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Synthesis and Evaluation of Benzoxazolinonic Imidazoles and Derivatives as Non-steroidal Aromatase Inhibitors

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New compounds were tested *in vitro* on aromatase activity in human placental and equine testicular microsomes. Equine aromatase, very well characterized biochemically, is used as a comparative model to understand the mechanism of aromatase inhibition. Among 15 molecules screened, 5 of them (11–15) strongly inhibit human and equine aromatases with IC_{50} values ranging from 13–85 nM and from 23–103 nM respectively. These results were corroborated by K_i/K_m values. Moreover, spectral studies showed a type II spectrum with both enzymes, which is characteristic of an interaction between the nitrogen atom of the molecule and the heme of the cytochrome P450. Compound 12, which has the lowest IC_{50} and K_i/K_m ratio, inactivates aromatase in a dose and time-dependent manner. This might be very important for the treatment of estrogen-dependent diseases such as breast cancer. Finally, MTT assays on E293 cells revealed that the molecules were not cytotoxic.

Keywords: Aromatase; Estrogens; Breast cancer; Inactivation; Cytochrome P450; Equine; CYP 19; Non-steroidal inhibitors; Inhibition

INTRODUCTION

Aromatase is the enzymatic complex formed by the specific cytochrome P450 aromatase (aromatase herein) and an ubiquitous flavoprotein NADPH cytochrome P450 reductase, which catalyses the aromatisation of androgens to estrogens. This enzyme is expressed in a broad array of tissues including some that are generally considered nonsteroidogenic.[1] Additionally, tumors from numerous sites such as breast,[2,3] gonads[4] and prostate[5] have been shown to express P450 aromatase. In postmenopausal women, androstenedione produced by the adrenal steroidogenic pathway is converted by peripheral and breast aromatase to estrone and then by the action of 17β-hydroxysteroid dehydrogenase type I to the potent estrogen, estradiol. Therefore, aromatase has been the target for the design of numerous inhibitors as agents in the treatment of breast cancer in postmenopausal women particularly after relapse.[6] Inhibition of aromatase reduces plasma estrogen levels and consequently the stimulus to growth of estrogen-dependent metastases. Over the last few years, a number of aromatase inhibitors have been introduced for such endocrine therapy.[7] Initially, aminoglutethimide found a clinical application as a possible therapeutic aromatase inhibitor for breast cancer.[8] However, it also inhibits the cholesterol side chain cleavage reaction catalysed by P450scc (cholesterol side chain cleavage enzyme), resulting in a deficiency of glucocorticoids and mineralocorticoids in addition to sex steroids. 4-Hydroxyandrostenedione (4-OHA) was subsequently utilized as a potent and specific steroidal inhibitor,[9] functioning as a mechanism based inhibitor or a suicide substrate and causing time-dependent inactivation in the presence of cofactors.[10] More recently, more potent and selective non-steroidal inhibitors of imidazole, triazole and tetrazole derivatives have been developed and clinically examined.[6] There have been many reports concerning development and clinical trials of new inhibitors for breast cancer patients but

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the response rate or the duration of remission with these drugs are far from satisfactory.[11] The need for clinical drugs with increased specificity, clinical efficiency and tolerability remains a challenge in the development of new compounds.[12,13] Discovery of new drugs may be realized by screening molecules derived from already known inhibitors or natural substrates of aromatase, but also with greater precision by taking into account our understanding of the enzyme active site structure.

Today's available model of human aromatase was deduced by default from comparative sequence homology studies with identified structures of soluble bacterial cytochrome P450 which have been crystallised.[14] To increase our knowledge of the enzyme structure, and consequently to obtain improved inhibitors, we considered that it was crucial to develop new mammalian comparative models. Thus, in our laboratory, we used equine testicular aromatase which is the only easy and available mammalian membrane-bound model, besides the human one, and well characterized at the biochemical level and cloned.[15–17] By comparing the inhibition effects of compounds on both human and equine enzymes and with the help of a molecular model, potent inhibitors of aromatase have been already designed by our group.[12,18] During our investigation towards the synthesis of new non steroidal aromatase inhibitors we decided to replace the indole heterocycle of some azoles derivatives recently described[19,20] by the benzoxazolinone heterocycle and some of its derivatives (benzothiazolinone, benzoxazinone).

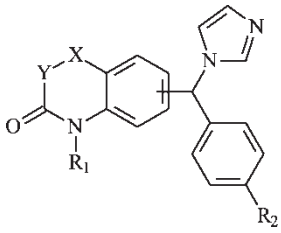
In this work, we evaluated this new series of compounds on aromatase inhibition and characterized their mechanism of action.

MATERIALS AND METHODS

General

All chemical products were obtained from Sigma (St Quentin Fallavier, France), [1β, 2β-^{3}H]-androstenedione from Dupont NEN (Les Ulis, France), and solvents from Carlo Erba (Val de Reuil, France) and sds (Peypin, France). Human embryonal kidney E293 cell line (ECACC number: 85120602) was provided by CERDIC (Sophia-Antipolis, France).

TABLE I Structures of compounds **11–15**

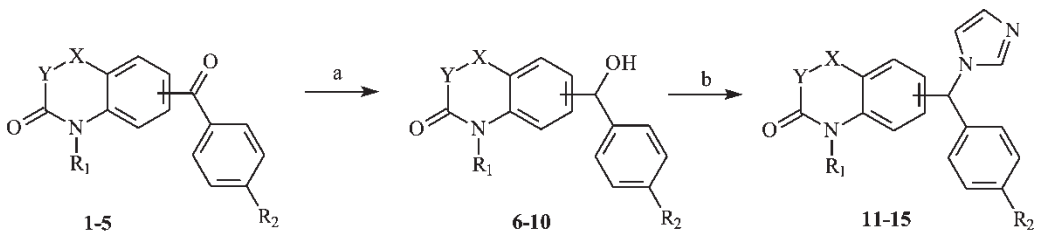


Compound	X	Y	R_1	R_2	Isomer
11	O	–	H	H	6
12	S	–	CH_3	H	6
13	O	–	CH_3	CN	6
14	O	–	CH_3	H	5
15	O	CH_2	CH_3	H	7

Chemistry

Melting points were determined on a Büchi SMP-535 apparatus and are uncorrected. Column chromatography was carried out using silica gel (silica gel 60, 70–230 Mesh, ASTM, Merck) with an appropriate solvent. IR spectra were recorded on a Vector 22 Bruker spectrophotometer. ^{1}H-NMR spectra were recorded on a Bruker AC 300 P spectrometer. Chemical shifts are reported in δ units (ppm) relative to $(Me)_4Si$. Elemental analyses (C, H, N) for final compounds were performed by CNRS Laboratories (Vernaison, France). Obtained results were within 0.4% of the theoretical values.

The synthesis of compounds **11–15** (Table I) is outlined in Scheme 1. Acylated derivatives **1–5** were obtained according to previously described procedures.[21–25] Reduction of the ketonic group of compounds **1–5** with sodium borohydride in methanol gave the secondary alcohols **6–10** in good yields. Treatment of these alcohols with 1,1′-carbonyldiimidazole in THF led to moderate yields of the azoles derivatives **11–15**.



Reagents: (a) $NaBH_4$, CH_3OH ; (b) carbonyldiimidazole, THF.

SCHEME 1 Synthesis of compounds **11–15** (see Materials and Methods).

General Procedure for the Synthesis of Hydroxy Derivatives (6–10)

The method adopted for the synthesis of (*R,S*)-6-[1-hydroxy-1-phenylmethyl]-1,3-benzoxazol-2(3*H*)-one (**6**) is described. Sodium borohydride (70 mmol) was added over a period of 15 min to a solution of 6-benzoyl-1,3-benzoxazol-2(3*H*)-one (**1**) (35 mmol) in methanol (150 mL). The reaction mixture was stirred for 1 h at room temperature, and acidified with 10% hydrochloric acid. Methanol was evaporated and the resulting precipitate was filtered, dried and recrystallised from methanol to give pure **6** in 75% yield: mp 143–144°C; IR (*ν*) 3280, 3150, 1750 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.69 (d, 1H, CH), 5.95 (d, 1H, OH), 7.10 (d, 1H, H_4), 7.15–7.50 (m, 6H, H_5, H phenyl), 7.60 (d, 1H, H_7), 10.00 (br s, 1H, NH, exchangeable with D_2O).

(*R,S*)-6-[1-Hydroxy-1-phenylmethyl]-3-methyl-1,3-benzothiazol-2(3*H*)-one (**7**)

Recrystallization from ethanol gave pure **7** in 84% yield; mp 127–129°C; IR (*ν*) 3400, 1640 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.40 (s, 3H, CH_3), 5.26 (d, 1H, CH), 5.50 (d, 1H, OH), 6.60–7.00 (m, 7H, H_4, H_5, H phenyl), 7.20 (d, 1H, H_7).

(*R,S*)-6-[1-Hydroxy-1-(4-cyanophenylmethyl)]-3-methyl-1,3-benzoxazol-2(3*H*)-one (**8**)

Recrystallization from ethyl acetate gave pure **8** in 90% yield; mp 145–146°C; IR (*ν*) 3440, 2227, 1752 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.42 (s, 3H, CH_3), 5.85 (d, 1H, CH), 6.25 (d, 1H, OH), 7.0–7.20 (m, 2H, H_4, H_5), 7.33 (d, 1H, H_7), 7.58 (d, 2H, $H_{3'}$, $H_{5'}$), 7.77 (d, 2H, $H_{2'}$, $H_{6'}$), 10.11 (br s, 1H, NH, exchangeable with D_2O).

(*R,S*)-5-[1-Hydroxy-1-phenylmethyl]-3-methyl-1,3-benzoxazol-2(3*H*)-one (**9**)

Recrystallization from ethanol–water (2:1) gave pure **9** in 88% yield; mp 106–109°C; IR (*ν*) 3464, 1741, 1620 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 3.31 (s, 3H, CH_3), 5.74 (d, 1H, CH), 5.98 (d, 1H, OH), 7.10 (dd, 1H, H_6) 7.17–7.24 (m, 2H, H_4, H_7), 7.32–7.38 (m, 5H, H phenyl).

(*R,S*)-7-(1-Hydroxy-1-phenylmethyl)-4-methyl-1,4-benzoxazin-3(4*H*)-one (**10**)

Recrystallization from ethanol–water (2:1) gave pure **10** in 84% yield; mp 116–121°C; IR (*ν*) 3443, 1657, 1614 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 3.23 (s, 3H, CH_3), 4.60 (s, 2H, H_2), 5.64 (d, 1H, CH), 5.89 (d, 1H, OH), 6.97 (d, 1H, H_5), 7.03–7.09 (m, 2H, H_6, H_8), 7.17–7.38 (m, 5H, H phenyl).

General Procedure for the Synthesis of Imidazole Derivatives (11–15)

The method adopted for the synthesis of (*R,S*)-6-[1-(imidazol-1-yl)-1-phenylmethyl]-1,3-benzoxazol-2(3*H*)-one (**11**) is described. Compound **6** (10 mmol) and *N,N'*-carbonyldiimidazole (10 mmol) in THF (60 mL) were stirred at reflux for 2 h. The resulting reaction mixture was evaporated under reduced pressure. The residue was taken up with water, acidified with an aqueous solution of 6 M HCl and extracted with ethyl acetate. The aqueous layer was basified to pH 9 with a 10% solution of potassium carbonate and extracted with ethyl acetate. The organic phase was washed with water, dried over anhydrous $MgSO_4$, and evaporated. The residue was purified by column chromatography (silica gel) with ethyl acetate–methanol (9:1) to give pure **11** in 65% yield: mp 193–195°C; IR (*ν*) 3422, 1774 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 6.90 (s, 1H, CH), 6.97–6.99 (m, 2H, H_4, H_5), 7.10–7.14 (m, 5H, H phenyl), 7.34–7.42 (m, 3H, H_7 and H_4, H_5 imidazole), 7.66 (s, 1H, H_2 imidazole), 11.77 (br s, 1H, NH, exchangeable with D_2O). Anal. ($C_{17}H_{13}N_3O_2$) C, H, N.

(*R,S*)-6-[1-(Imidazol-1-yl)-1-phenylmethyl]-3-methyl-1,3-benzothiazol-2(3*H*)-one (**12**)

Purification by column chromatography (silica gel) with ethyl acetate–methanol (8:2) gave pure **12** in 55% yield; mp 65–68°C; IR (*ν*) 1681 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.46 (s, 3H, CH_3), 6.57 (s, 1H, CH), 6.85–7.15 (m, 7H, H_4, H_5, H phenyl), 7.37–7.42 (m, 4H, H_7, and H imidazole). Anal. ($C_{18}H_{15}N_3OS$) C, H, N.

(*R,S*)-6-[1-(Imidazol-1-yl)-1-(4-cyanophenylmethyl)]-3-methyl-1,3-benzoxazol-2(3*H*)-one (**13**)

Purification by column chromatography (silica gel) with ethyl acetate–methanol (9:1) gave pure **13** in 54% yield; mp 85–87°C; IR (*ν*) 2228, 1780 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.43 (s, 3H, CH_3), 6.63 (s, 1H, CH), 6.83–6.98 (m, 4H, H_4, H_5 and $H_{4''}$, $H_{5''}$ imidazole), 7.16–7.20 (m, 3H, H_7,$H_{2'}$, $H_{6'}$), 7.44 (s, 1H, $H_{2'}$), 7.68 (d, 2H, $H_{3'}$, $H_{5'}$). Anal. ($C_{19}H_{14}N_4O_2$) C, H, N.

(*R,S*)-5-[1-(Imidazol-1-yl)-1-phenylmethyl]-3-methyl-1,3-benzoxazol-2(3*H*)-one (**14**)

Purification by column chromatography (silica gel) with ethyl acetate–methanol (8:2) gave pure **14** in 82% yield; mp 133–135°C; IR (*ν*) 1781 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.34 (s, 3H, CH_3), 6.58 (s, 1H, CH), 6.68 (d, 1H, H_4), 6.85–6.88 (m, 2H, H_6, H_7), 7.09–7.41 (m, 8H, H imidazole and phenyl). Anal. ($C_{18}H_{15}N_3O_2$) C, H, N.

(*R,S*)-7-[1-(Imidazol-1-yl)-1-phenylmethyl]-4-methyl-1,4-benzoxazin-3(4*H*)-one (**15**)

Purification by column chromatography (silica gel) with ethyl acetate–ethanol (8:2) gave pure **15** in 45% yield; mp 66–68°C; IR*ν* 1686 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.36 (s, 3H, CH_3), 4.62 (s, 2H, CH_2), 6.47 (s, 1H, CH), 6.75 (d, 1H, H_8), 6.79 (dd, 1H, H_6), 6.86–7.42 (m, 9H, H imidazole and phenyl), 7.42 (s, 1H, $H_{2''}$ imidazole). Anal. ($C_{19}H_{17}N_3O_2$) C, H, N.

Biochemistry

Preparation of Microsomes

Human placental and equine testicular microsomes were prepared as described previously.[26] In each instance the tissue with high specific aromatase activity was taken. Briefly, fresh tissue washed with 0.50 M KCl was first homogenized in 50 mM phosphate buffer pH 7.5 containing 0.25 M sucrose, 1 mM DTT, and 4 μM androstenedione in order to preserve the enzyme active site and then centrifuged at 20000 × *g*. The supernatant was further ultra centrifuged at 100000 × *g*. The final pellet was dissolved in the same buffer containing 20% glycerol, 1 mM DTT, 0.2 mM EDTA-4 Na, 4 μM androstenedione, and stored at −80°C until use. Protein concentration was evaluated according to Bradford[27]using bovine serum albumin as standard.

Inhibition Studies

Aromatase activity was evaluated by measuring ^3H_2O released from 200 nM [1β, 2β-^{3}H] androstenedione (a 2 μM substrate solution was prepared by adding 3.5 nmol of tritiated androstenedione with specific activity 1554 GBq/mmol, to 76.5 nmol non-radioactive androstenedione in 40 mL final volume of ethanol) at 37°C during 15 min according to Thompson and Siiteri[28] in the presence of various inhibitors from 0–12 μM. Reactions with microsomes (16 and 20 μg of human and equine microsomal proteins respectively for determination of IC_{50} values) were initiated by adding 60 μM $NADPH,H^+$ to a final volume of 0.5 mL Tris–maleate buffer, 50 mM pH 7.4, and stopped by adding 1 mL of chloroform. Steroids were then extracted by incubation with a charcoal–dextran solution (7:1.5%) and the radioactivity of the aqueous phase was measured as previously described.[26] Control incubation was realized by incubating microsomes, substrate and inhibitors without $NADPH,H^+$ under the same conditions. The results were the mean of triplicate experiments ± SD and were expressed as pmol estrogen formed/min mg microsomal proteins.

Kinetic Studies

Concentration range for inhibitors and substrate were respectively 0–15 and 3–24 nM. Aromatase activity was evaluated in the same Tris–maleate buffer by incubating 5 μg of microsomal protein with substrate and various inhibitors at 37°C during 12 min for human aromatase or 10 min for the equine enzyme. These times have been determined to evaluate aromatase activity at the exponential portion of the Michaelis–Menten plot. K_m and K_i values were determined graphically by using respectively LineweaverBurk and Dixon plots.

Inactivation Studies

Inactivation studies were carried out on 16 μg of human microsomal proteins by testing different incubation times (0–30 min) at 25°C with inhibitors and 60 μM $NADPH,H^+$ in 0.1 M Na-phosphate buffer, pH 7.5, containing 0.5 M sucrose as previously described.[16] Incubations were stopped by adding a charcoal–dextran mixture (2:1%) and after centrifugation, aromatase activity was assayed using 300 μL of the aqueous phase and labelled androstenedione as described above.

Spectral Studies

Absorbance of microsomal proteins (1 mg) in 1.5 mL of 50 mM Tris–maleate, pH 7.4, was measured with a Kontron–Uvikon 860 spectrophotometer. Difference absorption spectra were recorded at 37°C over 300–500 nm after addition of 100 μM of each inhibitor diluted in ethanol (16 μL). The spectrum of ethanol was comparable to the baseline (300–500 nm). The spectra of microsomes alone and of the inhibitor alone were subtracted from the spectrum of the incubation of microsomes with the inhibitor.

Cell Viability

The MTT reduction assay is an enzymatic test based on the activity of mitochondrial dehydrogenase enzymes. This test developed by Mosmann[29] has been adapted to evaluate the viability of E293 cells with inhibitors after various times of incubation (24, 48 and 96 h). Cells were grown-up on 24-wells cell culture plates in EMEM red phenol free media containing 2 mM glutamine, 100 U/ml of antibiotics (mix of penicillin, streptomycin and fungizon), 10% heat-inactived fetal calf serum and 1% non essential amino acid and 10 μM of inhibitors. After incubation, the medium was aspirated and 500 μL of MTT solution (stock solution of 5 mg MTT/mL PBS filtered through a 0.22 μm filter and diluted 5X in sterile EMEM medium) was added to each dish to be tested. Plates were incubated at 37°C during 3 h. Reactions were stopped by addition of an equal volume of isopropanol containing 0.04 N HCl. The plates were shaken for 30 min at room temperature. Absorbances were measured at 565 and 705 nm for the test and reference respectively.

Statistical Studies

Data were compared using a unilateral *t*-test (ANOVA).

RESULTS

The initial screening assays have revealed that 5 compounds (**11**–**15**) strongly inhibited both enzymes. The other molecules presented IC_{50} values more than 1 μM and were omitted from further analysis. For comparative purposes, we also included three compounds known as effective aromatase inhibitors, 4-OHA,[30] fadrozole[31] and MR20494.[12] As shown in Table II, we obtained 5 potent derivatives that inhibited human and equine aromatases with IC_{50} values ranging from 13–85 nM and from 23–103 nM respectively. Among them, compound **12** was the most potent with an IC_{50} which is at least 3 times lower than that of fadrozole ($p < 0.001$ and $p < 0.05$ for both enzymes respectively), whereas IC_{50} values of the four other derivatives (**11**, **13**–**15**) are in the same range as fadrozole. In order to characterize the binding within the active sites, aromatisation of androstenedione was measured at several inhibitor and substrate concentrations. Analysis of Dixon plots gave the apparent K_i values and the results obtained are summarized in Table II. It was found that compounds **11**–**15** were very potent inhibitors since their K_i/K_m ratio values, which represented their relative inhibitory potency, ranged from 0.08–0.36 and 0.61–1.03 for human and equine aromatases respectively. These values are in the same range as those for fadrozole (K_i/K_m: 0.06 and 0.40 for both enzymes), but lower than those for 4-OHA (K_i/K_m: 1.9 and 1) and MR20494 (K_i/K_m: 0.5 and 6.1). Moreover, compounds **12**–**14** had K_i values significantly lower for human than equine enzyme. However, the order of affinity was the same for both enzymes (K_i/K_m values: **12** < **14** < **13**). Results of these studies were plotted on typical Lineweaver-Burk plots where five compounds, **11**–**15**, exhibited a mixed inhibition (competitive and non-competitive inhibitions) for human and equine enzymes, except for **15** and **13**, which showed a non-competitive inhibition for the equine aromatase. The Lineweaver-Burk plots obtained for **14** and **15** are shown in Figure 1 (part A and B as representative of the two types of inhibition). The kinetic mechanisms of the new compounds were examined under rate-limiting conditions for aromatase.

To further investigate the mechanism of aromatase inhibition, interaction between the 5 potent inhibitors and human placental and equine testicular microsomes was studied spectroscopically. It has been shown that nitrogen coordination of amines to the heme-iron of cytochrome P-450 gives rise to a characteristic spectral species termed a type II spectra.[32] In our study, we showed a type II difference spectrum for each compound tested with human and equine aromatases. Figure 2 is representative of a type II spectra obtained for **14** with equine testicular microsomes.

The newly synthesized compounds were then tested for their ability to act as time-dependent inactivators on the human microsomal enzyme. For comparative purposes 4-OHA was included, previously reported to be a time-dependent inactivator of aromatase.[33] Time-dependent inactivation of human aromatase was observed when **12** was incubated with microsomes in the presence of NADPH (Figure 3) but compared with time-dependent inactivation caused by 4-OHA, it is likely that **12** is a weak inactivator.

Finally, the cytotoxic results obtained with the E293 cells revealed that our derivatives were not cytotoxic, since the mitochondrial succinate-dehydrogenase activity was not significantly reduced in the presence of 10 μM inhibitor (Figure 4). The absorbance increased with time of cell culture. However, this concentration (10 μM) did not lead to cellular death after 24, 48 or 96 h; in fact cells were moderately growing up to 48 h and faster until 96 h.

DISCUSSION

In this study, experiments were carried out on human placental and equine testicular microsomes

TABLE II IC_{50} and kinetic parameters of compounds **11**–**15**, and of positive controls (4-OHA, fadrozole and MR20494) as inhibitors of human and equine aromatases

	IC_{50} (nM)[a]		K_i/K_m[b]		Inhibition type	
Inhibitors	Human	Equine	Human	Equine	Human	Equine
4-OHA	450 ± 57	580 ± 28	1.90	1.00	C	C
fadrozole	55 ± 5	62 ± 6	0.06	0.40	C	C
MR20494	111 ± 6	1341 ± 12	0.50	6.10	C	C
11	85 ± 6	103 ± 7	0.36	1.03	Mixed	Mixed
12	13 ± 2*	23 ± 3*	0.08	0.61	Mixed	Mixed
13	46 ± 6	78 ± 1	0.23	0.97	Mixed	NC
14	46 ± 8	50 ± 10	0.18	0.93	Mixed	Mixed
15	50 ± 6	59 ± 9	0.28	0.66	Mixed	NC

C: competitive; NC: non-competitive; Mixed: competitive and non-competitive. [a]: 200 nM of [1β-2β^{3}H]-androstenedione was incubated with 20 μg of human placental microsomes or 16 μg of equine testicular microsomes for 15 min. [b]: An apparent K_i value was obtained from a Dixon plot in which the apparent K_m for androstenedione was 12.3 and 6.8 nM for human and equine enzymes respectively (see Materials and Methods for the incubation conditions). *: $p < 0.001$ compared to fadrozole.

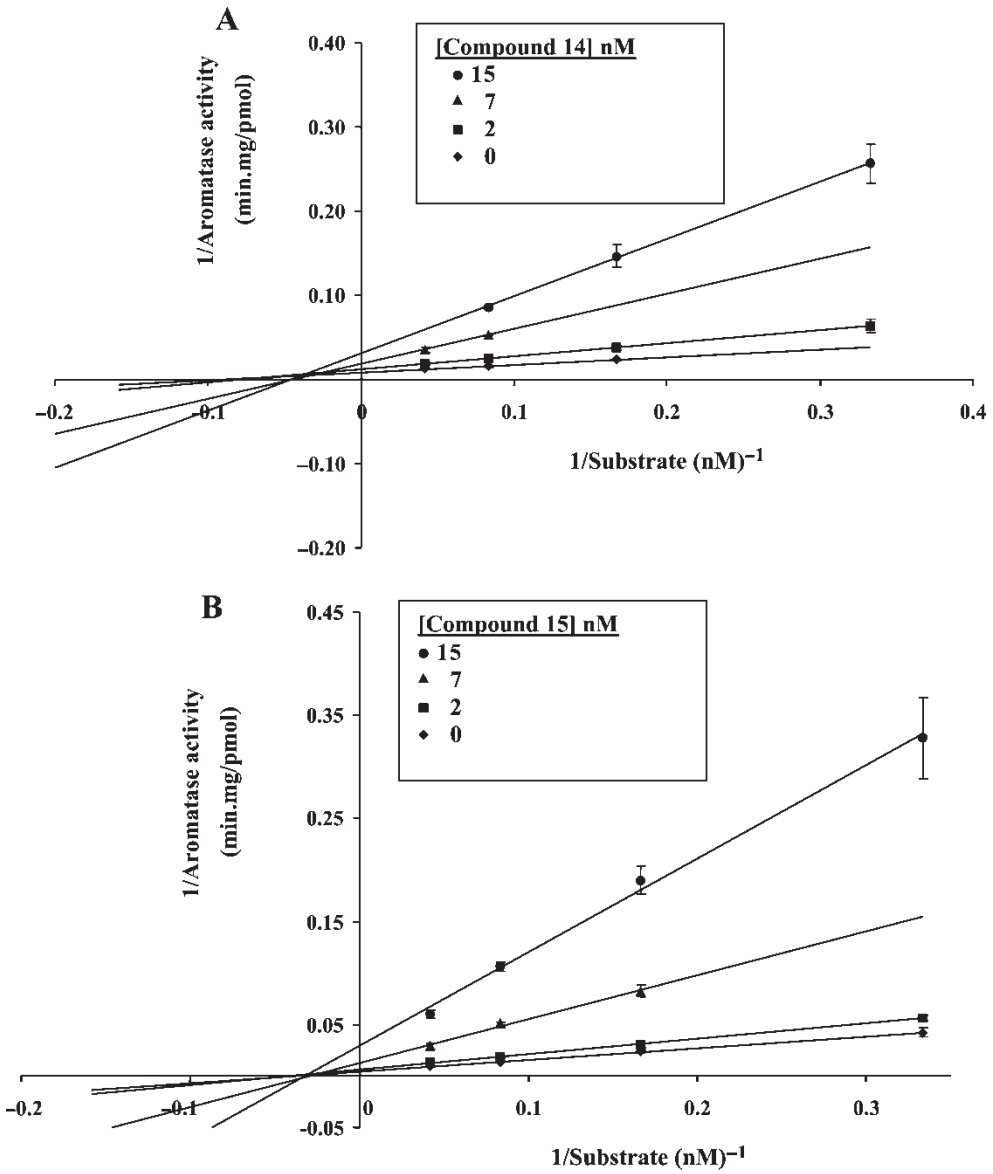


FIGURE 1 *Kinetic inhibition characteristics for human and equine aromatases*. Two types of aromatase inhibition are seen, with **14** and **15** respectively: a mixed type with the human enzyme (A) and a non-competitive with the equine model (B). Different concentrations of androstenedione (3, 6, 12 and 24 nM) were incubated with each enzyme and with 0 (◆), 2 (■), 7 (▲) and 15 (●) nM of inhibitor. Similar plots of mixed inhibitions were observed for all inhibitors with both enzymes except for **13** that showed a non-competitive inhibition with equine aromatase. Results are expressed as pmol estrogen formed $min^{-1}mg^{-1}$ microsomal proteins and are the mean of triplicate values ± S.D. Results are representative of three experiments showing similar profiles.

in order to evaluate a novel series of non-steroidal derivatives as potent aromatase inhibitors. Five of them, **11**–**15**, strongly inhibited human placental aromatase with an IC_{50} value ranging from 13–85 nM, **12** being the most potent. These values were significantly lower than those of MR20494 (IC_{50}: 111 ± 6 nM) and MR20492 (IC_{50}: 147 ± 1 nM), two indolizinone derivatives previously tested in our laboratory and known to be very potent aromatase inhibitors.[12] Moreover, these compounds were at least 5 times more potent than 4-OHA (IC_{50}: 450 ± 57 nM) and showed an inhibitor potency comparable or close to that of fadrozole (IC_{50}: 55 ± 5 nM). These differences could be explained by the benzothiazolinonic heterocycle, which is the main characteristic of **12**, and could present the most favorable stereo-electronic properties to facilitate access inside the active site. For the phenyl ring, and in contrast to other azoles series, we did not observe significant amelioration when a CN group was added (see **13**, Table I). On the other hand, no significant difference in activities was observed between the compounds **11** and **14** (see IC_{50} and K_i values) despite having a different substitution pattern on the benzoxazolinone ring. These interesting results were corroborated by K_i/K_m values

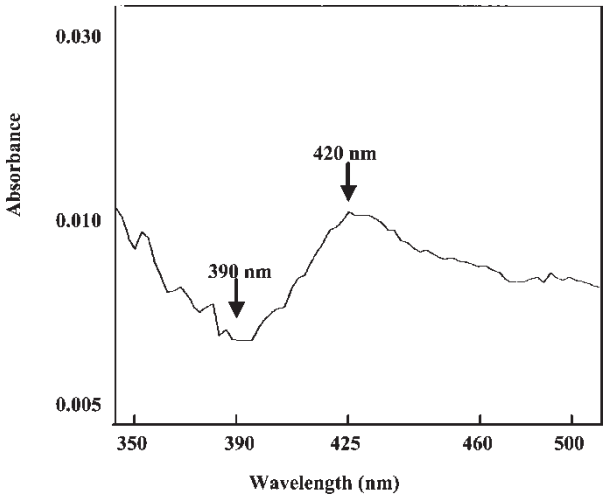


FIGURE 2 *Spectral studies of interactions between* ***14*** *and the aromatase active site*. Absorbance from 350 to 500 nm of microsomal proteins (1 mg) with 100 μM of inhibitor was measured at 37°C. The spectra of the microsomes or inhibitor alone were substracted from the spectrum measured during the incubation of microsomes with the chosen inhibitors. Results are representative of three experiments showing similar profiles with the equine enzyme. The interactions with the other compounds and the active site of human and equine aromatases gave essentially similar spectra (data not shown).

obtained for the five molecules. These compounds, tested *in vitro* on equine testicular microsomes, were also potent inhibitors of aromatase with an IC_{50} value ranging from 23–103 nM, but they were less potent on the equine than the human enzyme. These results were also confirmed by kinetic studies. The Lineweaver-Burk plot showed the type of inhibition and that the five molecules exert a mixed inhibition. This kind of inhibition is observed when the inhibitor is able to bind either the enzyme or the enzyme-substrate complex. However, a non-competitive inhibition was obtained only for **13** and **15** with the equine enzyme. These results together show the conformational differences between the human and equine models.[12,16,18] Studies of the interaction between the heme-iron and a substrate or an inhibitor has been conducted with cytochrome P450s,[34] as for example with aromatase.[18,28,35] In this study a type II spectrum, considered to be specific for an interaction between an heteroatom of the molecule and the heme-iron of the cytochrome was observed for all the molecules, with the minimal absorbance at 390 nm and a maximal one at 420 nm, for both the human and equine models. These results confirmed our previous studies in which it was shown that an inhibitor characterized by an interaction with the heme-iron is in general more potent.[18] Nevertheless, even if the interaction of these compounds with the heme-iron explains in part the mechanism of action, this stronger affinity for human aromatase underlines the complementarity of the inhibitors **11**–**15** with the active site, and the differences of structure between human and equine aromatase. Moreover, the *in vitro* properties of **12** as a potential non-steroidal inactivator are quite unique and promising.

In conclusion, 15 new compounds were tested here which were designed following up our previous work.[12,13,16,18] and five of them, **11**–**15**, were shown to be highly potent aromatase inhibitors. Since none of them has shown cytotoxicity on E293 cells, a further biological evaluation could confirm their selectivity profile, for example by testing with P450 17, P450 18 and P450scc.[36] Moreover, site-directed mutated aromatase could allow a better understanding of the inhibitor interactions. Our strategy used for several years concerning a detailed study of the structure–activity relationships, chemical synthesis, molecular modelling with

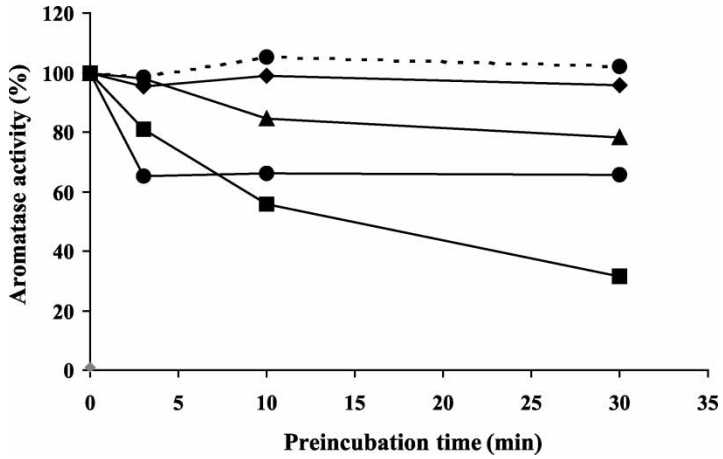


FIGURE 3 *Inactivation tests of human aromatase with compound* ***12***. Different concentrations of **12** (0 μM (◆), 0.5 μM (▲), 1 μM (●) were used with or without NADPH (–●–). Microsomal proteins (25 μg) from human placenta were preincubated with **12** or with 1 μM of 4-OHA (■) at 25°C during 0–30 min. 4-OHA was used as positive control. The preincubation was stopped by adding a charcoal/dextran mixture (2%:1%) and after centrifugation the aromatase activity was assayed using 300 μL of the aqueous phase. Results are expressed as % of control which was incubated with NADPH, H^+ and without inhibitor and are the mean of triplicate experiments ± S.D.

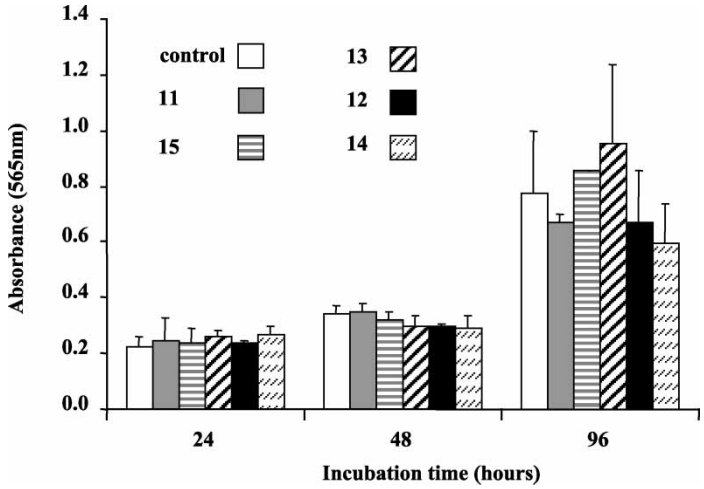


FIGURE 4 *Cell viability with the* **5** *new aromatase inhibitors*. Human embryonic E293 cells were chosen for their sensitivity. In 24-wells, 5×10^4 cells were treated with 10 μM inhibitors during 24, 48 or 96 h. Cytotoxicity was evaluated by measuring the transformation of the MTT into blue formazan product. The absorbance at reference wavelength (705 nm) was substracted from the test absorbance (565 nm) and the results were the mean of triplicate values ± S.D.

two different mammalian enzymes, and pharmacological studies, has shown its utility and efficiency in the development of more potent inhibitors of aromatase, which are promising in particular for the treatment of breast cancer.

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