PERGAMON

Journal of Steroid Biochemistry and Molecular Biology 70 (1999) 59–71

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

MR 20492 and MR 20494: two indolizinone derivatives that strongly inhibit human aromatase

P. Auvray[a], P. Sourdaine[a], S. Moslemi[a], G.-E. Séralini[a,*], P. Sonnet[b], C. Enguehard[b], J. Guillon[b], P. Dallemagne[b], R. Bureau[b], S. Rault[b]

[a] *IBBA, Laboratoire de Biochimie et Biologie Moléculaire, Université de Caen, Esplanade de la Paix, 14032 Caen cedex, France*
[b] *C.E.R.M.N., Laboratoire de Pharmacochimie, 1 rue Vaubénard, 14032 Caen cedex, France*

Received and accepted 26 February 1999

Abstract

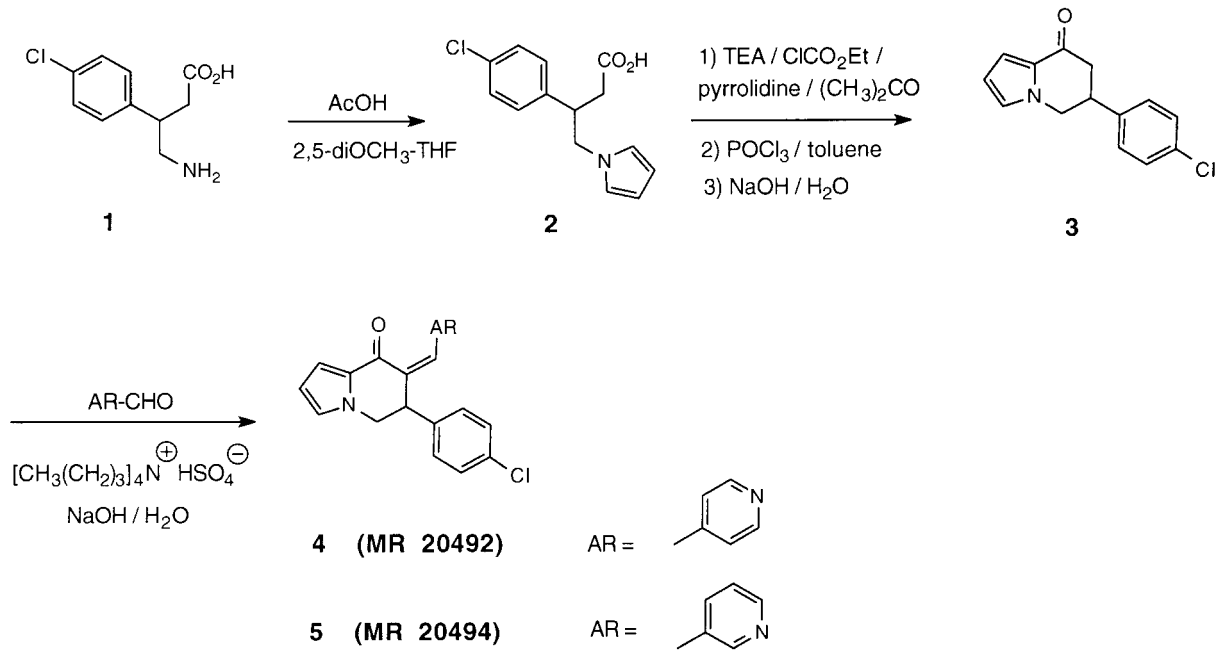
In this study, we describe the synthesis of a new family of indolizinone derivatives designed to fit an extrahydrophobic pocket within the active site of aromatase and to strongly inhibit human aromatase. This could help improve the specificity of the inhibitors. Equine aromatase, very well characterized biochemically, is used as a comparative model. Indeed, in a previous comparison between both human and equine aromatases, we described the importance of the interaction between the inhibitor and this pocket for the indane derivative MR 20814. MR 20492 and MR 20494 are more potent inhibitors of human aromatase (K_i/K_m: 1.0 ± 0.3 and 0.5 ± 0.3, respectively). The K_i/K_m for MR 20494 is slightly higher than that obtained for fadrozole (0.1 ± 0.0) and K_i/K_m for both indolizinone derivatives are lower than those obtained for 4-hydroxyandrostenedione (1.9 ± 0.8) and MR 20814 ($8.1 \pm .7$). These new compounds are not enzyme inactivators. Moreover, as indicated by the higher K_i/K_m values obtained with equine enzyme (9.0 ± 0.6 and 6.1 ± 1.6 for MR 20492 and MR 20494, respectively), both human and equine aromatase active sites appear to be structurally different. Difference absorption spectra study (350–500 nm) revealed that MR20492 and MR20494 were characterized by a combination of type-I and -II spectra with both enzymes. This result could be due to the isomerization of the molecule in polar solvent (*Z* and *E* forms). The evaluation of these new molecules, as well as 4-hydroxyandrostenedione and fadrozole, on aromatase activity in transfected 293 cell cultures evidenced a strong inhibition (IC_{50}: 0.20 ± 0.03 μM, 0.20 ± 0.02 μM and 0.50 ± 0.40 μM for MR 20494, fadrozole and 4-OHA, respectively) except for MR 20492 (3.9 ± 0.9 μM) and MR 20814 (10.5 ± 0.6 μM). These results proved that these molecules formed part of a promising family of potent inhibitors and that they penetrate 293 cells, without evidencing any cytotoxicity in Hela cells with MTT assay. This is thus encouraging for the development of new drugs for the treatment of estrogen-dependent cancers, these molecules also constitute new tools for understanding the aromatase active site.

1. Introduction

Aromatase is the enzymatic complex responsible for estrogen biosynthesis, formed by the specific cytochrome P450 aromatase (aromatase herein) and an ubiquitous flavoprotein NADPH cytochrome P450 reductase. The study of structure–activity relationships of aromatase inhibitors is very helpful for evaluating the active site structure of the enzyme. Moreover, its inhibition constitutes an approach for studying the roles of estrogen biosynthesis in various physiological or pathological processes, such as estrogen-dependent cancers. To avoid inhibition of other enzymes, the selectivity of the inhibitors is crucial. However, nonselective aromatase inhibitors, like nonsteroidal aminoglutethimide [1,2], which is a well known and long-used therapeutic agent, could affect enzymes controlling the production of other steroids and induce significant side effects. Recently, many new potent and selective inhibitors have been designed and are now under clinical development. Some of these are: (i) 4-hydroxyandrostenedione (4-OHA) or formestane, a

* Corresponding author. Tel.: +33-2-3156-5489; fax: +33-2-3156-5320.

0960-0760/99/$ - see front matter
PII: S0960-0760(99)00093-X



Scheme 1. Synthesis of compounds **1-5**.

very specific steroidal inhibitor used in therapy [3], (ii) fadrozole or CGS 16949A, highly potent in vitro [4], (iii) vorozole, highly specific for aromatase [5,6], (iv) two recent potent clinically used inhibitors [7], anastrozole (Arimidex) [8] and letrozole (Femara) [9]. The first generation inhibitor aminoglutethimide and second and third generation inhibitors, like formestane and fadrozole, were found to inhibit in vivo aromatization by 85–93%. The novel aromatase inhibitors letrozole and anastrozole inhibit in vivo aromatization by 97–99%. However, the response rate or the duration of remission with these drugs are far from satisfactory [10]. Indeed, in phase III clinical trials, fadrozole, vorozole, anastrozole, 4-OHA and letrozole caused overall response rates of only 14–16% [11,12], 17% [13], 24–27% [8], 33% [12] and 36% [14], respectively. Many studies have currently been undertaken to evaluate several new families of in vitro aromatase inhibitors [15–28] illustrating the considerable resources invested in the development of drugs that may provide a better response rate from patients. The need for clinical drugs with increased specificity, clinical efficiency and tolerability remains a challenge in the development of new compounds [12,29].

Recently, we used a new approach [30] based on the molecular modeling of the enzyme active site to design new inhibitors. This was helped, for the first time, by the comparison of two mammalian enzymes very well characterized at the biochemical level and cloned: the human [31–35] and equine [36–44] aromatases. As a matter of fact, we compared the inhibition effects of indane derivatives (MR 20814: 3-amino-5-methoxy-2-(pyridin-4-ylmethyl)inden-1-one and MR 20818: 3-amino-2-(pyridin-4-ylmethyl)-5,6,7-trimethoxy)inden-1-one) on both human and equine aromatases, in placental and testicular microsomes, respectively. The comparison of these enzymes and their differential interactions with the indane derivative MR 20814 [30], shown by the kinetic results and molecular modeling, suggested that the amino group of this molecule could occupy the entrance of an extrahydrophobic pocket located in the active site according to actual models [34,45,46]. Moreover, Laughton et al. [45] postulated that the alkylation of the glutarimide nitrogen of 3-alkyl-3(4-pyridyl)piperidine-2,6-dione led to a better inhibition of the aromatase activity by increasing the interaction with the extrahydrophobic pocket. These previous results allowed us to orientate the synthesis of a new family of indane derivatives among which MR 20496 (3-phenyl-2-(pyridin-4-ylmethylen)indan-1-one) was the most potent with an IC_{50} value of 0.8 ± 0.1 μM [47]. To check the existence of the extrahydrophobic pocket, we postulated that the phenyl group of MR 20496 could occupy it within the aromatase active site, and this could reinforce the interaction between a nitrogen atom of the inhibitors and the heme iron atom of the enzyme. This hypothesis and all our previous results led us to develop a new family of molecules belonging to the indolizinone series, that we consider to be a structural compromise between the benzocycloalkenes-type [48] and the 'azole'-type (like fadrozole and vorozole) aromatase inhibitors. Some of

the compounds tested herein appeared to be more potent than 4-OHA and comparable in vitro to fadrozole. This new family could thus provide information concerning the mammalian aromatase active site, and consequently be of interest for the design of new inhibitors in estrogen-dependent cancer treatment (Scheme 1).

2. Materials and methods

2.1. Materials

All chemical products were obtained from Sigma (St Quentin Fallavier, France) or GibcoBRL (Cergy Pontoise, France). Polyethylenimine was prepared in ddH_2O at 10 mM, pH 7.0. [1β,2β-^{3}H]-androstenedione was from Dupont NEN (Les Ulis, France), Coomassie brilliant blue G-250 dye from BioRad (Ivry/Seine, France), solvents from Carlo Erba (Val de Reuil, France) and from sds (Peypin, France), human embryonal kidney 293 cells (ECACC number: 85120602) and human cervix epithelial carcinoma Hela cells (ECACC number: 93021013) from CERDIC (Sophia-Antipolis, France), JM109 bacterial strain from Promega (Charbonnières, France), culture media from BioWhittaker (Gagny, France), Thermo Sequenase Kit from Amersham (Les Ulis, France), pCMV plasmid from Invitrogen (NV Leek, The Netherlands) and Qiagen Plasmid Maxi Kit from Qiagen (Courtaboeuf, France). Human aromatase cDNA was kindly provided by E.R. Simpson (Dallas, USA). The oligonucleotide primers sense A (5′-^{555}CCATTGACG TCAATGGGAGTT^{575}-3′) and antisense B (5′-^{1169}TAAGGCTTTGCGCATGACCAAG^{1148}-3′) were from EUROBIO (Les Ulis, France).

2.2. Chemistry

Melting points were determined on a Kofler block and are uncorrected. IR spectra were recorded on a Unicam Mattson 1000 spectrometer. NMR spectra (^{1}H, ^{13}C, ^{1}H-COSY, NOE) were recorded at 400 or 100 MHz with tetramethylsilane as an internal standard using a JEOL JNM-LA 400 spectrometer. Mass spectra were recorded on a JEOL D300 instrument using direct inlet system and electron impact ionization. Silica gel 60 (70–230 mesh) was used for column chromatography. Elemental analyses (C, H, N) were performed by INSA, Rouen-France, and agreed with the proposed structures within ±0.3% of the theoretical values.

2.2.1. Preparation of **4** and **5** (general method)

A solution of sodium hydroxide (0.48 g, 12 mmol) and tetrabutylammonium hydrogen sulfate (catalytic amount; 0.135 g, 0.4 mmol) in water (3 ml) was added to a solution of 6-(4-chlorophenyl)-5,6,7,8-tetrahydroindolizin-8-one **3** (1 g, 4 mmol) and pyridine-4-carboxaldehyde (0.47 g, 4.4 mmol) in methylene chloride (40 ml), was added. The reaction mixture was stirred at room temperature for 2 h. After addition of methylene chloride (80 ml), the organic layer was separated, washed with water, dried over calcium chloride and evaporated to dryness. A silica-gel column was used to purify the residue with cyclohexane/ethyl acetate (70/30) as eluent.

2.2.2. (Z) 6-(4-chlorophenyl)-7-(pyridin-4-ylmethylene)-5,6,7,8-tetrahydroindolizin-8-one **4** (MR 20492)

Yellow crystals (65%), mp 238°C, IR (KBr) 1665 cm^{-1} (CO), ^{1}H NMR (400 MHz, $CDCl_3$) 8.50 (d, J = 5.6 Hz, 2H, H2′ and H6′), 7.94 (s, 1H, Hα), 7.26 (d, J = 8.0 Hz, 2H, H3″ and H5″), 7.17 (m, 1H, H3), 7.10 (m, 4H, H3′, H5′, H2″ and H6″), 6.72 (m, 1H, H1), 6.25 (m, 1H, H2), 4.59 (m, 1H, H6), 4.45 (dd, J = 12 and 3.5 Hz, 1H, H5a), 4.31 (dd, J = 12 and 2.5 Hz, H5b), ^{13}C NMR ($CDCl_3$) 176.2 (C-8), 150.1 (C-2′ and C-6′), 142.6 (C-4′), 138.9 (C-7), 137.1 (C-1″), 133.4 (C-8a), 130.6 (C-α), 129.1 (C-2″ and C-6″), 128.5 (C-3″ and C-5″), 128.2 (C-4″), 126.8 (C-3), 123.2 (C-3′ and C-5′), 116.5 (C-1), 111.8 (C-2), 50.8 (C-5), 42.1 (C-6), analysis calculated for $C_{20}H_{15}N_2OCl$: C, 71.74, H, 4.51, N, 8.36, found: C, 71.59, H, 4.52, N, 8.19.

2.2.3. (Z) 6-(4-chlorophenyl)-7-(pyridin-3-ylmethylene)-5,6,7,8-tetrahydroindolizin-8-one **5** (MR 20494)

Yellow oil (45%), IR (KBr) 1670 cm^{-1} (CO), ^{1}H NMR (400 MHz, $CDCl_3$) 8.48 (s, 1H, H2′), 8.44 (m, 1H, H6′), 7.93 (s, 1H, Hα), 7.45 (d, J = 4.0 Hz, 1H, H4′), 7.20 (m, 3H, H5′, H3″ and H5″), 7.11 (m, 1H, H3), 7.05 (d, J = 7.5 Hz, 2H, H2″ and H6″), 6.63 (m, 1H, H1), 6.18 (m, 1H, H2), 4.53 (m, 1H, H6), 4.39 (dd, J = 12 and 3.6 Hz, 1H, H5a), 4.24 (dd, J = 12 and 2.4 Hz, H5b), ^{13}C NMR ($CDCl_3$) 176.5 (C-8), 150.1 (C-6′), 149.7 (C-2′), 139.0 (C-7 and C-4′), 136.3 (C-1″), 135.7 (C-3′), 133.4 (C-8a), 130.7 (C-α), 129.3 (C-2″ and C-6″), 128.6 (C-3″, C-4″ and C-5″), 126.6 (C-3), 123.4 (C-5′), 116.3 (C-1), 111.6 (C-2), 50.9 (C-5), 42.2 (C-6), analysis calculated for $C_{20}H_{15}N_2OCl$: C, 71.74, H, 4.51, N, 8.36, found: C, 71.67, H, 4.39, N, 8.25.

The synthesis of other compounds used in this study have previously been described [30,47].

2.3. Preparation of microsomes

Human placental and equine testicular microsomes were prepared as previously described [30,37]. Briefly, in each instance the available tissue presenting the

highest specific aromatase activity was utilized. Fresh tissue was 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 20,000*g*. The supernatant was further ultracentrifuged at 100,000*g* and 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 [49] using bovine serum albumin as standard, and Coomassie brilliant blue as dye-reagent.

2.4. Inhibition studies with microsomes

Aromatase activity was evaluated by measuring ^3H_2O released from 200 nM [1β,2β-^{3}H]-androstenedione (specific activity: 1554 GBq/mmol) at 37°C for 15 min [30,32], in the presence of various inhibitors from 0 to 15 μM. Reactions with microsomes (195 μg 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 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 [37]. Control incubations were realized by incubating microsomes, substrate and inhibitors without NADPH,H^+ in the same conditions. Results were the mean of triplicate experiments ± S.D. and were expressed as pmol estrogen formed/min. mg microsomal proteins.

2.5. Kinetic studies

Concentration range was 0–600 nM for inhibitors and 6–100 nM for substrate. Aromatase activity was evaluated at the linear portion of the Michaelis–Menten plot by incubating 5 μg (human) or 4 μg (equine) microsomal proteins with substrate and various inhibitors, at 37°C for 12 min with human aromatase and 8 min with the equine enzyme. K_m and K_i values were determined graphically by using, respectively, Lineweaver–Burk and Dixon representations.

2.6. Inactivation studies

Inactivation studies were carried out with 26 μg (human) or 20 μg (equine) microsomal proteins by testing different incubation times (0–30 min) at 25°C with 10 μM inhibitors and 60μM NADPH,H^+ in 0.1 M Na-phosphate buffer, pH 7.5, containing 0.5 M sucrose according to Moslemi and Séralini [42]. An activated charcoal-dextran mixture (2%/1%, 100 μl) was added to the incubation and kept at 4°C for 5 min. The solution was then centrifuged for 10 min at 350*g*. Aromatase activity was assayed with 300 μl of the aqueous phase and labeled androstenedione as described above.

2.7. Spectral studies

Absorbance of microsomal proteins (1.5 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 350–500 nm after addition of 100 μM of each inhibitor diluted in ethanol (16 μl). The spectrum of ethanol alone was comparable to the baseline (350–500 nm). The spectra of the microsomes and of the molecule alone were subtracted from the spectrum of microsomes plus molecule.

2.8. pCMV-human aromatase cDNA construction

Human aromatase cDNA (2920 bp) [33] was previously cloned into pUC18 (2.7 kb) with two fragments, λgt10 *Hin*dIII–*Eco*RI in 5′ end (240 bp) and *Eco*RI–*Bgl*II in 3′ end (900 bp). This construction was partially digested with *Eco*RI and the 2920 bp fragment *Eco*RI–*Eco*RI subcloned into the pCMV *Eco*RI site. Orientation was then checked by PCR amplification with the primers sense A and antisense B which are specific for pCMV and the cDNA, respectively (the amplicon length was 1359 bp) and by sequencing. The pCMV–cDNA was purified from transformed JM109 bacterial strain by using the Qiagen Plasmid Maxi Kit. The length, the concentration and the purity of the plasmid-cDNA construction were verified by 1% agarose electrophoresis and ethidium bromide staining.

2.9. 293 cells culture and transfection

Human embryonal kidney 293 cells were grown at 37°C in red phenol free EMEM medium supplemented with 2 mM glutamine, 10% new born calf serum (supreme serum), 1% nonessential amino acid under an atmosphere of 5% CO_2 and 95% air. Fifty thousand cells were grown to 50% confluence on 24-wells cell culture plates 18 h before transfection, washed with serum-free cell culture medium, supplemented with 500 μl serum-free medium and transiently transfected with 2 μg pCMV–human aromatase cDNA using a modification of the method of Boussif et al. [50]. Briefly, 2 μg pCMV–cDNA (corresponding to 6 nmol of phosphate) and 54 nmol of polyethylenimine were separately diluted with 50 μl 150 mM NaCl, incubated 10 min at room temperature under laminar fume hood, mixed together, then further incubated 10 min at room temperature and added to each well. Cells

were incubated 3–4 h at 37°C and then supplemented with 500 μl medium containing 10% supreme serum. After a further 18 h incubation, cells were washed with serum-free medium and the aromatase activity measured 'in cell'.

2.10. Aromatase activity and inhibition "in cell"

In this study, we evaluated aromatase activity 'in cell' according to Zhou et al. [51] by measuring ^3H_2O released from 200 nM [1β,2β-^{3}H]-androstenedione. Briefly, cells were washed with serum-free culture medium. The dessicated radioactive substrate supplemented with 1 μM progesterone (used to inhibit the possible endogenous 5α-reductase activity) and 0–10 μM inhibitor, were mixed with serum-free culture medium and added to each well. Cells were incubated at 37°C under 5% CO_2 atmosphere for 45 min. After incubating culture plates 5 min on ice, 1 ml culture medium was sampled and mixed with 1 ml chloroform. Steroids were extracted by incubation with a 1 ml charcoal dextran solution (7%/1.5%) and the radioactivity of the aqueous phase was measured as previously described [37]. Control incubation was realized by transfecting in the same conditions the pCMV plasmid alone instead of the pCMV–cDNA plasmid. The results were the mean of triplicate experiments ± S.D. and were expressed as the percentage of control.

2.11. Hela cells culture and cytotoxicity study

Hela cells were grown at 37°C in red phenol free EMEM medium in the same conditions previously described. In the trypan blue exclusion test, 100,000 cells were plated on day 0 and grown to 50% confluence on six-wells cell culture plates, treated (day 1) with 10 μM inhibitor during 24, 48 and 72 h (fresh culture medium containing 10 μM inhibitor was added daily). Cells were harvested by 0.25% trypsin-EDTA digestion, treated with trypan blue and counted using a hematocytometer. The results were the mean of triplicate experiments ± S.D. and were expressed as cells/ml culture medium. In the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, 50,000 cells were plated on 24-wells cell culture plates and treated in the same conditions as previously described for the trypan blue exclusion test. The method of Denizot and Lang [52] was adapted to our model. Briefly, MTT (5 mg/ml in PBS) was diluted to 1 mg/ml in culture medium. Culture supernatant was then removed by inverting, flicking and blotting the plate. MTT (250 μl) solution was added to the cells, gently shaken and incubated 3 h at 37°C. The plates were finally inverted, flicked and blotted to remove the untransformed MTT. DMSO (250 μl) was added to dissolve the formazan formed by the transformation of the substrate MTT by the mitochondrial succinate-dehydrogenase. This solution was then removed and added to 1 ml PBS before being evaluated by spectrophotometer with MTT as reference (this reference was incubated in the same conditions of the assay but without cells). The wavelengths were determined for our model (see Results and the legend of Fig. 6).

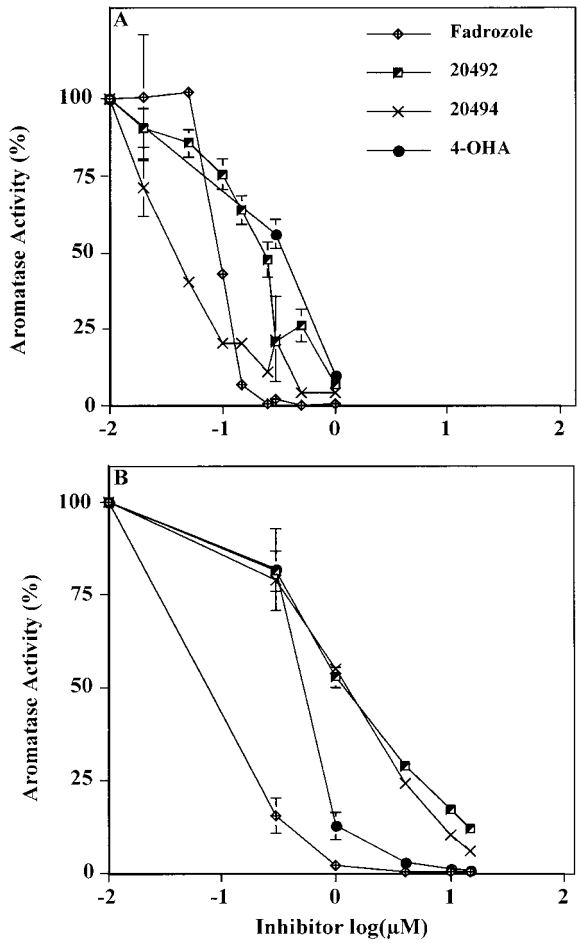


Fig. 1. Inhibition of human (A) and equine (B) aromatases by MR 20492 and MR 20494 in comparison with 4-OHA and fadrozole. Aromatase activity was evaluated by measuring ^3H_2O released from 200 nM [1β,2β-^{3}H]-androstenedione at 37°C for 15 min, in the presence of inhibitors MR 20492 and MR 20494 and fadrozole or 4-OHA used as controls. The inhibition of aromatase in human placental (195 μg) and equine testicular (20 μg) microsomes was performed in the presence of 60 μM NADPH,H$^+$. The blank was realized without adding NADPH,H$^+$. The results are expressed as percentage to a standard control which was incubated with NADPH,H$^+$ and without inhibitor, and are the mean of triplicate experiments ± S.D.

2.12. Statistical study

A *t*-test with unpaired values was performed with the Statview demo program version 4.5.3 (Abacus Concepts).

Table 1
Human (H) and equine (E) aromatase inhibition with the compounds used in this study. 4-OHA, fadrozole and MR 20814 are indicated for comparison. K_m values with androstenedione were 10.7 and 6.2 nM for human and equine enzymes, respectively. See legends of Figs. 1 and 2 for details

Compounds	IC_{50} (μM)		K_i (nM)		K_i/K_m	
	H	E	H	E	H	E
Fadrozole	0.059 ± 0.029	0.283 ± 0.161	0.6 ± 0.4	2.5 ± 0.0	0.1 ± 0.0	0.4 ± 0.0
4-OHA	0.450 ± 0.057	0.580 ± 0.243	20.2 ± 8.8	6.2 ± 0.0	1.9 ± 0.8	1.0 ± 0.0
MR 20814	3.493 ± 1.167	> 10	86.2 ± 7.8	577.7 ± 36.9	8.1 ± 0.7	93.9 ± 6.0
MR 20492	$0.147 \pm 0.013^{a,c}$	$1.28 \pm 0.002^{a,b,c}$	$10.3 \pm 3.3^{a,c}$	$58.3 \pm 4.2^{a,b,c}$	$1.0 \pm 0.3^{a,c}$	$9.0 \pm 0.6^{a,b,c}$
MR 20494	0.111 ± 0.063^{c}	$1.341 \pm 0^{a,b,c}$	$5.5 \pm 2.6^{a,c}$	$39.4 \pm 10.6^{a,b,c}$	0.5 ± 0.3^{c}	$6.1 \pm 1.6^{a,b,c}$

[a] $p < 0.05$ compared to fadrozole.
[b] $p < 0.05$ compared to 4-OHA.
[c] $p < 0.05$ compared to MR 20814.

3. Results

3.1. Chemistry

The synthesis of the aromatase inhibitors was achieved starting either from indanones or from indolizinones. Involvement of baclofen **1** in a Clauson–Kaas reaction led to pyrrolylbaclofen **2** which was subsequently cyclized under Vilsmeier conditions into the phenyltetrahydro indolizinone **3** [53]. The final tested compounds **2**–**6** were some pyridinyl derivatives resulting from an aldolization reaction run from the indolizinone **3**. On the other hand, reaction of **3** with pyridinecarboxaldehyde, in a heterogenous system (dichloromethane/aqueous sodium hydroxide solution) and in the presence of tetrabutyl-ammonium sulfate as catalyst, yielded the pyridinylmethylene-indolizinones **4**, **5** (MR 20492 and MR 20494, respectively). The assignment of the *Z* configuration of **4** was made on the basis of NOE experiments. Specifically, irradiation of the methylene proton in **4** led to a NOE with both H-2′ and H-6′. Furthermore **4**, in d6-DMSO solution suffered, after a week at room temperature, a partial isomerization into its *E* form whose ^{1}H NMR spectrum exhibited a shielded methylene signal at 6.88 ppm (7.80 ppm for the *Z* form) confirming the initial configuration. Configurations of **5** were determined by analogy between their ^{1}H NMR spectra and those of **4**.

3.2. Biological results on human aromatase in placental microsomes

In this study, we tested the inhibitory effect of newly synthesized indolizinone derivatives on the human aromatase activity. We obtained two potent molecules **4** (MR 20492) and **5** (MR 20494) that inhibited aromatase (Fig. 1A) with an IC_{50} of 0.147 ± 0.013 μM and 0.111 ± 0.063 μM, respectively (Table 1). The IC_{50}

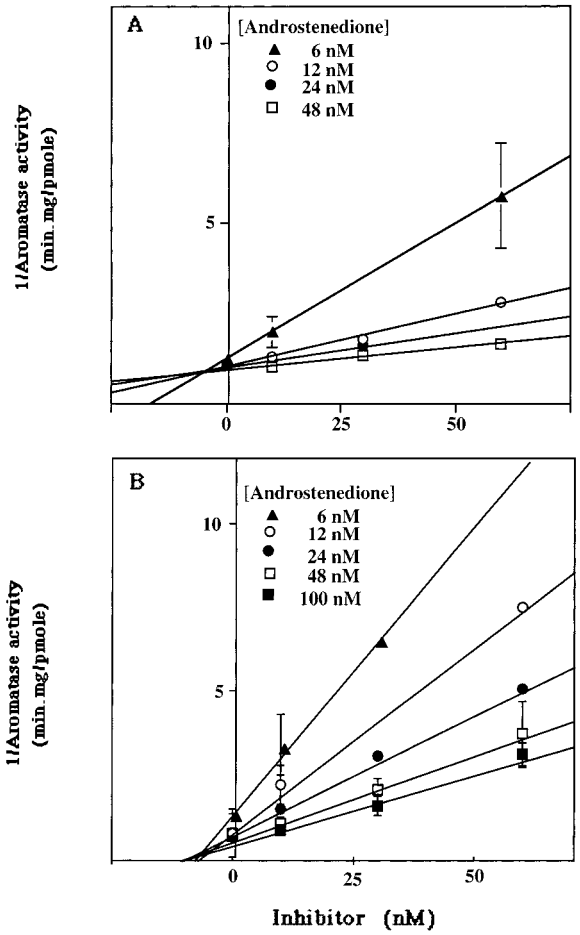


Fig. 2. Kinetic inhibition studies with MR 20492 (A) and MR 20494 (B) on human aromatase. Aromatase activity was evaluated by incubating 5 μg of placental microsomal proteins with [1β,2β-^{3}H]-androstenedione (6–100 nM) and with 0 to 60 nM of each compound at 37°C during 12 min. K_i were determined graphically by Dixon-plot. Results are expressed as pmol estrogen formed/min.mg microsomal proteins and are the mean of triplicate values ± S.D. Results are representative of one from three experiments showing similar profiles.

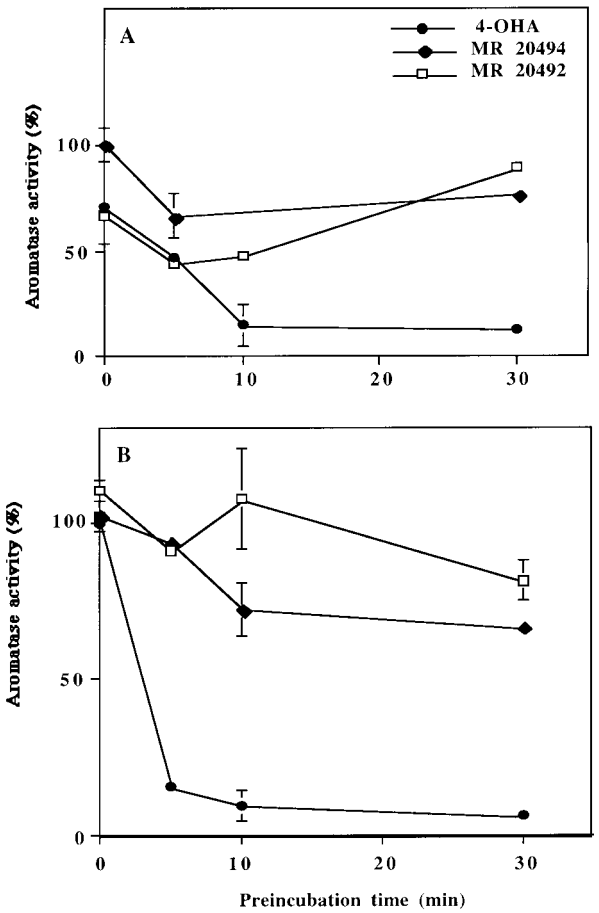


Fig. 3. Preincubation tests of human (A) and equine (B) aromatases with MR 20492, MR 20494 or 4-OHA. Microsomal proteins from human placenta (26 μg, (A)) or equine testes (20 μg, (B)) were incubated with 10 μM of MR 20492, MR 20494 or 4-OHA and 60 μM $NADPH,H^+$ at 25°C during 0–30 min. 4-OHA was used as control. Incubations were stopped by adding a charcoal/dextran mixture (2%/1%) and after centrifugation aromatase activity was assayed with 300 μl of the aqueous phase. Results are expressed as % to a standard control which was incubated with $NADPH,H^+$ and without inhibitor and are the mean of triplicate experiments ± S.D.

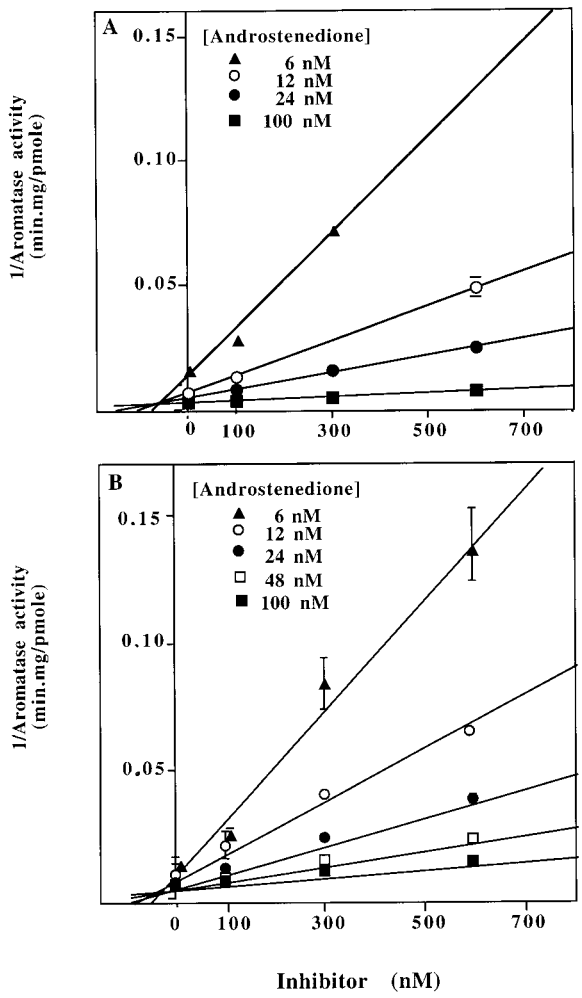


Fig. 4. Kinetic inhibition studies with MR 20492 (A) and MR 20494 (B) on equine aromatase. Testicular microsomal proteins (4 μg) were incubated with 6–100 nM [1β,2β-^{3}H]-androstenedione and with 0–600 nM MR 20492 (A) or MR 20494 (B) at 37°C during 8 min. Results are expressed as pmol estrogen formed/min mg microsomal proteins and are the mean of triplicate values ± S.D. Results are representative of one from three experiments showing similar profiles.

value for MR 20494 was not significantly different from that obtained for fadrozole (0.059 ± 0.029 μM), which is currently in phase III clinical trials, and both indolizinone derivatives have an inhibitory potency 3 to 4-fold greater than 4-OHA (0.450 ± 0.057 μM), which is clinically available. Moreover, MR 20492 and MR 20494 were 24 to 31 times more potent than the indane derivative MR 20814 (3.493 ± 1.167 μM). A kinetic study was performed and is summarized in Table 1. These new indolizinone derivatives presented a competitive inhibition (Fig. 2) but they are not inactivators in contrast to 4-OHA (Fig. 3A). Furthermore, we showed that compounds MR 20492 and MR 20494 were very potent inhibitors since their K_i/K_m ratio values, which represented their relative inhibitory potency, were 1.0 ± 0.3 and 0.5 ± 0.3, respectively. However, these results were slightly higher than those for fadrozole ($K_i/K_m = 0.1 \pm 0.0$). Furthermore, K_i/K_m values for both indolizinone derivatives were 2 to 4-fold lower than for 4-OHA (1.9 ± 0.8). However, these compounds presented an inhibitory potency greater than MR 20814.

3.3. Biological results on equine aromatase in testicular microsomes

In order to understand better the active site of human aromatase by comparative study and to design new more efficient inhibitors, we tested the indolizinone derivatives on the equine enzyme (Fig. 1B). The IC_{50} values obtained were 1.28 ± 0 μM and 1.341 ± 0 μM with MR 20492 and MR 20494, respectively, which were lower than the values evidenced for MR

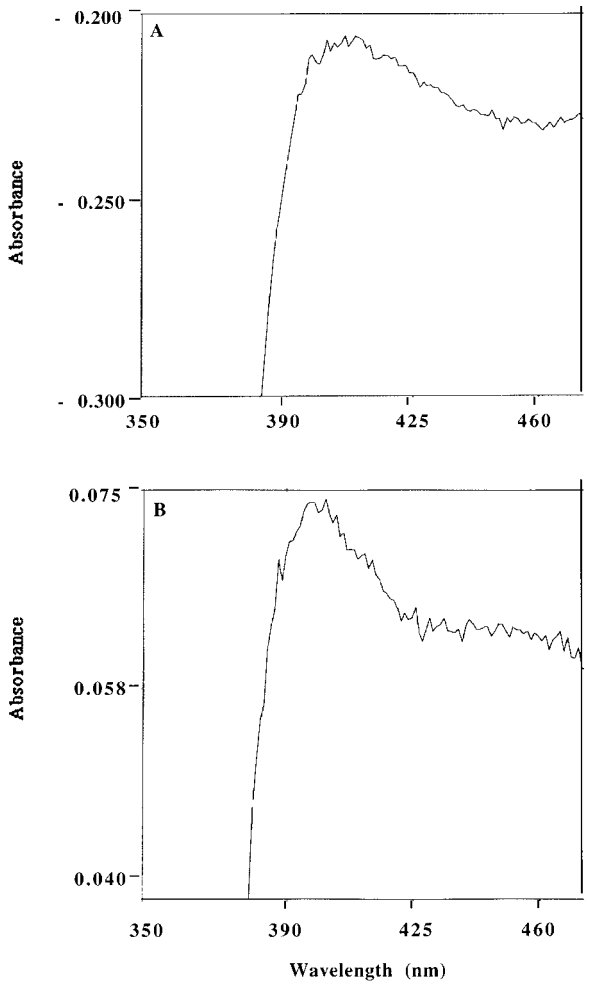


Fig. 5. Spectral studies of interactions between MR 20492 (A) or MR 20494 (B) and the human aromatase active site. Absorbance from 350 to 500 nm of microsomal proteins (1.5 mg) with 100 μM of inhibitor was measured at 37°C. The spectra of the microsomes or inhibitor alone were subtracted from the spectrum measured during the incubation of microsomes with the chosen molecule. Results are representative of one from three experiments showing similar profiles. The interactions between MR 20492 or MR 20494 and the active site of equine aromatase gave essentially similar spectra (data not shown).

20814, but higher than the values obtained for 4-OHA and fadrozole (0.580 ± 0.243 μM and 0.283 ± 0.161 μM, respectively), and much higher than values obtained with the human enzyme. The differential inhibitory potencies were reinforced by K_i/K_m ratio values which are 9 and 12 times greater (for MR 20492 and MR 20494, respectively) than those obtained with human aromatase (Table 1). As observed for the human enzyme, these two inhibitors were not inactivators of the equine enzyme (Fig. 3B) and led to a competitive inhibition (Fig. 4).

3.4. Spectral study on human and equine aromatases

In this context, in contrast with our previous results [30], we did not evidence a type I or type II spectrum for MR 20492 and MR 20494 but another spectrum characterized by a single absorbance maximum at 400–410 nm which characterizes, in the same manner, both human (Fig. 5) and equine (data not shown) enzymes.

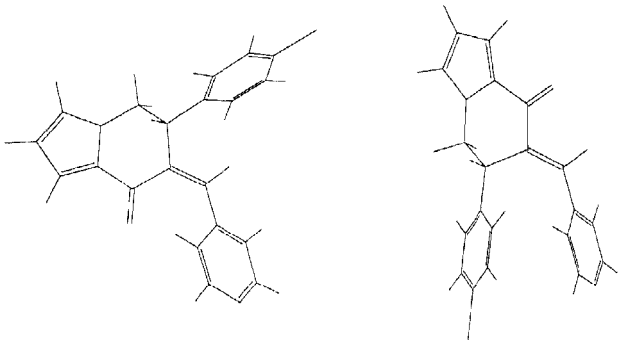


Fig. 6. Conformations of the *Z* (left) and *E* (right) forms for the compound 15 (MR 20492, *R* configuration). The conformations were obtained with the Discover software (MSI). Optimization of the geometry was carried out with the steepest descents and conjugate gradient methods (CVFF force field) with a gradient of 0.01 kcal/(mol/Å) for the convergence criteria.

3.5. Aromatase inhibition in cell culture

In this experiment, we tested inhibitory potencies of indolizinone in cell cultures, in comparison with MR 20814 [30], fadrozole and 4-OHA used as known potent inhibitors of aromatase. MR 20492 and MR 20494 were tested in human embryo kidney 293 cells transfected with human aromatase cDNA. As shown in Table 2, MR 20494, like fadrozole and 4-OHA, inhibited aromatase activity in cells in the same order of magnitude as was observed for microsomes (for microsomes, $IC_{50} = 3.5 \pm 1.2$ μM for MR 20814; Table 1). However, MR 20492 and MR 20814 were slightly

Table 2
Inhibition of human aromatase activity in 293 cells transfected with human aromatase cDNA. 50,000 cells in 24-wells plates were transfected with 2 μg pCMV-human aromatase cDNA with polyethylenimine as transfecting agent [55]. Aromatase activity was evaluated by measuring ^3H_2O released from 200 nM [1β,2β-^{3}H]-androstenedione incubated in culture medium at 37°C–5% CO_2 atmosphere for 45 min in the presence of different inhibitors. Results are expressed as % ± S.D. to a standard control which was incubated without inhibitor in the same conditions and are the mean of four experiments

Compounds	IC_{50} (μM)
Fadrozole	0.20 ± 0.02
4-OHA	0.50 ± 0.40
MR 20814	1045 ± 0.60[a]
MR 20494	0.20 ± 0.03
MR 20492	3.90 ± 0.93[a]

[a] $p < 0.05$ compared to IC_{50} obtained with microsomes.

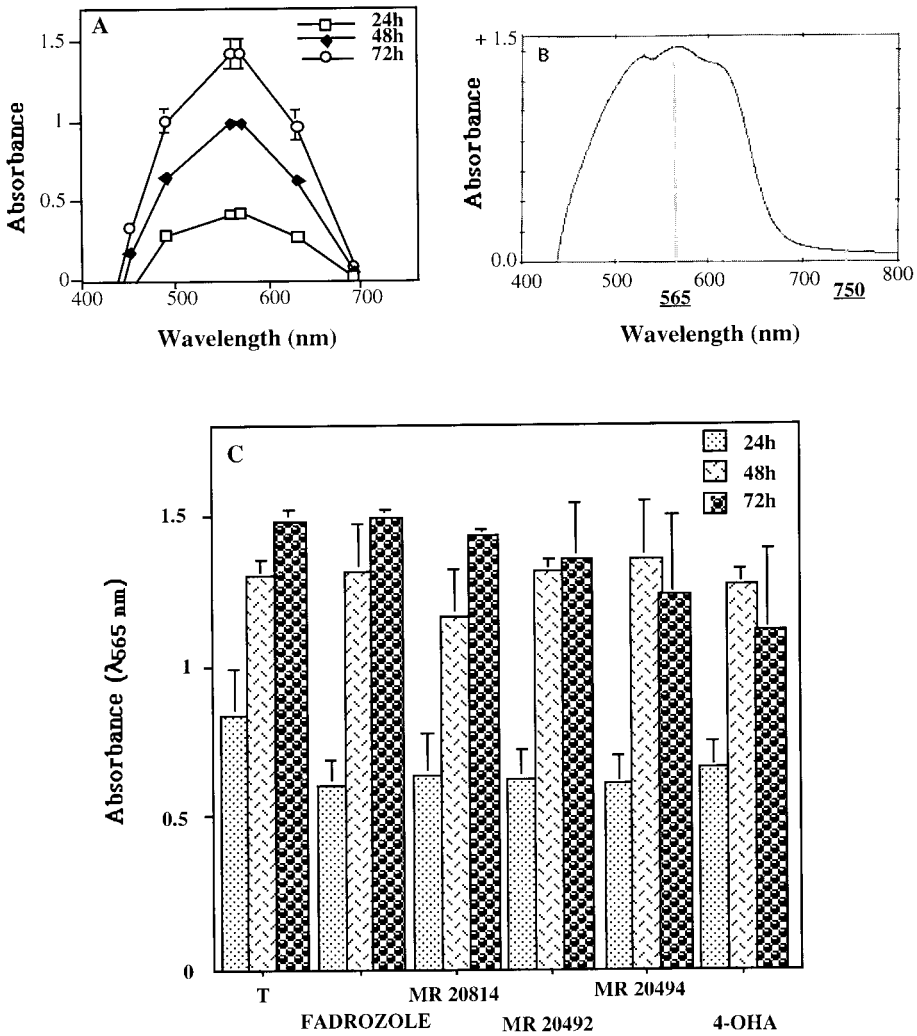


Fig. 7. Cytotoxicity study of new human aromatase inhibitors on Hela cells. 50,000 cells in 24-wells plates were treated with 10 μM inhibitors during 24, 48 or 72 h. (A) The MTT assay was adapted to Hela cells and the formazan production was performed at 24, 48 and 72 h between 405 and 690 nm (the MTT was used as reference). (B) The formazan spectrum was evidenced at 72 h of cell culture and two wavelengths were evaluated: 565 and 750 nm for the test and reference, respectively. (C) Cytotoxicity was evaluated by measuring the transformation of the mitochondrial succinate-dehydrogenase substrate (MTT) in blue formazan product. The absorbance at reference wavelength was subtracted from the test absorbance and results were the mean of triplicate values ± S.D. Fadrozole, 4-OHA, MR 20814 and MR 20818 [35] were also used as controls. T corresponded to cells without inhibitor. No absorbance was significantly different compared to the control T.

less potent (3.9 ± 0.9 μM and 10.4 ± 0.6 μM, respectively).

3.6. Cytotoxicity in cell culture

The results obtained with the 293 cells revealed that the molecules MR 20492 and MR 20494 were not cytotoxic since no dead cell was observed in this experiment. Moreover, we tested the cytotoxicity of these compounds, as well as the potent indane derivative inhibitor MR 20814, on the human cervix epitheloid carcinoma Hela cells by using the trypan blue exclusion test (data not shown). In this type of study, the usual concentration of inhibitor which is supposed to not affect cell survival is 1 μM [54]. This concentration did not lead to cellular death with this method and even at a concentration of 10 μM no cytotoxicity was evidenced, either with indane derivatives or with indolizinone derivatives on Hela cells during culture times of 24, 48 or 72 h. In fact, cells were fast growing up to 48 h and more moderately until 72 h. This method was modified by using a spectrophotometer and optimized to our Hela cells by testing different wavelengths during 24, 48 and 72 h of culture. As shown in Fig. 7A, the absorbance increased proportionally to the time of cell culture. Moreover, Fig. 7B allowed determination of the test wavelength (565 nm) and reference wavelength (750 nm). As the maximal absorbance of DMSO was at 503 nm [55], it did not interfere with the chosen test wavelength. The results obtained with

the MTT assay (Fig. 7C) confirmed our previous results since none of the tested molecules showed a cytotoxic effect.

4. Discussion

In this study, we screened the effect of indolizinone derivatives on human and equine aromatases in placental and testicular microsomes, respectively. We obtained two molecules, MR 20492 and MR 20494, which are very potent inhibitors. Indeed, IC_{50} and K_i/K_m values obtained for MR 20494 evidenced that this molecule was 694 times more potent than aminoglutethimide and slightly less potent than fadrozole which is in phase III clinical trials. However, MR 20494 and MR 20492 were not inactivators. Moreover, our comparative results between models also confirm conformational differences between the active sites of human and equine aromatases. Indeed, as described in our previous work [30], we suggested that the chlorophenyl group could interact with the extrahydrophobic pocket within the active site and that the N atom of the pyridyl group could be close to the heme iron. The interaction of these new inhibitors with this pocket could be stronger with the human aromatase than the interaction previously described [30] with the amino group of MR 20814, and could then explain the higher inhibition observed. Moreover, with the equine aromatase, the chlorophenyl group of MR 20492 and MR 20494 could strongly interact with the 'acidic pocket' previously described [30] and formed by D309, D476 and N475. This interaction could remove the pyridyl group from the heme iron and then render the inhibitor less potent in comparison to the human enzyme.

The study of the interaction between the heme iron and a substrate or an inhibitor has currently been conducted with cytochromes P450 [56], as for example with aromatase [30,31,57,58]. In our previous study, a type II spectrum, considered as specific for an interaction between nitrogen atom of the molecule and the heme iron of the cytochrome, with minimal absorbance at 390 nm and maximal absorbance at 420 nm, was observed between the indane derivative 3-amino-5-methoxy-2-(pyridin-4-ylmethyl)inden-1-one (MR 20814) and human aromatase, whereas a type I spectrum (inverted absorbance) was observed for equine aromatase. In this study, the spectra produced by the interaction between MR 20492 or MR 20494 and the active site of both enzymes showed an unexpected and unique type spectrum since it was characterized by an absorbance peak at 400–410 nm. One hypothesis to explain this profile is that a combination of type I and type II spectra was obtained. A previous study showed the possibility of getting two different types of spectra in relation to the substituents on pyridine [59]. The 2-benzoylpyridine showed exclusively a reverse type I spectrum and pyridine and its derivatives substituted at the 3- or 4-positions with phenyl or benzoyl groups showed exclusively a type II spectrum. By considering the *Z* form for the compounds MR 20492 and MR 20494, the type II spectrum should be obtained. However, if the *E* form and the *R* configuration are considered, the pyridinic nitrogen is sterically hindered, by the chlorophenyl group, from access to the heme iron. This situation could lead to a reverse type I spectrum (see Fig. 6). The isomerization of the compound MR 20492 (or MR 20494) was checked and indeed, in polar solvent, this fact was observed leading to a mixture of *E* and *Z* forms explaining the profile for the UV spectra obtained during the conditions of the biological evaluations. Thus, we suggest that the inhibition results are due to a combination of both geometric isomer activities. However, we previously showed with indane derivatives [30] that an inhibitor characterized by an interaction with the heme iron (type II spectrum) was more potent. We thus suggest that MR 20492 and MR 20494 could be more efficient if the isomer proportion tended to the 'type II spectrum' one.

In the present study, we evidenced in vitro two potent inhibitors of human aromatase in placental microsomes, MR 20492 and MR 20494. As our molecules could also be developed as drugs, it would be useful to evaluate their ability to penetrate cells. As shown in Table 2, MR 20494 seemed to be an interesting candidate since it remained highly potent in cell cultures like fadrozole. These results suggest that MR 20494, more easily than MR 20492, was able to pass through the cellular membrane, suggesting that it was not or little metabolized before reaching its specific target.

Moreover, in order to evaluate the cytotoxicity of these compounds, we adapted the MTT assay to our model. Although the trypan blue exclusion test is currently used in cytotoxicity studies, this method only revealed membrane permeability and not the intracellular damages [60]. Mosmann [61] then developed a quantitative colorimetric assay to detect mammalian cell survival and proliferation based on the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). This assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cells since the tetrazolium ring is cleaved by active mitochondria. This method was validated by comparison with the [^{3}H]-thymidine uptake method [52], and Carmichael et al. [55] demonstrated that it was a fast and efficient method for studying the cytotoxicity of compounds in cell culture. Mosmann [61] previously determined a test wavelength of 570 nm and a reference wavelength of 630 nm (this value correlates with the baseline) whereas Denizot

and Lang [52] used 560 nm test and 690 nm reference wavelengths, and Carmichael et al. [55] a 540 nm test wavelength when using DMSO as solvent. All these authors used this MTT assay by reading on an enzyme-linked immunosorbent assay reader and with different cells. Our results, using a test wavelength at 565 nm and a reference wavelength at 750 nm, allowed us to show that neither MR 20492 nor MR 20494 were cytotoxic after 72 h incubation with Hela cells.

In conclusion, these results are promising with regard to the development of new drugs as we evidenced two new original inhibitors which are able to strongly inhibit human aromatase in placental microsomes and in cell culture. They allow a better modeling and understanding of the aromatase active site by comparison with the equine enzyme, and this new design will be tested by site-directed mutagenesis studies. It would be interesting to examine the effects of MR 20492 and MR 20494 on other steroidogenic enzymes, and in vivo.

Acknowledgements

This work was initiated and developed through a grant from the Ligue Nationale Contre Le Cancer (Comité de la Manche) for the biological studies and a studentship to P.A., and recently supported by the Ligue Nationale Contre Le Cancer, Comité du Calvados, for the chemical syntheses. The Conseil Général du Calvados and Fonds FEDER supported the equine studies. We wish to thank Dr. B. Plainfossé for providing horse tissues, M.J. Simon and S.Galopin for technical assistance.

References

[1] A.M. Brodie, Overview of recent development of aromatase inhibitors, Cancer Res. 42 (1982) 3312s–3314s.
[2] M.J. Carella, N.V. Dimitrov, V.V. Gossain, L. Srivastava, D.R. Rovner, Adrenal effects of low-dose aminoglutethimide when used alone in postmenopausal women with advanced breast cancer, Metabolism 43 (1994) 723–727.
[3] L.R. Wiseman, K.L. Goa, Formestane: a review of its pharmacological properties and clinical efficacy in the treatment of postmenopausal breast cancer, Drugs Aging 9 (1996) 292–306.
[4] R.J. Santen, P. Langecker, S.J. Santner, S. Sikka, J. Rajfer, R. Swerdloff, Potency and specificity of CGS-16949A as an aromatase inhibitor, Endocr. Res. 16 (1990) 77–91.
[5] P.C. De Jong, J. Van de Ven, H.W. Nortier, I. Maitimu-Smeele, T.H. Donker, J.H. Thijssen, P.H. Slee, R.A. Blankenstein, Inhibition of breast cancer tissue aromatase activity and estrogen concentrations by the third-generation aromatase inhibitor vorozole, Cancer Res. 57 (1997) 2109–2111.
[6] L.R. Wiseman, C.M. Spencer, Vorozole, Drugs Aging 11 (1997) 245–250.
[7] New aromatase inhibitors for breast cancer. Drug Ther. Bull., 35 (1997) 55–56.
[8] A.U. Buzdar, S.E. Jones, C.L. Vogel, J. Wolter, P. Plourde, A. Webster, The Arimidex Study Group, A phase III trial comparing anastrozole (1 and 10 mg), a potent and selective aromatase inhibitor, with megestrol acetate in postmenopausal women with advanced breast carcinoma, Cancer 79 (1997) 730–739.
[9] J.N. Ingle, P.A. Johnson, V.J. Suman, J.B. Gerstner, J.A. Mailliard, J.K. Camoriano, H. GesmeD, C.L. Loprinzi, A.K. Hatfield, L.C. Hartmann, A randomized phase II trial of two dosage levels of letrozole as third-line hormonal therapy for women with metastatic breast carcinoma, Cancer 80 (1997) 218–224.
[10] P.E. Lonning, Aromatase inhibition for breast cancer treatment, Acta Oncol. 35 (1996) 38–43.
[11] R.C. Stein, M. Dowsett, J. Davenport, A. Hedley, H.T. Ford, J.C. Gazet, R.C. Coombes, Preliminary study of the treatment of advanced breast cancer in postmenopausal women with the aromatase inhibitor CGS 16949A, Cancer Res. 50 (1990) 1381–1384.
[12] W.R. Miller, Aromatase inhibitors: where are we now?, Br. J. Cancer 73 (1996) 415–417.
[13] S.R. Johnston, I.E. Smith, D. Doody, S. Jacobs, H. Robertshaw, M. Dowsett, Clinical and endocrine effects of the oral aromatase inhibitor Vorozole in postmenopausal patients with advanced breast cancer, Cancer Res. 54 (1994) 5875–5881.
[14] G. Bisagni, G. Cocconi, F. Scaglione, F. Fraschini, C. Pfister, P.F. Trunet, Letrozole, a new oral non-steroidal aromastase inhibitor in treating postmenopausal patients with advanced breast cancer. A pilot study, Ann. Oncol. 7 (1996) 99–102.
[15] R.W. Hartmann, M. Frotscher, D. Ledergerber, G.A. Wachter, G.L. Grun, T.F. Sergejew, Synthesis and evaluation of azole-substituted tetrahydronaphtalenes as inhibitors of P450 arom, P450 17, and P450 TxA2, Arch. Pharm. 329 (1996) 251–261.
[16] M. Kudoh, Y. Susaki, Y. Ideyama, T. Nanya, M. Mori, H. Shikama, T. Fujikura, Inhibitory effect of a novel non-steroidal aromatase inhibitor, YM511 on the proliferation of MCF-7 human breast cancer cell, J. Steroid Biochem. Mol. Biol. 58 (1996) 189–194.
[17] D. Lesuisse, J.F. Gourvest, O. Benslimane, F. Canu, C. Delaisi, B. Doucet, C. Hartmann, J.M. Lefrancois, B. Tric, D. Mansuy, D. Philibert, G. Teutsch, Structure-activity relationships of a new family of steroidal aromatase inhibitors. 1. Synthesis and evaluation of a series of analogs related to 19-[(methylthio)methyl]androstenedione (RU54115), J. Med. Chem. 39 (1996) 757–772.
[18] V.C.O. Njar, J. Duerkop, R.W. Hartmann, Novel 19-(cyclopropylamino)-androst-4-en-3,17-dione: a mechanism-based inhibitor of aromatase, J. Enzyme Inhib. 10 (1996) 47–56.
[19] G.A. Wachter, R.W. Hartmann, T. Sergejew, G.L. Grun, D. Ledergerber, Tetrahydronaphthalenes: influence of heterocyclic substituents on inhibition of steroid enzymes P450 arom and P450 17, J. Med. Chem. 39 (1996) 834–841.
[20] E. Bajetta, N. Zilembo, S. Barni, C. Noberasco, A. Martinetti, L. Ferrari, G. Schieppati, R. Buzzoni, A. Jirillo, M.D. Amichetti, M. Aprile, G. Comella, E. Bichisao, G.F. Bolelli, A. Attili, E. Bombardieri, The Italian Trials in Medical Oncology (I.T.M.O.) group, A multicentre, randomized, pharmacokinetic, endocrine and clinical study to evaluate formestane in breast cancer patients at first relapse: endocrine and clinical results, Ann. Oncol. 8 (1997) 649–654.
[21] J.G. Blanco, R.R. Gil, C.I. Alvarez, L.C. Patrito, S. Genti-Raimondi, A. Flury, A novel activity for a group of sesquiterpene lactones: inhibition of aromatase, FEBS Lett. 409 (1997) 396–400.

[22] M. Le Borgne, P. Marchand, M. Duflos, B. Delevoye-Seiller, S. Piessard-Robert, G. Le Baut, R.W. Hartmann, M. Palzer, Synthesis and in vitro evaluation of 3-(1-azolylmethyl)-1H-indoles and 3-(1-azolyl-1-phenylmethyl)-1H-indoles as inhibitors of P450 arom, Arch. Pharm. 330 (1997) 141–145.
[23] M. Numazawa, M. Oshibe, S. Yamaguchi, 6-Alkylandrosta-4,6-diene-3,17-diones and their 1,4,6-triene analogs as aromatase inhibitors: structure–activity relationships, Steroids 62 (1997) 595–602.
[24] M. Okada, T. Yoden, E. Kawaminami, Y. Shimada, M. Kudoh, Y. Isomura, H. Shikama, T. Fujikura, Studies on aromatase inhibitors. I. Synthesis and biological evaluation of 4-amino-4H-1,2,4-triazole derivatives, Chem. Pharm. Bull. 44 (1996) 1871–1879.
[25] M. Okada, T. Yoden, E. Kawaminami, Y. Shimada, M. Kudoh, Y. Isomura, Studies on aromatase inhibitors. II. Synthesis and biological evaluation of 1-amino-1H-1,2,4-triazole derivatives, Chem. Pharm. Bull. 45 (1997) 333–337.
[26] M. Okada, T. Yoden, E. Kawaminami, Y. Shimada, M. Kudoh, Y. Isomura, Studies on aromatase inhibitors. III. Synthesis and biological evaluation of [(4-bromobenzyl)(4-cyanophenyl)amino]azoles and their azine analogs, Chem. Pharm. Bull. 45 (1997) 482–486.
[27] M. Okada, T. Yoden, E. Kawaminami, Y. Shimada, M. Kudoh, Y. Isomura, Studies on aromatase inhibitors IV. Synthesis and biological evaluation of *N*,*N*-disubstituted-5-aminopyrimidine derivatives, Chem. Pharm. Bull. 45 (1997) 1293–1299.
[28] M. Takahashi, T. Kayo, T. Karakida, S. Nakagawa, M. Kato, S. Matsuno, Y. Koide, M. Sakato, S. Kawashima, Potent and selective aromatase inhibitor: in vitro and in vivo studies with s-triazine derivative SEF19, Endocr. Res. 23 (1997) 1–13.
[29] A. Howell, S. Downey, E. Anderson, New endocrine therapies for breast cancer, Eur. J. Cancer 32A (1996) 576–588.
[30] P. Auvray, S. Moslemi, P. Sourdaine, G.E. Séralini, C. Enguehard, P. Dallemagne, P. Sonnet, R. Bureau, S. Rault, Evidence for new potent nonsteroidal human aromatase inhibitors and comparison with equine aromatase inhibition for an understanding of the mammalian active site, Eur. J. Med. Chem. 33 (1998) 451–462.
[31] E.A. Thompson, P.K. Siiteri, Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione, J. Biol. Chem. 249 (1974) 5364–5372.
[32] E.A. Thompson, P.K. Siiteri, The involvement of human placental microsomal cytochrome P-450 in aromatization, J. Biol. Chem. 249 (1974) 5373–5378.
[33] C.J. Corbin, S. Graham-Lorence, M. McPhaul, J.I. Mason, C.R. Mendelson, E.R. Simpson, Isolation of a full-length cDNA insert encoding human aromatase system cytochrome P450 and its expression in nonsteroidogenic cells, Proc. Natl. Acad. Sci. USA 85 (1988) 8948–8952.
[34] S. Graham-Lorence, B. Amarneh, R.E. White, J.A. Peterson, E.R. Simpson, A three-dimensional model of aromatase cytochrome P450, Protein Sci. 4 (1995) 1065–1080.
[35] E.R. Simpson, Y. Zhao, V.R. Agarwal, M.D. Michael, S.E. Bulun, M.M. Hinshelwood, S. Graham-Lorence, T. Sun, C.R. Fisher, K. Qin, C.R. Mendelson, Aromatase expression in health and disease, Recent Prog. Horm. Res. 52 (1997) 185–213.
[36] J.L. Gaillard, P. Silberzahn, Aromatization of 19-norandrogens by equine testicular microsomes, J. Biol. Chem. 262 (1987) 5717–5722.
[37] T. Dintinger, J.L. Gaillard, I. Zwain, R. Bouhamidi, P. Silberzahn, Synthesis and aromatization of 19-norandrogens in the stallion testis, J. Steroid Biochem. 32 (1989) 537–544.
[38] J.L. Gaillard, Equine testicular aromatase: substrates specificity and kinetic characteristics, Comp. Biochem. Physiol. 100B (1991) 107–115.
[39] A. Tomilin, E. Komarova, S. Moslemi, P. Auvray, P. Sourdaine, M.A. Drosdowsky, G.E. Séralini, Molecular cloning and characterization of equine cytochrome P450 aromatase, in: IV International Aromatase Conference, Tahoe City, CA, 1996 Abst. A22.
[40] J. Almadhidi, G.E. Séralini, J. Fresnel, P. Silberzahn, J.L. Gaillard, Immunohistochemical localization of chromosome P450 aromatase in equine gonads, J. Histochem. Cytochem. 43 (1995) 571–577.
[41] J. Almadhidi, S. Moslemi, M.A. Drosdowsky, G.E. Séralini, Equine cytochrome P450 aromatase exhibits an estrogen 2-hydroxylase activity in vitro, J. Steroid Biochem. Mol. Biol. 59 (1996) 55–61.
[42] S. Moslemi, G.E. Séralini, Inhibition and inactivation of equine aromatase by steroidal and non-steroidal compounds: a comparison with human aromatase inhibition, J. Enz. Inhib. 12 (1997) 241–254.
[43] S. Moslemi, A. Vibet, V. Papadopoulos, L. Camoin, P. Silberzahn, J.L. Gaillard, Purification and characterization of equine testicular cytochrome P-450 aromatase: comparison with the human enzyme, Comp. Biochem. Physiol. 118B (1997) 217–227.
[44] S. Moslemi, P. Auvray, P. Sourdaine, M.A. Drosdowsky, G.E. Séralini, Structure–function relationships for equine and human aromatases: a comparative study, Ann. NY Acad. Sci. 839 (1998) 576–577.
[45] C.A. Laughton, R. Mckenna, S. Neidle, M. Jarman, R. McCague, Crystallographic and molecular modeling studies on 3-ethyl-3-(4-pyridyl)piperidine-2,6-dione and its butyl analogue, inhibitors of mammalian aromatase. Comparison with natural substrates: prediction of enantioselectivity for *N*-alkyl derivatives, J. Med. Chem. 33 (1990) 2673–2679.
[46] D. Zhou, L.L. Cam, C.A. Laughton, K.R. Korzekwa, S. Chen, Mutagenesis study at a postulated hydrophobic region near the active site of aromatase cytochrome P450, J. Biol. Chem. 269 (1994) 19501–19508.
[47] P. Sonnet, P. Auvray, J. Guillon, P. Dallemagne, R. Bureau, S. Rault, S. Moslemi, P. Sourdaine, S. Galopin, G.E. Séralini, Design and synthesis of a new type of nonsteroidal human aromatase inhibitors, Bioorg. Med. Chem. Lett. 8 (1998) 1041–1044.
[48] R.W. Hartmann, H. Bayer, G. Grun, Aromatase inhibitors. Synthesis and structure–activity studies of novel pyridyl-substituted indanones, indans, and tetralins, J. Med. Chem. 37 (1994) 1275–1281.
[49] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.
[50] O. Boussif, F. Lezoualc'h, M.A. Zanta, M.D. Mergny, D. Scherman, B. Demeneix, J.P. Behr, A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine, Proc. Natl. Acad. Sci. USA 92 (1995) 7297–7301.
[51] D.J. Zhou, D. Pompon, S.A. Chen, Stable expression of human aromatase complementary DNA in mammalian cells: a useful sytem for aromatase inhibitor screening, Cancer Res. 50 (1990) 6949–6954.
[52] F. Denizot, R. Lang, Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability, J. Immunol. Methods 89 (1986) 271–277.
[53] P. Dallemagne, P. Sonnet, C. Enguehard, S. Rault, A convenient route to new phenyltetrahydroindolizines, J. Heterocyclic Chem. 33 (1996) 1689–1694.
[54] R.W. Brueggemeier, N.E. Katlic, Effects of the aromatase inhibitor 7a-(4′-amino)phenylthio-4-androstene-3,17-dione in

MCF-7 human mammary carcinoma cell culture, Cancer Res. 47 (1987) 4548–4551.
[55] J. Carmichael, W.G. DeGraff, A.F. Gazdar, J.D. Minna, J.B. Mitchell, Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing, Cancer Res. 47 (1987) 936–942.
[56] J.B. Schenkman, Studies on the nature of the type I and type II spectral changes in liver microsomes, Biochem. 9 (1970) 2081–2091.
[57] J.A. Canick, K.J. Ryan, Cytochrome P-450 and the aromatization of 16 alpha-hydroxytestosterone and androstenedione by human placental microsomes, Mol. Cell. Endocr. 6 (1976) 105–115.
[58] J.N. Wright, G. Slatcher, M. Akhtar, 'Slow-binding' sixth-ligand inhibitors of cytochrome P-450 aromatase: studies with 19-thiomethyl- and 19-azido-androstenedione, Biochem. J. 273 (1991) 533–539.
[59] A.D.N. Vaz, M.J. Coon, H. Peegel, K.M.J. Menon, Substituted pyridines: nonsteroidal inhibitors of human placental aromatase cytochrome P-450, Drug Metab. Dispos. 20 (1992) 108–111.
[60] J.A. Cook, J.B. Mitchell, Viability measurements in mammalian cell systems, Anal. Biochem. 179 (1989) 1–7.
[61] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immun. Methods 65 (1983) 55–63.