of the 4-OH group, closely superimposable to the 3-OH group of 17β-estradiol, known to be essential for the activity. The presence of an additional 2-OH groups on the benzoic acid counterpart results in the reduction or loss of activity, probably as a result of a different conformation deriving from the intramolecular hydrogen bond or steric hindrance. It is worthwhile to note that amide **2** could be considered the "inverted" amide of the active entry **3** and this inversion of the carboxylic acid and the amine moiety results in the nearly complete loss of the activity; the 4-OH group should be located in the benzoic acid moiety, the latter playing the role of the A ring of 17β-estradiol.

Conclusions

In conclusion, we prepared a series of *N*-phenylbenzamides with the aim to produce a minimal structure that retains the estrogen pharmacophores hallmark. Here we demonstrated that the one-step condensation of an aminophenol with hydroxybenzoic acids results in simple amides that can satisfy some of the key structural features required in the interaction with ERs. In particular, compound **3**, presenting a structure that can be easily superimposed to the natural ER ligand, shows the higher agonistic activity, confirming the quality of the structural design. The simplicity of the reported scaffold paves the way for a wide array of potential structural variations, through a fine-tuning of the substitution pattern and following the known guidelines for the design of ER ligands. The formation of amide bond is simple, reliable and easily automated, opening the possibility for large combinatorial screening on this scaffold. Pharmacology benefits from the use of combinatorial chemistry and high throughput assay screen exploited to find new molecules with defined characteristics. Here, we show that an almost unlimited combination of different substructures, maintaining few pharmacophores hallmarks in molecules presenting elevated versatility, could be used to generate large libraries that can be produced and tested in an automated manner, with a potential impact in the field of drug discovery.

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Declaration of interest

The authors report no conflicts of interest.

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