Enzymic aromatization of 6-alkyl-substituted androgens, potent competitive and mechanism-based inhibitors of aromatase

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To gain insight into the relationships between the aromatase inhibitory activity of 6-alkyl-substituted androgens, potent competitive inhibitors, and their ability to serve as a substrate of aromatase, we studied the aromatization of a series of 6 α - and 6 β -alkyl (methyl, ethyl, n-propyl, n-pentyl and n-heptyl)-substituted androst-4-ene-3,17-diones (ADs) and their androsta-1,4-diene-3,17-dione (ADD) derivatives with human placental aromatase, by gas chromatography–mass spectrometry. Among the inhibitors examined, ADD and its 6 α -alkyl derivatives with alkyl functions less than three carbons long, together with 6 β -methyl ADD, are suicide substrates of aromatase. All of the steroids, except for 6 β -n-pentyl ADD and its n-heptyl analogue as well as 6 β -n-heptyl AD, were found to be converted into the corresponding 6-alkyl oestrogens. The 6-methyl steroids were aromatized most efficiently in each series, and the aromatization

rate essentially decreased in proportion to the length of the 6-alkyl chains in each series, where the 6 α -alkyl androgens were more efficient substrates than the corresponding 6 β isomers. The $V_{\max}$ of 6 α -methyl ADD was approx. 2.5-fold that of the natural substrate AD and approx. 3-fold that of the parent ADD. On the basis of this, along with the facts that the rates of a mechanism-based inactivation of aromatase by ADD and its 6 α -methyl derivative are similar, it is implied that alignment of 6 α -methyl ADD in the active site could favour the pathway leading to oestrogen over the inactivation pathway, compared with that of ADD. The relative apparent K_m values for the androgens obtained in this study are different from the relative K_i values obtained previously, indicating that there is a difference between the ability to serve as an inhibitor and the ability to serve as a substrate in the 6-alkyl androgen series.

INTRODUCTION

Aromatase is a cytochrome *P*-450 enzyme, classified as cytochrome *P*-450 XIX A1, responsible for the conversion of the 4-en-3-one androgens androst-4-ene-3,17-dione (androstenedione, AD) and testosterone into the oestrogens oestrone and oestradiol respectively [1–3]. Aromatization of the androgens is achieved via three sequential oxygenations [4], the eventual loss of the angular methyl group at C-19 [5–9] and the elimination of the 1 β ,2 β hydrogens [8,10–13], resulting in the aromatization of the A-ring of the androgen molecule to form oestrone. Two of these oxygenations seem to occur at the C-19 position. Considerable speculation continues as to the mechanism of attack of the third molecule of oxygen. A leading theory for the third step proposes a nucleophilic attack of the haem–ferric peroxide species on the 19-aldehyde to produce a 19-hydroxy-19-ferric peroxide intermediate [8,14–16].

There seems to be a major clinical role for methods of controlling oestrogen levels, not least in the treatment of established oestrogen-dependent breast and endometrial cancers [17–20]. The inhibition of aromatase is one route to such control. A number of potent aromatase inhibitors, analogues of the substrate AD, have been described; it has been established that the introduction of a double bond at C₍₁₎–C₍₂₎ of AD causes a mechanism-based inactivation of aromatase [21]. We have synthesized 6 α - and 6 β -alkyl (C₁ to C₈)-substituted ADs [22,23] or androsta-1,4-diene-3,17-diones (ADDs) [24], some of which are among the most potent competitive or mechanism-based inhibitors reported so far. The structure–activity relationships between various substituents at C-6 and their inhibitory activities

have revealed that aromatase has a hydrophobic binding pocket with a limited accessible volume. This pocket is situated in the region corresponding to the β -side rather than the α -side at the C-6 position of AD or ADD. In contrast, both the length and the configuration of a straight alkyl chain at C-6 of ADD have an important role in the process of the mechanism-based inactivation of aromatase caused by the ADD derivatives.

Thus it was of interest to know whether 6-alkyl ADs and 6-alkyl ADDs can serve as a substrate of aromatase that results in the formation of 6-alkyl oestrogens, from the viewpoint of a correlation between their ability to serve as substrate and their ability to serve as inhibitor or inactivator. This paper describes the kinetic studies of aromatization of the 6 α - and 6 β -alkyl-substituted ADs and their ADD analogues with placental aromatase. A GC–MS analysis of the aromatization showed that the 6 α -alkyl steroids were aromatized more efficiently than the corresponding 6 β isomer, and elongation of the 6-alkyl group essentially decreased the conversion rate in proportion to its length in each series. Neither the apparent K_m nor $V_{\max}$ values for the aromatization of the alkyl androgens was correlated with the inhibition constants (K_i values).

EXPERIMENTAL

Materials

The 6 α - and 6 β -alkyl-substituted derivatives of AD, ADD and oestradiol (alkyl = methyl, ethyl, n-propyl, n-pentyl, n-heptyl) (Figure 1) were synthesized by previously reported methods

Abbreviations used: AD, androst-4-ene-3,17-dione; ADD, androsta-1,4-diene-3,17-dione.

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Figure 1 Structures of 6-alkyl-substituted androgens and oestrogens

[22–25]. [2,4,16,16,17 α - $^3\text{H}_5$]Oestradiol ($^3\text{H}_3$, 1.0%; $^3\text{H}_4$, 5.4%; $^3\text{H}_5$, 92.8%; $^3\text{H}_6$, 0.8%) was also prepared with known methods [26,27]. [1 β - ^3H]AD (specific radioactivity 27.5 Ci/mmol; ^3H distribution 76% at 1 β) was obtained from New England Nuclear (Boston, MA, U.S.A.). NADPH was purchased from Kohjin Co. (Tokyo, Japan), NaBH_4 from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and bistrimethylsilyltrifluoroacetamide from Tokyo Kasei Kogyo Co. (Tokyo, Japan). The anti-aromatase monoclonal antibody, MAb3-2C2, prepared by Dr. Y. Osawa's group [28,29] with the support of a grant from the National Institute of Child Health and Human Development (HD04945), was kindly donated.

Enzyme preparation

Human term placental microsomes (sedimented after 60 min at 105000 *g*) were obtained as described by Ryan [30]. They were washed once with 0.05 mM dithiothreitol solution, freeze-dried and stored at -20°C . No significant loss of activity occurred for 2 months.

Aromatization studies with gas chromatography–MS (GC–MS)

Incubations were done in a shaking water bath at 37°C in air with 30 ml Erlenmeyer flasks. Each contained 6.7 μM of AD, ADD or their 6-alkyl analogues, 114 μM NADPH, 500 μg of protein of placental microsomes, 100 μl of methanol and 67 mM phosphate buffer, pH 7.4, in a total volume of 2.1 ml. For each experiment, four parallel incubations were performed. After 5 or 30 min of incubation, an internal standard [6 β -ethyloestradiol (50–200 ng) for all the experiments except that in which 6 β -ethyl AD and its ADD analogue were used as substrates, where 25–100 ng of oestradiol was used as an internal standard] was added to each incubation mixture and extracted twice with 4 ml of ethyl acetate. The organic layer was evaporated under reduced pressure to give a residue, which was dissolved in methanol (100 μl). NaBH_4 (500 μg) was added to the methanolic solution at 0°C . After 30 min of reaction, the reaction mixture was diluted with water (3 ml) and then placed in a Sep-Pak C_{18} cartridge (Waters, Milford, MA, U.S.A.). After a wash with water (5 ml) followed by 10% (v/v) methanol (5 ml), the steroid fraction was eluted with 80% (v/v) methanol (5 ml). The ster-

oid fraction was evaporated to dryness under reduced pressure to yield a residue, which was then dissolved in 150 μl of benzene/methanol (19:1, v/v) and placed in a Sephadex LH 20 column (7.3 cm $\times$ 0.6 cm internal diam.; Pharmacia, Uppsala, Sweden). The column was developed with the same solvent mixture, where each aromatized product was obtained in 1–14 ml fractions. The recovery rates for all the 6-alkyloestradiols with these procedures were in the range 50–57%.

In the above incubation experiments with 6 α - and 6 β -methyl ADs, ADD and 6 α -methyl and 6 α -ethyl ADDs, 25 and 100 μg of protein of placental microsomes were also employed to determine the linearity of their aromatization rates. In the incubation experiments with AD and ADD, [2,4,16,16,17 α - $^3\text{H}_5$]oestradiol (100 ng), instead of the 6 β -ethyl oestrogen, was also employed as the internal standard.

For the kinetic analysis, the incubation mixture contained various concentrations of each 6-alkyl androgen, 25 μg of the microsomal protein, 60 μM NADPH, 100 μl of methanol and the 67 mM phosphate buffer, pH 7.4, in a 30 ml Erlenmeyer flask in a total volume of 1.0 ml. The mixture was incubated at 37°C for 5 min in air. The aromatized product was purified as described above. When [1 β - ^3H]AD was used as the substrate in the kinetic study, the aromatization rate was determined by measuring the amount of tritiated water released from the labelled substrate into the incubation medium during aromatization, by the method previously reported [31].

Derivatization of the aromatized products, 6-alkyloestradiols

The aromatized product, 6-alkyloestradiol, obtained from the Sephadex LH 20 column chromatography was dissolved in dry pyridine (30 μl). Bistrimethylsilyltrifluoroacetamide (30 μl) was added separately to the solution and the mixture was heated at 60°C for 30 min; the solvent was then removed under a stream of nitrogen gas. The residue was dissolved in anhydrous hexane (25 μl), and 2 μl portions of the solution were subjected to analysis.

GC–MS

A Finnigan MAT SSQ GC–MS instrument (San Jose, CA, U.S.A.) was used. Gas chromatographic conditions were: column, 30 m $\times$ 0.250 mm internal diam. fused silica DB5 (J & W Scientific, CA, U.S.A.); column temperature, from 50°C to 250°C at $25^\circ\text{C}/\text{min}$ and then at $10^\circ\text{C}/\text{min}$ to 280°C ; carrier gas, He at a flow rate of 1 ml/min. MS conditions were: ionization energy, 70 eV; ion source temperature, 150°C .

The quantitative analysis of the aromatized product was performed with a selected ion-monitoring method; the molecular ion was the base peak ion for all of the 6-alkyl oestrogens and oestradiol, then the amount of the oestrogen produced was obtained by the relative abundance of the molecular ion of the product to that of the internal standard.

RESULTS

Aromatization studies were initially conducted on 6-alkyl-substituted AD and ADD analogues to determine whether they are converted into the corresponding oestrogens by human placental aromatase. It was postulated that the aromatization product would be a mixture of 6-alkyloestrone and its 17 β -reduced oestrogen, 6-alkyloestradiol, produced by the action of 17 β -hydroxysteroid dehydrogenase [32,33], in each incubation. Thus the aromatization product was converted into 6-alkyloestradiol on treatment with NaBH_4 [32] in each experiment, which was

Figure 2 Mass spectra of the aromatized products obtained by incubation of 6 α -methyl AD and 6 β -ethyl ADD with human placental microsomes and their authentic samples

The 6-alkyl androgens (6.7 μ M) were incubated separately with the microsomes (500 μ g of protein) for 30 min at 37 $^{\circ}$ C. The products were converted into the 17 β -hydroxy derivatives by reduction with NaBH₄ and then analysed as the corresponding 6-alkyloestradiol bistrimethylsilyl ethers using GC-MS, as described in the Experimental section. (A, B) The product from 6 α -methyl AD (A) and the authentic 6 α -methyloestradiol (B). (C, D) The product from 6 β -ethyl ADD (C) and the authentic 6 β -ethyloestradiol (D). The 6-alkyl oestrogens produced from the other 6-alkyl androgens were also identical with the corresponding authentic samples (results not shown).

then purified and analysed as the bistrimethylsilyl ether by GC-MS (electron-impact mode). Mass spectra and retention times of the aromatized products of all of the androgens, except for 6 β -n-heptyl AD and the 6 β -n-pentyl and 6 β -n-heptyl ADDs, for which aromatization was not detected, were identical with those of the corresponding authentic 6-alkyloestradiols respectively (Figure 2). The mass spectra of the bistrimethylsilyl ethers of all the aromatized products showed a molecular ion (M^+) as the base peak with characteristic ions of $M^+ - 131$ and, with the exception of the 6-methyl and 6-n-pentyl products, m/z 325 and 229.

The aromatization rates of 6 α - and 6 β -methyl ADs, ADD and 6 α -methyl and 6 α -ethyl ADDs at a concentration of 6.7 μ M increased linearly with increasing amounts of placental microsomes (up to 500 μ g of protein); furthermore, only in the experiments using the AD analogues, the conversion of substrate increased linearly with incubation time (up to 30 min). The aromatization rates of these AD and ADD analogues were first determined in the experiments with a 30 min incubation time and 500 μ g of placental microsomes. The conversions of all the substrates were less than 13 % (Table 1). The aromatization rate of AD to oestradiol (120 pmol/min per mg of protein), obtained

Table 1 Aromatization of 6-alkyl ADs and 6-alkyl ADDs with human placental microsomes with a 30 min incubation time

All steroids (6.7 μ M) were incubated separately with human placental microsomes (500 μ g of protein) at 37 °C for 30 min in the presence of NADPH (114 μ M) in 2.1 ml of 67 mM phosphate buffer, pH 7.4. The aromatized products were analysed as the bistrimethylsilyl derivatives by GC-MS with an internal standard in each case, as described in the Experimental section. Aromatization of AD was determined with 6 β -ethyloestradiol (expt. 1) or [3 H] α -oestradiol (expt. 2) as the internal standard or by the radiometric assay (expt. 3). Results are means $\pm$ S.D. (n = 4). Abbreviation: n.d., not detected (less than 0.12 pmol/min per mg of protein).

6-Alkyl substituent (R)	Aromatization rate (pmol/min per mg of protein)	
	6 α	6 β
AD series		
Methyl	106 $\pm$ 6.5	39 $\pm$ 7.7
Ethyl	25 $\pm$ 2.0	2.3 $\pm$ 0.35
n-Propyl	4.7 $\pm$ 0.21	0.77 $\pm$ 0.08
n-Pentyl	4.5 $\pm$ 0.45	0.21 $\pm$ 0.04
n-Heptyl	0.18 $\pm$ 0.036	n.d.
H (AD), expt. 1	120 $\pm$ 12	
H (AD), expt. 2	115 $\pm$ 11	
H (AD), expt. 3	128 $\pm$ 11	
ADD series		
Methyl	114 $\pm$ 8.0	7.3 $\pm$ 0.73
Ethyl	30 $\pm$ 0.6	1.5 $\pm$ 0.15
n-Propyl	35 $\pm$ 3.0	0.40 $\pm$ 0.10
n-Pentyl	13.5 $\pm$ 2.5	n.d.
n-Heptyl	0.55 $\pm$ 0.09	n.d.
H (ADD)	26 $\pm$ 2.6	

Table 2 Effect of the anti-aromatase antibody on aromatization of AD and 6 α -methyl AD

Human placental microsomes (25 μ g of protein) were preincubated with the anti-aromatase monoclonal antibody (MAb 3-2C2) (50 μ g of IgG protein) for 45 s at 37 °C. The aromatization rate then was determined under the kinetic analysis conditions described in the Experimental section. Results are means $\pm$ S.D. (n = 4).

Substrate	Relative aromatization rate (%)	
	Without antibody	With antibody
AD	100	37 $\pm$ 4.0
6 α -Methyl AD	100	33 $\pm$ 3.3

in the experiment with 6 β -ethyloestradiol as an internal standard for the GC-MS analysis, was similar to that (115 pmol/min per mg of protein) obtained in the experiment with [3 H] α -oestradiol as the internal standard and also to that (128 pmol/min per mg of protein) obtained by the radiometric assay with [1 β - 3 H]AD as a substrate (Table 1). The rates of AD aromatization over a range of AD concentrations between 60 and 240 nM, measured by the GC-MS method with [3 H] α -oestradiol as the internal standard, corresponded well to those measured by the radiometric assay (see Table 4). These reveal that the GC-MS method employed in this study is reliable.

The aromatization of 6 α -methyl AD was effectively blocked in the presence of the anti-aromatase monoclonal antibody, as seen in the inhibition of the aromatization of the natural substrate AD (Table 2). Some 6-alkyl ADDs are known to be mechanism-based inhibitors of aromatase that cause a time-dependent loss of

Table 3 Aromatization of 6-alkyl ADD with human placental microsomes with a 5-min incubation time

All steroids (6.7 μ M) were incubated separately with human placental microsomes (500 μ g of protein) for 5 min, as described in Table 1. Results are means $\pm$ S.D. (n = 4). Abbreviation: n.d., not determined.

6-Alkyl substituent (R)	Aromatization rate (pmol/min per mg of protein)	
	6 α	6 β
Methyl	314 $\pm$ 6.1	23 $\pm$ 1.8
Ethyl	67 $\pm$ 5.8	2.2 $\pm$ 0.43
n-Propyl	71 $\pm$ 5.8	0.60 $\pm$ 0.15
n-Pentyl	18 $\pm$ 2.0	n.d.
n-Heptyl	0.62 $\pm$ 0.10	n.d.
H (ADD)	97 $\pm$ 3.5	

the aromatase activity during the incubation [24]. Therefore, to minimize the time-dependent loss of the initial aromatization rate, a short (5 min) incubation time was employed for the aromatization of a series of ADD analogues (Table 3). The aromatization rates obtained with the short incubation time were higher than those obtained with the longer incubation time only in the case of the suicide substrates. When ADD and its 6 α -methyl derivative at a 6.7 μ M concentration were separately preincubated with the placental microsomes in the presence of NADPH at 37 °C for 5 min, the remaining aromatase activity was approx. 95% of the initial rate obtained with no preincubation in each case. Moreover, when a 3 min incubation time was employed for the aromatization of ADD, its aromatization rate (100 $\pm$ 9.6 pmol/min per mg of protein, n = 4) was not significantly different from that obtained in the 5 min incubation experiment. On the basis of these observations, the aromatization rate of the suicide substrate, obtained from the 5 min incubation experiment, would be comparable with the initial rate.

The aromatization of 6 α - and 6 β -methyl ADs, 6 α -ethyl AD, and 6 α -alkyl ADDs having a methyl, ethyl or n-propyl group at C-6 as well as 6 β -methyl ADD, which are good substrates for aromatase, was further studied to characterize the affinity (K_m) for the enzyme with a 5 min incubation time. All seven steroids showed a typical saturation curve with increasing substrate concentration in each case (Figure 3), and the Lineweaver-Burk plot gave the apparent K_m and V_{max} values shown in Table 4.

DISCUSSION

A series of 6-alkyl-substituted AD and ADD analogues were aromatized by human placental microsomes to the corresponding 6-alkyl oestrogens. The conversion of 6 α -methyl AD into 6 α -methyloestradiol was efficiently blocked by an anti-aromatase antibody (Table 2), as previously reported for AD [34].

The introduction of a methyl group at the C-6 β position of AD or ADD markedly decreased the capacity to serve as a substrate of aromatase to about one-third or one-quarter that of the parent AD or ADD, respectively (Tables 1 and 3). In contrast, the introduction of a methyl group at the 6 α -position produced an approx. 4-fold higher aromatization of ADD but had no significant effect on the aromatization rate of AD. Elongation of the 6 α - and 6 β -methyl groups decreased the aromatization essentially in proportion to the carbon number of the straight alkyl chain at C-6 in the AD and ADD series. This effect was more pronounced in the 6 β -alkyl series than in the 6 α -alkyl series.

Figure 3 Kinetic analysis of aromatization of 6 α -methyl AD and 6 α -methyl ADD with human placental aromatase

Various concentrations of 6 α -methyl AD (**A**) and its ADD analogue (**B**) were incubated with microsomes (25 μ g of protein) for 5 min at 37 $^{\circ}$ C and the aromatization rates were analysed by GC-MS with an internal-standard method as described in the Experimental section. Each experiment was performed in duplicate; the variation between determinations was less than 10%.

The conversion of the 1,4-diene steroid, ADD, by placental aromatase into oestrone has previously been examined under conditions with a prolonged incubation time (30 or 60 min) and a large quantity of placental microsomes (more than 3 mg of protein) to demonstrate that the capacity of ADD to serve as a substrate of the enzyme is about one-quarter of that of AD [30,35–37]. When we employed a 5 min incubation time for the aromatization experiment with the 6-alkyl ADD analogues, the rate of aromatization was approx. 2.5-fold that observed in the experiments with the 30 min incubation for the suicide substrates ADD and its 6 β -methyl, 6 α -methyl, 6 α -ethyl and 6 α -n-propyl derivatives. There was no significant difference in the aromatization rates between the 5 and 30 min incubation experiments

Table 4 Kinetic analysis of aromatization of selected 6-alkyl androgens with human placental microsomes

All incubations were performed at 37 $^{\circ}$ C for 5 min with various concentrations of the 6-alkyl steroid and human placental microsomes (25 μ g of protein) in the presence of NADPH (60 μ M), as described in the Experimental section. Apparent K_i values obtained previously [22,24] are also listed for comparison. In the previous experiments, the apparent K_m value for AD was approx. 20 nM.

Steroid	K_i from [22,24] (nM)	K_m (nM)	V_{max} (pmol/min per mg of protein)
6 α -Methyl AD	5.6	9.8	120
6 β -Methyl AD	11	25	60
6 α -Ethyl AD	4.7	34	18
6 α -Methyl ADD	54	232	250
6 β -Methyl ADD	43	132	20
6 α -Ethyl ADD	19	89	52
6 α -n-Propyl ADD	22	98	81
ADD	43	56	105
AD*		30	130
AD†		25	120

* These values were obtained by radiometric assay.

† These values were obtained by GC-MS with [3 H] α -oestradiol as an internal standard.

with the ADD derivatives with the 6 β -ethyl, 6 β -n-propyl, 6 α -n-pentyl and 6 α -n-heptyl groups, which are not suicide substrates (Table 3). Interestingly, 6 α -methyl ADD was aromatized approx. 2.5-fold more efficiently than the natural substrate AD. To our knowledge, this compound is the first to show such a high capacity to serve as a substrate of aromatase. Because the rates of the time-dependent inactivation of aromatase caused by ADD and its 6 α -methyl derivative are similar [24], it is presumed that active-site alignment of 6 α -methyl ADD could favour the pathway leading to oestrogen over the inactivation pathway, compared with that of ADD.

To gain further insights into the binding aspects of the 6-alkyl-substituted AD and ADD derivatives at the active site of aromatase, the aromatization of the AD derivatives having 6 α - and 6 β -methyl and 6 α -ethyl groups as well as ADD and its 6 α -methyl, 6 β -methyl, 6 α -ethyl and 6 α -n-propyl analogues were then analysed kinetically (Table 4). The apparent K_m and V_{max} values for ADD were 56 nM and 105 pmol/min per mg of protein respectively, showing that ADD is not as unsuitable a substrate for aromatase as previously reported (1.3 μ M for K_m and 14 pmol/min per mg of protein for V_{max}) [37], possibly owing to the long incubation time (30 min) used previously.

The apparent K_m values for ADD as well as 6 α -methyl AD and its 6 β -isomer were very similar to the corresponding K_i values obtained by the inhibition study [24]; in contrast, the K_m values for 6 β -methyl and 6 α -ethyl ADs and 6-alkyl ADD analogues with one to three carbons at the 6 α position were approx. 3–5 times the corresponding K_i (Table 4). Thus there is no significant correlation between the ability to serve as an inhibitor and the ability to serve as a substrate in the 6-alkyl androgen series. Furthermore, with the exception of 6 β -methyl ADD, the aromatization rates of 6-alkyl ADD analogues causing time-dependent inactivation were higher than those not causing it. This suggests that there might be a significant correlation between the aromatization reaction and production of a reactive electrophile binding irreversibly to the active site during the inactivation process, although the structure of the electrophile is currently uncertain [21,38].

On the basis of previous findings on the relative binding affinities of some 6-alkyloestradiols for the oestrogen receptor in lamb uterine cytosol [39], it is likely that the binding of 6-alkyloestrones to the oestrogen receptor would be very low.

The aromatization of the 6-alkyl-substituted AD and ADD analogues, potent competitive and/or mechanism-based inhibitors of aromatase, has been studied extensively with GC-MS. The length and configuration of the 6-alkyl group have a critical role in the cause of the catalytic function of aromatase, probably through a steric interaction of the alkyl substitution with the binding pocket situated around the C-6 position in the active site. The present results will be valuable for understanding both the alignment of the aromatase inhibitors in the active site of aromatase and also the spatial structure of the active site.

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