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INHIBITION AND INACTIVATION OF EQUINE AROMATASE BY STEROIDAL AND NON-STEROIDAL COMPOUNDS. A COMPARISON WITH HUMAN AROMATASE INHIBITION ☆

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In order to approach the detailed structure–function relationships of aromatase, we studied the inhibitory and inactivatory potencies of several steroidal androstenedione analogues (**1**: 4-hydroxyandrostenedione, **2**: 4-acetoxyandrostenedione and **3**: 7α-(4′-amino)phenylthio-4-androstene-3, 17-dione) and non-steroidal imidazole derivatives (**4**: ketoconazole, **5**: miconazole and **6**: fadrozole) on equine aromatase in placental microsomes, a well established mammalian model. Human placental microsomes and the purified enzyme from equine testis were also used to compare inhibition by **1** and **2**. In equine microsomes, all compounds tested exhibited a competitive inhibition, with K_i values of 4.1, 26 and 1.8 nM for **1**, **2** and **3**, and of 2400, 1.4 and 4 nM for **4**, **5**, and **6**, respectively. The K_m for androstenedione, the substrate mainly used in these studies, was 1.8 ± 0.13 nM. The three non-steroidal derivatives did not inactivate equine aromatase, but **1** and **2** acted as comparable inactivators to a much higher degree than **3**. Compound **1** inhibited in a similar manner (89–94%) purified or equine and human microsomal aromatases, whereas **2** inhibited microsomal aromatase more efficiently in the horse than in man (92% and 33% inhibition, respectively). There was only a 40% inhibition with **2** on the purified equine enzyme, which is no more in the natural membrane environment. The comparisons between equine and human microsomal aromatases allow precise functional and structural differences to be observed with these enzymes.

Keywords: Aromatase; Aromatase-inhibitors; Equine; Testis; Placenta; Structure–function

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INTRODUCTION

Aromatase is the key enzyme in the biosynthesis of estrogens, the latter being important in development, reproduction and sex hormone-dependent diseases such as breast cancer.[1–4] Equine aromatase is a very particular model: it is responsible for the elevated estrogen synthesis by the horse testis, where it is present in high concentration in the Leydig cells.[5] It is the only other mammalian model, besides the human one, which has been purified (in the mg range from one testis), biochemically characterized in detail,[6] and cloned.[7] The 3-dimensional structure of human aromatase, a membrane-bound complex in mammals, has been proposed on the basis of comparisons with procaryotic cytosoluble models.[8] Thus any information on other mammalian active sites could help in modeling studies and understanding the catalytic mechanism. On the other hand, the development and evaluation of selective inhibitors or inactivators of aromatase, to specifically block the estrogen synthesis, may be useful for controlling pathologic conditions associated with estrogen dependency.[9,10] Several aromatase inhibitors and inactivators have been synthesized and evaluated using human placental aromatase in microsomes as the enzyme source.[11–18]

Our group has previously determined differences between the human and equine aromatases such as a differential affinity for androgens and 19-norandrogens, and effects of ions on aromatization,[19–25] and also new biochemical similarities such as the capacity to synthesize catecholestrogens.[26]

In order to examine the structure–function relationships in this enzyme, we studied the inhibitory and inactivatory potencies of some steroidal androstenedione analogues (**1**, **2** and **3**) and non-steroidal imidazole derivatives (**4**, **5** and **6**) on equine aromatase, in comparison with the human enzyme.

MATERIALS AND METHODS

Chemicals

[4-^{14}C]-testosterone (56.9 Ci/mmol) and [4-^{14}C]-androstenedione (59 Ci/mmol) were obtained from Amersham, Les Ulis, France. [1β,2β-^{3}H]-androstenedione (43.1 Ci/mmol) and [1β-^{3}H]-androstenedione (50 Ci/mmol) were purchased from Du Pont-New England Nuclear (Les Ulis, France). Steroids were purchased from Steraloids (Wilton, NH, USA) or Sigma (Saint-Quentin-Fallavier, France). Fadrozole (CGS-16949A) was a gift of

Ciba-Geigy (Basel, Switzerland). Solvents and chemicals of analytical grade were obtained from Sigma and Merck (Darmstadt, Germany). Steroidal and non-steroidal structures of inhibitors are described in Figure 1.

Aromatase Activity and Protein Concentration

Aromatase activity was estimated by the evaluation of the tritiated water formed during aromatization of tritiated androstenedione.[1] This substrate was used for all inhibition studies, testosterone was also tested as substrate. Protein concentration was measured according to Bradford's method.[27]

Inhibition Studies

These have been performed both with microsomes and the purified enzyme. For the preparation of microsomes, full-term placentas from thoroughbred mares and from non-smoking healthy women were collected immediately after delivery and rinsed extensively with 0.15 M KCl at 4°C. Microsomes were prepared by differential centrifugation as previously described,[22] re-suspended in 50 mM Tris-maleate buffer, pH 7.4 containing 40 mM niacinamide, 1 mM dithiothreitol and 20% glycerol and stored at −78°C. Incubation with various amounts of microsomes from human or equine placentas were performed in 0.5 ml of 50 mM Tris-maleate buffer pH 7.4 at 30°C for 25 min, in the presence or absence of different concentrations of inhibitors **1** and **2**. The reaction was initiated by the addition of 0.3 mM NADPH to microsomes and [1β-^{3}H]-androstenedione (for concentrations depending on experiments, see figure legends), and stopped by the addition of 2 ml chloroform. A 7% charcoal suspension (0.5 ml) containing 1.5% dextran T-70 was added and the mixture was gently shaken. After centrifugation (2700 × g for 10 min), the radioactivity of 0.5 ml supernatant was measured for ^3H_2O release. For the kinetic studies, incubations of 1.5 µg of equine placental microsomal proteins with or without inhibitor (**1**–**6**) were carried out in 0.5 ml of 50 mM Tris-maleate buffer pH 7.4 at 15°C for 5 min under initial velocity conditions, with [1β-^{3}H]-androstenedione and 0.3 mM NADPH. Aromatase activity was then measured as detailed above.

In order to study the effect of **1**, the classical inhibitor of human aromatase, on different steroid synthesis from 0.05 or 0.50 µM of [4-^{14}C]-androstenedione or [4-^{14}C]-testosterone respectively in equine placental microsomes, 100 µg of protein was incubated with these substrates, in 0.5 ml of 50 mM Tris-maleate buffer pH 7.4 at 30°C for 20 min with 0.3 mM NADPH, and in the presence or absence of 1 µM **1**. Steroids were

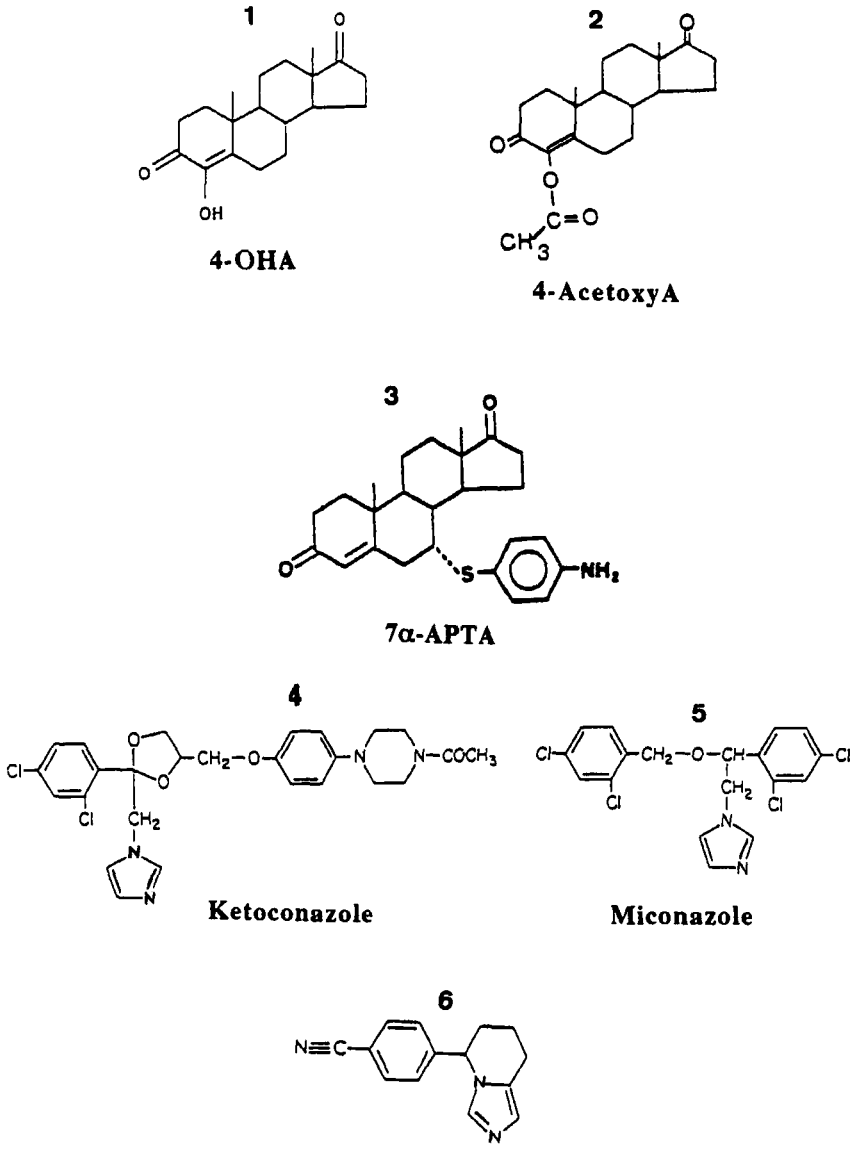

FIGURE 1 *Steroidal (androstenedione analogues,* **1**–**3***) and non-steroidal (imidazole derivatives,* **4**–**6***) inhibitors and inactivators of aromatase used in this work.* **1**: 4-hydroxyandrostenedione (4-OHA); **2**: 4-acetoxyandrostenedione (4-AcetoxyA); **3**: 7α-(4′-amino)phenylthio-4-androstene-3,17-dione (7α-APTA); **4**: ketoconazole; **5**: miconazole; **6**: fadrozole.

extracted from the incubation mixture with 4×3 ml diethyl ether. The extracts were chromatographed on 0.25 mm silica-gel plates in cyclohexane/ethylacetate (1 : 1, v/v). After autoradiography, the spots corresponding to identified metabolites were scraped off and their radioactivity was measured.

For the studies with the purified enzyme, equine cytochrome P-450$_{arom}$ was purified to homogeneity from 5 year old horse testes by five chromatographic steps: on aminohexyl-sepharose 4B, concanavaline A, hydroxyapatite, DEAE-sepharose CL-6B and a second hydroxyapatite column.[28] The activity of equine aromatase was reconstituted (13 nM) with 60 nM of purified rat liver reductase obtained as previously described[29] and incubated at 20°C for 30 min in 50 mM Tris-maleate buffer pH 7.4 and 0.3 mM NADPH, in the presence or absence of different concentrations of inhibitors **1** and **2**.

Inactivation Studies

Equine microsomes (200 µg proteins) were pre-incubated for different times at 15°C in 50 mM Tris-maleate buffer pH 7.4 containing 0.25 M sucrose with 0.3 mM NADPH, in the presence or absence of inhibitors **1**–**6**. An activated charcoal–dextran suspension (2% : 1%, 100 µl) was added to the incubation at 4°C for 5 min, the solution was then centrifuged for 10 min at $350 \times g$. An aliquot of 60 µl of supernatant was removed and incubated at 25°C for 20 min with 1 µM [1β,2β-^{3}H]-androstenedione and 0.3 mM NADPH, in 0.5 ml of 50 mM Tris-maleate buffer pH 7.4.

RESULTS AND DISCUSSION

Inhibition with Steroidal Compounds

Generally, from literature reports and results obtained in our laboratory, androstenedione, which is a more hydrophobic substrate than others, presents the best affinity for equine and human aromatases (K_m for androstenedione and testosterone with equine aromatase, obtained from gonadal and placental tissues, ranged between 2 and 13 nM and 16 and 30 nM, respectively, data not shown). Thus, the effectiveness of an aromatase inhibitor, having a structural analogy with androstenedione may come, at least in part, from its potential hydrophobicity.

With microsomal equine aromatase, the K_m for androstenedione was 1.8 ± 0.13 nM ($n = 6$). The studies demonstrated a competitive inhibition

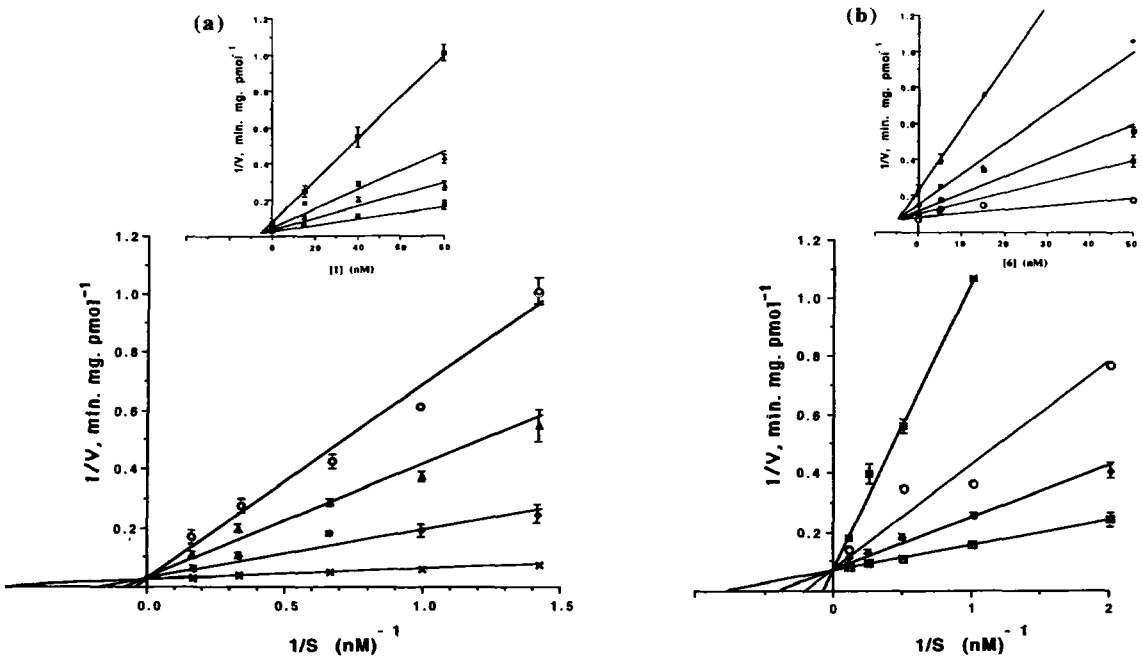

FIGURE 2 *Kinetic inhibition by* (a) **1** *and* (b) **6** *of androstenedione aromatization with equine placental microsomal aromatase.* Double-reciprocal plots of reaction velocity with (a): 0.7–6 nM [1β-^{3}H]-androstenedione and 0 (×), 15 (◆), 40 (▲), 80 (○) nM of **1**; and (b): 0.5–8 nM [1β-^{3}H]-androstenedione and 0 (□), 5 (◆), 15 (○), 50 (*) nM of **6**. Microsomal protein (1.5 µg) was incubated in 0.5 ml of 50 mM Tris-maleate buffer pH 7.4, at 15°C for 5 min with 0.3 mM NADPH. Aromatase activity was assessed by the evaluation of tritiated water formed during the enzymatic reaction, from labeled androstenedione ($n = 3$). *Inset*: Dixon-plot representation of the same data. The inhibition experiments with all other inhibitors (**2**, **3**, **4** and **5**) gave essentially similar plots.

for **1** (Figure 2(a)), with very similar results for **2** and **3** (data not shown). The corresponding K_i values, calculated from Dixon analysis, were 4.1, 26 and 1.8 nM for **1**, **2** and **3**, respectively. With 2 μM of **1**, microsomal equine and human aromatases were inhibited in a comparable manner (94% and 89% inhibition, respectively), whereas the same concentration of **2** inhibited equine aromatase more efficiently than human aromatase (92% and 33% inhibition, respectively, Figures 3(a) and (b)). The differential effect of

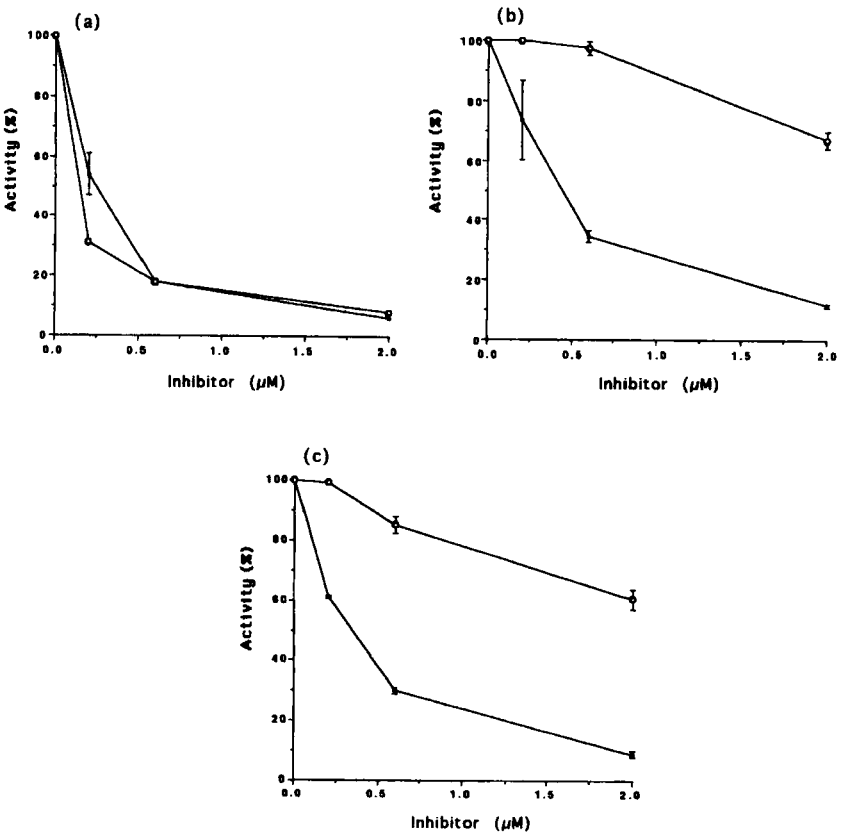

FIGURE 3 *Inhibition by steroidal inhibitors* **1** *and* **2** *of* (a) *equine and* (b) *human microsomal aromatases and* (c) *purified equine testicular aromatase.* 200 nM of [1β-^{3}H]-androstenedione was used as substrate with 0.3 mM NADPH, in 0.5 ml of 50 mM Tris-maleate buffer pH 7.4. (a) and (b): microsomal protein (100 μg) was incubated at 30°C for 25 min with different concentrations of **1** (■) and **2** (○). (c): purified equine aromatase (13 nM) was incubated at 20°C for 30 min with different concentrations of **1** and **2**. In this case, 60 nM of purified rat reductase was added before incubation to reconstitute the enzymatic complex. Aromatase activity was assessed by the evaluation of tritiated water formed from labeled androstenedione ($n = 3$).

2 on microsomal equine and human aromatases suggests that the presence of the hydrophobic group, 4-acetoxy (-$OCOCH_3$), on C4 of androstenedione, results in a more difficult binding of this inhibitor within the active site of the human enzyme. In order to exert its inhibitory effect on microsomal aromatase, **2** could need to be hydrolyzed first: it has been shown that human placental microsomes possess an esterase activity capable of hydrolyzing **2** into **1**, so that **2** is converted from a compound of low affinity into a compound with a higher affinity.[30] Furthermore, the addition of this hydrophobic group also decreases the affinity for microsomal equine aromatase (K_i of 4.1 and 26 nM for **1** and **2**, respectively). However, this reduction of affinity could be compensated by the high solubility of the inhibitor in microsomes, since at saturating concentrations it inhibits microsomal equine aromatase as effectively as **1** (92% and 94% inhibition, respectively, Figure 3(a)). This is supported by the differential effect of **2** on equine microsomal placental aromatase and on the purified testicular enzyme (92% and 40% inhibition, respectively, Figures 3(a) and (c)). Consequently, studies with a purified enzyme *in vitro* that is normally membrane bound *in vivo*, do not appear to give real estimations of the inhibitory potencies of compounds. However, microsomal preparations may give more appropriate results in comparable studies.

The presence of an (4′-amino)phenylthio group on the 7α-position of androstenedione (inhibitor **3**) results in good affinity for both human and equine aromatases. Indeed, a comparison between the K_i/K_m ratios, which reflect the relative inhibitory potencies of inhibitors, obtained in this work (for equine) and from literature (for human),[13,31] show that equine aromatase presents approximately the same affinity for **3** as does human aromatase (K_i/K_m of 1.0 and 0.3–0.4, respectively).

Specificity of Inhibition

It is well established that **1** is a specific inhibitor of human aromatase.[32] In this work, we show that, irrespective of the substrate, this inhibitor reduces aromatase and 19-hydroxylase activities of the equine placental microsomal preparation (Table I). This reinforces the hypothesis that 19-hydroxylations and aromatization of androgens are mediated by a single cytochrome P-450 mono-oxygenase enzyme.[33] It is clear that **1** has no effect on 17β-hydroxysteroid oxidoreductase activity since testosterone accumulates when androstenedione is incubated with **1** (Table I). Furthermore, androstenedione also accumulates when testosterone is incubated with **1** (Table I). Other experiments, with the equine species in our laboratory, have shown

TABLE I *Effect of* **1** *on* [4-^{14}C]-*testosterone or* [4-^{14}C]-*androstenedione metabolism with equine placental microsomes.* Equine placental microsomes (100 μg of protein) were incubated at 30°C for 20 min with different concentrations of substrates (0.05–0.5 μM) in the presence (+) or absence (−) of 1 μM of **1**. After extraction, steroids were analyzed on TLC and results are expressed as the percentage of substrate conversion into each steroid. Values presented are the mean of two determinations and the SD were within a range of ± 7% of the values indicated. 19-*OHT*: 19-hydroxytestosterone; 19-*OHA*: 19-hydroxyandrostenedione; *T*: testosterone; Δ_4: androstenedione; E_1: estrone; E_2: estradiol; ND: not detected

Substrates (μM)	*Compound* **1** (1 μM)	*Steroids* (%)					
		19-*OHT*	19-*OHA*	*T*	Δ_4	E_1	E_2
T							
0.05	−	2.2	ND	90.9	ND	0.7	6.2
	+	ND	ND	100.0	ND	ND	ND
0.50	−	11.3	ND	58.3	6.2	4.1	20.1
	+	ND	ND	90.8	8.1	0.3	0.8
Δ_4							
0.05	−	ND	2.6	1.9	84.7	10.0	0.8
	+	ND	ND	2.7	96.8	0.2	0.3
0.50	−	ND	23.5	17.5	23.9	31.5	3.6
	+	ND	3.3	31.0	62.9	1.6	1.2

that **1** did not affect 17α-hydroxylase/$C_{17,20}$-desmolase activity.[34] Thus, **1** appears to be a specific aromatase inhibitor in the equine species, like in human. From this point of view, both human and equine aromatases present comparable active sites.

Inactivation Studies

Compound **1** acts as a potent enzyme-activated inhibitor on both equine and human aromatases, in the presence of NADPH. The presence of the substrate androstenedione in the pre-incubation medium protects aromatases from inactivation caused by **1**, indicating the involvement of the active-site in the inactivation process (data not shown). The steroidal compounds **1** and **2** (2 μM) act as equivalent inactivators on equine aromatase, and are much more potent than **3** (Figure 4). Interestingly, published results with human microsomes are noticeably different.[30] Indeed, the comparison between inactivatory potencies deduced from k_{inact}/K_I ratios[†] indicates that **1** inactivates human aromatase 14-fold better than 2 (k_{inact}/K_I of 0.054 and 0.004, respectively). The comparison between the inactivatory potencies of **1** on equine (this work) and human[30] aromatases suggests that this

[†] k_{inact} is the first-order rate constant (s^{-1}) for conversion of the reversible complex to the irreversibly inhibited enzyme and K_I is the inactivator concentration required to produce a half-maximal rate of inactivation.

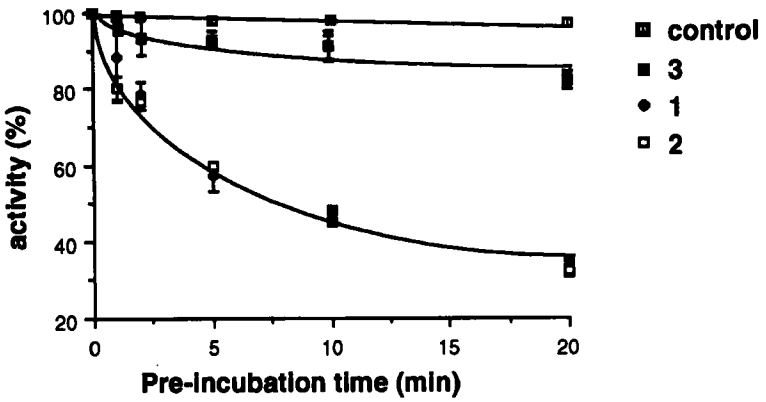

FIGURE 4 *Inactivation of equine placental microsomal aromatase activity with different steroidal inhibitors.* Microsomal protein (200 μg) was pre-incubated with 2 μM of each inhibitor (**1**, **2**, or **3**) or without (controls). Inhibitors were then removed from microsomes and an aliquot was incubated with labeled androstenedione as described in Materials and Methods. Each study ($n = 3$) at time 0 is considered as having 100% of enzymatic activity.

compound inactivates human aromatase 27-fold more efficiently than the equine enzyme (k_{inact}/K_I of 0.054 and 0.002, respectively). The half-life ($t_{1/2}$) of equine aromatase in the presence of **1** at saturating concentration was 3.5 min (Figure 5). This was comparable to the result obtained by Klein *et al.*[35] (3.2 min), and of the same order as the results obtained by Covey and Hood[30] (1.3 min) and Henderson *et al.*[36] (2.6 min). The biphasic kinetics observed with **1** or **2** (Figures 4 and 5) could be explained by the generation of a metabolite during the inactivation process, which could compete with the inactivator for the active site of aromatase. Indeed, it is generally accepted that the plot of log percent of remaining enzyme activity versus pre-incubation times often shows pseudo first-order kinetics, as an evidence for time-dependent inactivation. However, non-pseudo first-order kinetics (biphasics) can also be observed when an inactivator generates a metabolite that binds much more tightly to the enzyme than the inactivator does.[37]

Inhibition Studies with Non-Steroidal Compounds

The inhibition kinetic studies of equine aromatase indicated a competitive reversible inhibition for the non-steroidal compounds **4**, **5** and **6**, as with human aromatase.[38–40] The K_i values with equine aromatase, calculated from Dixon analysis, were 2400, 1.4 and 4 nM for **4**, **5** and **6**, respectively (Figure 2(b) and data not shown). Equine aromatase exhibits a higher

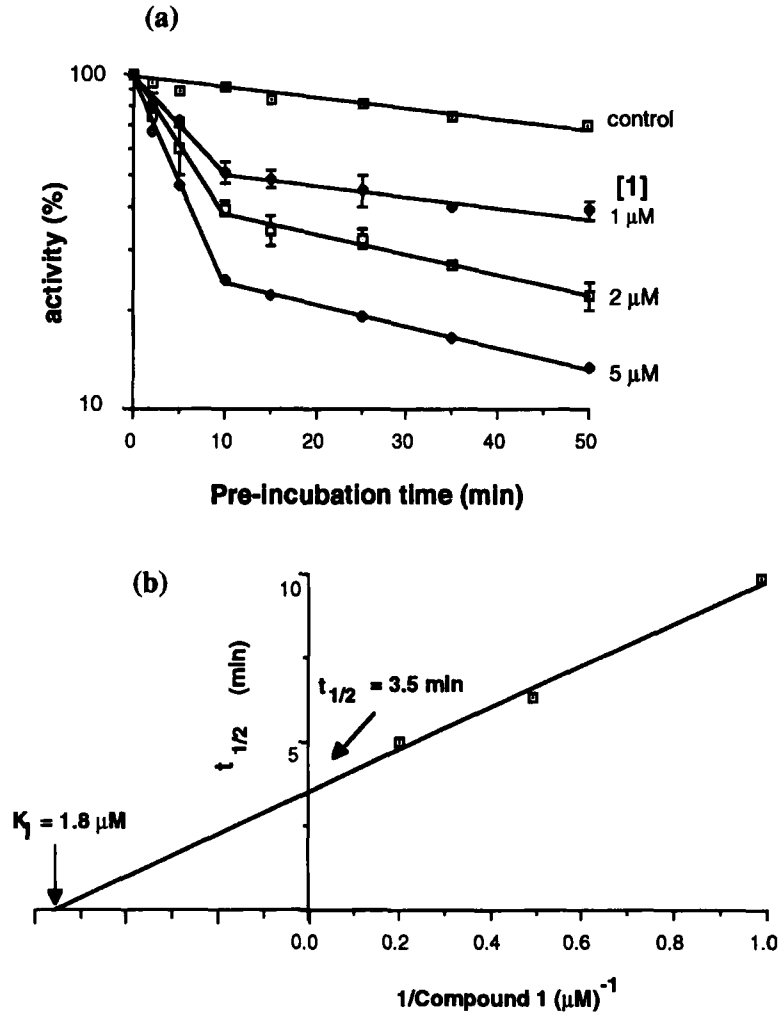

FIGURE 5 (a): *Time- and concentration-dependent inactivation by* **1** *of equine placental microsomal aromatase.* (b): *Determination of enzyme half-life* ($t_{1/2}$) *and* K_I *of* **1** *with equine placental microsomal aromatase.* Each study ($n = 3$) at time 0 is considered as having 100% of enzymatic activity.

affinity for **5** than for **6** (K_i/K_m of 0.78 and 2.22, respectively), but the human enzyme has a higher affinity for **6** than for **5** (K_i/K_m of 0.01–0.02 and 0.25, respectively).[39–41] Moreover, human aromatase[42] possesses a higher affinity for **4** than equine aromatase (K_i/K_m of 100 and 1333, respectively) but this compound exhibits a non-competitive inhibition with human aromatase when testosterone is used as the substrate.[42] All these

results indicate that both equine (the present work) and human aromatases[39–42] bind **5** and **6** more tightly than **4**. However, the inhibition mechanism with non-steroidal compounds appears to be different from the mechanism with steroidal compounds. Some non-steroidal inhibitors such as imidazole derivatives, having a heteroatom possess the capacity to bind by co-ordination to the heme iron of the cytochrome P-450. It has been reported that the mechanism of aromatase inhibition by imidazole involves the N-3 of imidazole ring.[43–45] Several authors have reported a strong inhibition of cytochrome P-450 by imidazole derivatives having one or more aromatic ring(s) linked to the N-1 substituent.[38,46–48] These authors suggested that this strong inhibition may be the result of the formation of a hydrophobic binding between the aromatic ring(s) of the inhibitor and a lipophilic region of the cytochrome P-450, close to the heme iron. It has been also suggested by Banting *et al.*[49] that analogues of 3-substituted-(unsubstituted)-3-(4′-aminophenyl)pyrrolidine-2,5-diones act by co-ordination of the nitrogen lone-pair of electrons with a vacant site of the heme iron to form a covalent bond. The inhibition mechanism by **4**, **5** and **6** of equine aromatase could involve the N-3 of the imidazole ring and/or the aromatic ring(s) linked to the N-1 substituent of these compounds (see Figure 1 for the structures). In contrast to the 3 steroidal compounds, the non-steroidal ones did not inactivate the equine enzyme. Since to our knowledge inactivators have often substrate-like structures, it could be interesting to develop specific non-steroidal inactivators in order to interfere less with the endocrine system and some progress has been made in this direction.[50]

In conclusion, the inhibitory and the inactivatory potencies of androstenedione analogues and imidazole derivatives were evaluated on equine aromatase, and compared with effects on the human enzyme. The different inhibitory and inactivation potencies exerted by these steroidal and non-steroidal compounds will allow for identification of functional and structural differences between equine and human aromatases. Modeling studies of both equine and human active sites together with these inhibitors are now in progress, since both enzymes have been cloned and biochemically characterized. Such data could help an understanding of the active-site structure of this mammalian membrane-bound enzyme, and to design new inhibitors or inactivators against estrogen-dependent diseases including breast cancer.

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