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EVALUATION OF 6,7-AZIRIDINYL STEROIDS AND RELATED COMPOUNDS AS INHIBITORS OF AROMATASE (P-450$_{arom}$)

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Inhibitors of human placental aromatase (P-450$_{arom}$) may be useful in treating estrogen-dependent diseases (e.g. breast cancer). Some 6,7-aziridinyl steroids and related compounds (fused steroidal oxiranes, azidohydrins and an azide) were evaluated as inhibitors of this enzyme. Although the 6,7-aziridines and their N- derivatives are poor inhibitors of the enzyme (IC_{50} values 49–>>250 μM), most of the other compounds are modest inhibitors (IC_{50} values 3.0–15.0 μM), while 6β-azido-7α-acetoxyandrost-4-ene-3,17-dione (**10**) is a potent inhibitor of the enzyme (IC_{50} value = 0.4 μM, K_i = 14 nM). The difference in inhibitory potency between **10** and the parent compound, 6β-azido-7α-hydroxyandrost-4-ene-3,17-dione (**9**), (IC_{50} value = 47 μM, K_i = 294 nM) is striking and unexpected. The inhibitory potency of **10** is comparable to that of formestane (4-hydroxyandrost-4-ene-3,17-dione, 4-OHA, **16**) (IC_{50} value = 0.6 μM, K_i = 9 nM), an inhibitor of aromatase which recently has been approved for clinical use in breast cancer treatment. Our most active inhibitors, **10** and 7α-azido-6β-hydroxyandrost-4-ene-3,17-dione (**11**) (at concentrations of 125 μM each) did not inhibit the rat 17α-hydroxylase/$C_{17,20}$-lyase (17α-lyase) enzyme.

KEY WORDS: Human placental aromatase (P-450$_{arom}$), 6,7-substituted analogues of androstenedione, aromatase inhibitors

INTRODUCTION

The developement and study of inhibitors of aromatase remains an important task because of their potential therapeutic value in the treatment of estrogen-dependent diseases, for example, breast cancer.[1–3] Very recently, formestane (4-hydroxyandrostenedione, 4-OHA, **16**), a potent aromatase inhibitor has been approved for clinical use in breast cancer treatment in the United Kingdom and a number of other countries.[4] Several 6- and 7-substituted analogues of androstenedione (AD) are powerful inhibitors of human placental aromatase.[5–7] In addition, 6α,7α-cyclopropyl-3-desoxyandrostenedione has recently been shown to be a potent aromatase inhibitor.[8] These literature precedents prompted the present studies in which we have evaluated 6α,7α- and 6β,7β-aziridinyl steroids and related compounds

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(fused steroidal oxiranes, azidohydrins and an azide) as inhibitors of human placental aromatase.[9]

MATERIALS AND METHODS

Chemical Synthesis

We have recently reported[10] the synthesis of the following compounds: 6α,7α-aziridinylandrost-4-ene-3,17-dione (**1**) and its acetyl and tosyl derivatives (**2** and **3** respectively); 6β,7β-aziridinylandrost-4-ene-3,17-dione (**4**) and its acetyl and tosyl derivatives (**5** and **6** respectively); 6α,7α-oxiranylandrost-4-ene-3,17-dione (**7**); 4α,5α-oxiranylandrost-6-ene-3,17-dione (**8**); 6β-azido-7α-hydroxyandrost-4-ene-3,17-dione (**9**); 6β-azido-7α- acetoxyandrost-4-ene-3,17-dione (**10**);7α-azido-6β-hydroxyandrost-4-ene-3,17-dione (**11**); 7α-azido-6β-acetoxyandrost-4-ene-3,17-dione (**12**). The synthesis of the following compounds have been described previously: 6-azidoandrosta-4,6-diene-3,17-dione (**13**),[11] androsta-4,6-diene-3,17-dione (**14**).[12] 6α,7α-difluoromethylandrost-4-ene-3,17-dione (**15**),[8] and 4-hydroxyandrost-4-ene-3,17-dione (**16**).[13] The structures of these compounds (**1–16**) are shown in Chart 1.

Preparation of Aromatase

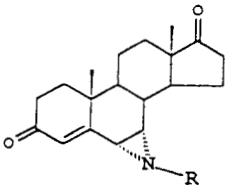
The enzyme was obtained from the microsomal fractions of freshly delivered human term placenta according to the method of Thompson and Siiteri.[14] The microsomes were resuspended in a minimum volume of phosphate buffer (0.05 M, pH = 7.4) and stored at −70°C. No loss of activity was observed within four months. Protein concentration was measured according to Lowry *et al.*[15]

Inhibition of Aromatase

Enzyme activity was monitored using the tritiated water method of Thompson and Siiteri.[14] The incubations were performed as described previously.[16] The tritiated water formed during the conversion of [1β,2β-^{3}H]-testosterone to estradiol was determined after separation of the steroids by dextran-coated charcoal (DCC). Following centrifugation, the radioactivity of a 200 μl supernatant aliquot was determined. For the determination of IC_{50} values, compounds were tested in six appropriate concentrations. The molar concentration causing 50% inhibition of aromatase activity was determined by plotting the percentage inhibition *vs* the concentration of inhibitor on a semilog plot.

The K_i values were determined using the same procedure as described above but with modifications as follows: concentrations of [^{3}H]-testosterone 0.05–0.4 μM; microsomal protein, 20–40 μg/incubation, 1 mM NADP and NADPH generating system (glucose-6-phosphate, 10 mM and glucose-6-phosphate dehydrogenase, 1 EU). The incubations (0.25 ml) with and without inhibitor were carried out for 15 min at 30°C under initial velocity conditions. The K_i values were calculated from the Lineweaver-Burk plots (e.g. Figure 1) and K_m value for testosterone (substrate) was also determined.

CHART 1



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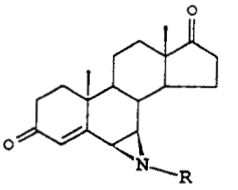

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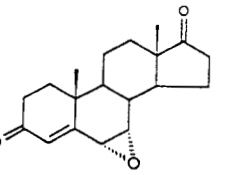

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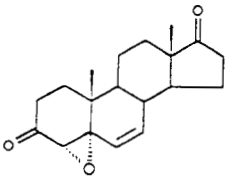

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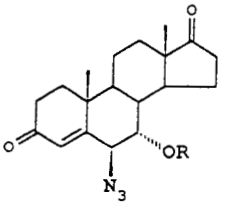

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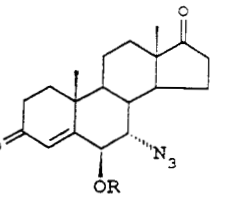
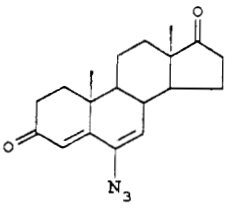


11, R = H
12, R = Ac



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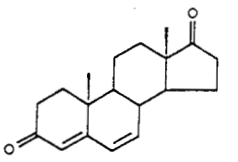

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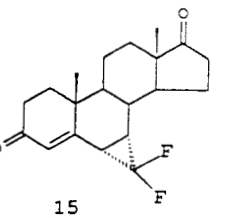

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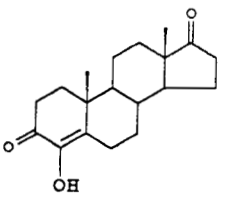

16

The assay for irreversible inhibition of aromatase was performed as previously described,[16] by incubating microsomal aromatase with NADPH and inhibitor (10 μM) for 30 min. After separation of the inhibitor (addition of DCC followed by centrifugation), aromatase activity in the supernatant was measured at 8, 16 and 24 min intervals. The substrate used in this assay was [^{3}H]-testosterone (0.5 μM).

Inhibition of 17α-Hydroxylase/17,20-Lyase

This assay was performed as recently reported by Sergejew and Hartmann.[17]

RESULTS AND DISCUSSIONS

The effectiveness of the compounds (**1–13**) as inhibitors of aromatase was tested by determining the concentration of the steroid which results in 50% inhibition (IC_{50} value) of the rate of estrogen synthesis catalysed by the enzyme. The compounds exhibit a broad range of inhibition constants (from 0.4 – $\gg$ 250 μM, Table 1). In addition, the IC_{50} values of some known aromatase inhibitors, androsta-4,6-diene-3,17-dione (**14**), 6α,7α-difluoromethylandrost-4-ene-3,17-dione (**15**) and 4-hydroxyandrost-4-ene-3,17-dione (4-OHA, **16**) were determined for comparison (Table 1).

TABLE 1
Aromatase inhibition by analogues of androstenedione.

Compound	IC_{50}, μM[a,b]	K_i, nM[c,d]
1	$\gg$250	nd
2	65	nd
3	93	nd
4	49	nd
5	>250	nd
6	54	nd
7	39	nd
8	15	nd
9	47	294±16
10	0.4	14±2
11	3	86±9
12	23	nd
13	4.6	nd
for comparison		
Androsta-4,6-diene-3,17-dione (**14**)	2	nd
6α,7α-Difluoromethylandrostenedione (**15**)	0.9	nd
4-Hydroxyandrostenedione (**16**)	0.6	9±1

[a]Substrate: 2.5 μM [1β,2β-^{3}H] testosterone; [b]Mean values of at least 4 experiments. The deviations were within ±10%; [c]K_m (testosterone): 86±10 nM. nd = not determined; [d]Average of 3 experiments.

The IC_{50} values of the isomeric aziridines, 6α,7α-aziridinylandrost-4-ene-3,17-dione (**1**) and the 6β,7β-isomer (**4**) and their corresponding derivatives [N-acetates (**2** and **5**), N-tosylates (**3** and **6**) respectively] show that they are poor aromatase inhibitors. However, differences in their inhibitory potency due to aziridine ring stereochemistry are observed. Considering that these aziridines are structurally similar to the 6α,7α-cyclopropane derivatives of androst-4-ene-17-one and androst-4-ene-3,17-dione, which are potent aromatase inhibitors,[8] the observed poor inhibitory potency of the aziridines may be due to their polar and/or basic nature. However, the poor inhibitory activity also exhibited by the 6α,7α-epoxide [**7**, IC_{50} = 39 μM, (aziridines and epoxides of similar structure differ strongly in their basicities[18])] suggest that the lack of inhibition of the enzyme by the 6,7-aziridines may not be due to the basicity of the aziridine group. Substitution of an aziridine or oxirane ring for hydrogen atoms at C-6 and C-7 of AD would cause significant structure perturbation. This structural feature may be responsible for their poor binding, although the exact stereochemical aspects of steroid binding is not known. Presumably, a hydrophobic three-membered ring at the 6- and 7-positions of AD (as observed with the 6α,7α-cyclopropane derivatives[8]) is important for tight binding to the enzyme.

4α,5α-oxiranylandrost-6-ene-3,17-dione (**8**) is a modest inhibitor (IC_{50} = 15.0 μM) of the enzyme. Androsta-4,6-diene-3,17-dione (**14**) is a good inhibitor (IC_{50} = 2.0μM) suggesting that C4-5 unsaturation is important for binding.[19] 6-Azidoandrosta-4,6-diene-3,17-dione (**13**) has an inhibitory potency (IC_{50} = 4.0 μM) comparable with that of the parent androsta-4,6-diene-3,17-dione (**14**, IC_{50} = 2.0 μM).

6β-azido-7α-hydroxyandrost-4-ene-3,17-dione (**9**) is a poor inhibitor (IC_{50} = 47 μM) of the enzyme. However, acetylation of the 7α-OH in **9** to give the 7α-*O*-acetyl compound, **10** causes a 118-fold increase in the inhibitory potency, resulting in the best inhibitor (IC_{50} = 0.4 μM) among the compounds tested. On the other hand, the opposite result was observed for the isomeric 7α-azido-6β-hydroxyandrost-4-ene-3,17-dione (**11**) in which acetylation of the 6β-OH in **11** to give the 6β-*O*-acetyl compound, **12** causes an 8-fold decrease in the inhibitory potency. The factor(s) underlying the potent inhibitory property of compound **10** is unknown at this time. Whether this is due to the increase in the 7α-substituent's size and/or favourable electronic effects of the acetyl substituent remains to be determined.

Following these initial enzyme studies, the isomeric azidohydrins **9** and **11** and the acetyl derivative of **9**, compound **10** were evaluated further to determine the apparent K_i values (from Lineweaver-Burk plots; e.g. Figure 1). The apparent K_i values are presented in Table 1 and show that all three compounds are competitive inhibitors of human placental microsomal aromatase. The inhibitory potency of compound **10** which has a K_i value of 14 nM is comparable with that of the clinically used aromatase inhibitor, 4-OHA (**16**), which has a K_i value of 9 nM under the same assay conditions.[20]

To further define the mechanism of inhibition of compounds **9–11** we performed an assay for irreversible inhibition. In this assay we also examined 4-OHA (an inhibitor which irreversibly binds to aromatase[21]) and aminoglutethimide (AG, a strictly competitive reversible aromatase inhibitor). Human placental microsomes were separately incubated with the inhibitors for 30 minutes as previously described.[16] After removal of the inhibitors (by DCC treatment followed by centrifugation),

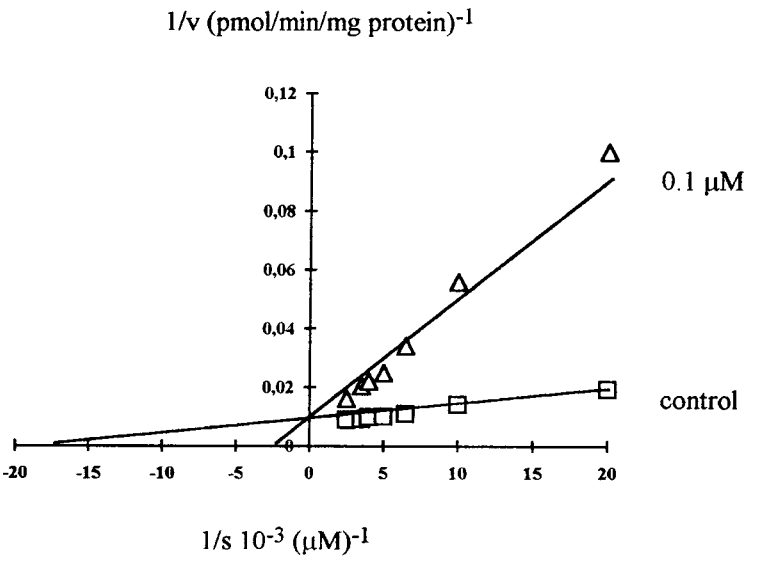

FIGURE 1 Lineweaver-Burk analysis (1/v *vs* 1/s) of azidohydrin acetate **10** at 0.1 μM. The inhibition experiments with other compounds, **9**, **11** and **16** gave plots that were essentially the same as shown above. A computer programme: easy plot[tm] for microsoft windows version 2.22 was used to obtain the plots.

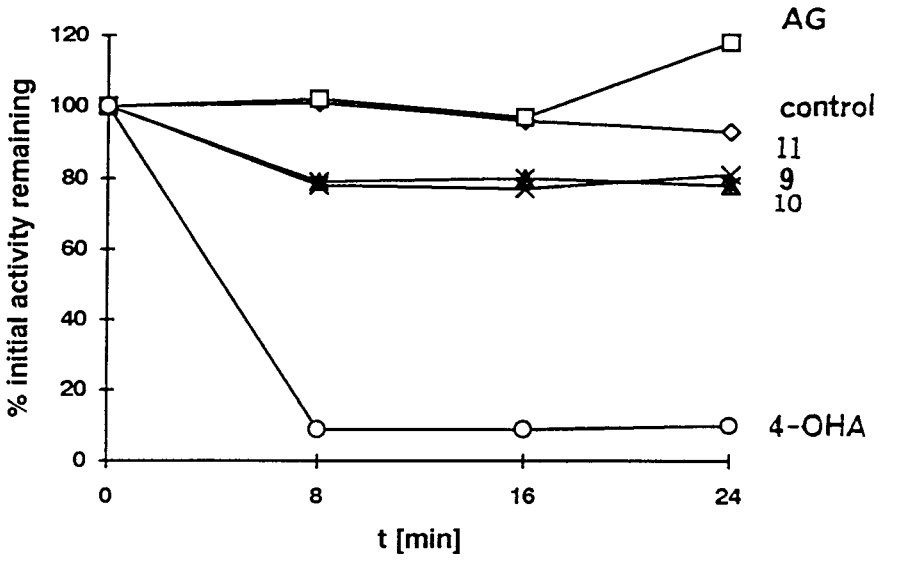

FIGURE 2 Irreversible inhibition studies with compounds **9–11**, AG and 4-OHA. Time course decrease in aromatase activity after treatment of the enzyme with inhibitors followed by removal of inhibitors from the microsomes by DCC treatment and centrifugation. Each point represents an average of three replicates with four experiments and the deviations were within a range of ±8%.

the enzyme activity was assayed over 24 minutes shown in Figure 2. The results show as expected that AG caused no irreversible inhibition of the enzyme while 4-OHA caused approx. 90% irreversibility. Each of the azidohydrin compound caused approx. a 20% irreversible inhibition of the enzyme.

Specificity of inhibition as well as intrinsic biological activity are important considerations regarding aromatase inhibitors.[22] Since it is known that some aromatase inhibitors also inhibit other cytochrome P-450-dependent hydroxylases of steroid biosynthesis[2] [e.g. 17α-hydroxylase/$C_{17,20}$-lyase (17α-lyase)], we examined whether our most active inhibitors exhibit this property too. Compounds **10** and **11** did not inhibit the rat 17α-lyase enzyme at concentrations of 125 μM each, which are 313- and 28-fold greater, respectively, of their aromatase inhibitory IC_{50} values (data not shown).

In conclusion, the present studies have identified 6β-azido-7α-acetoxyandrost-4-ene-3,17-dione (**10**, the 7α-acetyl derivative of azidohydrin **9**, a poor aromatase inhibitor) as a potent inhibitor of human placental aromatase. It would clearly be of interest to evaluate other 7α-derivatives (esters and ethers of various sizes) of **9** for aromatase inhibitory activity.

Acknowledgements

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