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Effect of Aromatase Inhibitors on Estrogen 2-Hydroxylase in Rat Liver

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The effect of aromatase inhibitors, 4-hydroxyandrostenedione, CGS 16949A and aminoglutethimide on the inhibition of estrogen 2-hydroxylase activity in rat liver microsomes *in vitro* and on its induction *in vivo* has been examined. Estrogen 2-hydroxylase was found to have over twice the affinity for estradiol compared to estrone. Using high pressure liquid chromatography and employing estradiol as a substrate, the IC_{50} values were 2.2, 98, 110 and 908 μM for the reference compound ketoconazole and the aromatase inhibitors, 4-hydroxyandrostenedione, CGS 16949A and aminoglutethimide, respectively. Similar IC_{50} values were obtained using estrone as a substrate and by a tritiated water method employing estradiol as a substrate. The K_m value for estrogen 2-hydroxylase with estradiol as a substrate using a tritiated water method was 4.3 μM with a V_{max} of 11.89 nmol/h/mg. Ketoconazole, CGS 16949A and aminoglutethimide exhibited non-competitive inhibition whereas 4-hydroxyandrostenedione appeared to be a competitive inhibitor of estrogen 2-hydroxylase. The K_i values were 2.6, 72, 114 and 958 μM for ketoconazole, 4-hydroxyandrostenedione, CGS 16949A and aminoglutethimide, respectively. All three aromatase inhibitors were weak inhibitors of estrogen 2-hydroxylase as compared to the reference drug, ketoconazole. Following treatment of rats with aminoglutethimide (40 mg/kg/day; i.p.; for 3 days), estrogen 2-hydroxylase activity was increased by 28 and 30% using estradiol and estrone as substrates, respectively. Following treatment of rats with CGS 16949A (2 mg/kg/day; p.o.; for 3 days), the corresponding increase in estrogen 2-hydroxylase activity was 48 and 44%. The results of this study indicate that the aromatase inhibitors, aminoglutethimide and CGS 16949A are only weak inhibitors of estrogen 2-hydroxylase activity *in vitro* and show no evidence of inhibition *in vivo*. On the contrary, there was some evidence to suggest that both aminoglutethimide and CGS 16949A induce estrogen metabolism following repeated administration. Therefore, aminoglutethimide and CGS 16949A may lower estrogen levels not only by primarily inhibiting their synthesis but also by inducing the metabolism of estrogens.

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INTRODUCTION

The conversion of androgens to estrogens is mediated by aromatase, an enzyme complex which involves a NADPH–cytochrome *P*-450-reductase and a cytochrome *P*-450. Inhibition of aromatase can be achieved by steroidal compounds which block the active site of the enzyme or by non-steroidal compounds consisting of nitrogen heterocyclic derivatives which bind to the iron of the heme moiety and to the apoprotein of the enzyme. Since aromatase belongs to a class of cytochrome *P*-450 mixed function oxidase enzymes, inhibitors of aromatase may inhibit the activity of other enzymes which are dependent on the cytochrome *P*-450 system.

In hormone-dependent breast cancer, it is known that estrogens play an important part in promoting tumor growth. Treatment of breast cancer patients with aromatase inhibitors lowers the level of circulating estrogens [1] and so results in the regression of tumors [2].

Aminoglutethimide was the first aromatase inhibitor to be successfully used in the treatment of postmenopausal women with estrogen-dependent breast cancer. Aminoglutethimide, however, has the disadvantage that it suppresses adrenal glucocorticoid synthesis as a result of inhibiting 20,22 desmolase, 11β- and 21-hydroxylase [3]. Aminoglutethimide has also been shown to be an inducer of certain hepatic mixed function monoxygenases, increasing the metabolism of several drugs in man [4, 5].

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The estrogens, estradiol and estrone are mainly metabolized in the liver to 2-hydroxyestrogens by the enzyme estrogen 2-hydroxylase [6]. Thus the aim of the present study was to examine the effects of aminoglutethimide and two other aromatase inhibitors, 4-hydroxyandrostenedione and CGS 16949A (Fig. 1) on estrogen 2-hydroxylase in rat liver microsomes *in vitro* using estradiol and estrone as substrates. Ketoconazole, an inhibitor of several cytochrome *P*-450-dependent enzymes and a potent inhibitor of estrogen 2-hydroxylase [7] was used as a reference compound. Inhibitors of cytochrome *P*-450 *in vitro* may also act as inducers of this enzyme complex following repeated administration *in vivo* [8]. Therefore, the effect of aminoglutethimide and CGS 16949A on the induction of estrogen 2-hydroxlase in rats *in vivo* was also examined.

MATERIALS AND METHODS

[2-^{3}H]Estradiol (15 Ci/mmol) was purchased from New England Nuclear (Dreieich, Germany). Unlabeled estradiol, 2-hydroxyestradiol, estrone, 2-hydroxyestrone and mestranol were purchased from Steraloids Inc. (Wilton, U.S.A.). Ketoconazole was a gift from Janssen (Beerse, Belgium). CGS 16949A, 4-hydroxyandrostenedione and aminoglutethimide were synthesized at Ciba-Geigy. All biochemicals and solvents were purchased from E. Merck (Darmstadt, Germany).

In the inhibition study, washed liver microsomes were produced from untreated female Sprague–Dawley rats by differential centrifugation. In the induction study, groups of rats were treated with either distilled water (control), aminoglutethimide (40 mg/kg/day, i.p. for 3 days) or CGS 16949A (2 mg/kg/day, p.o. for 3 days). Both drugs have been shown to suppress the formation of tumors in rats around these dose regimens [9]. Rats were sacrificed 48 h after the last dose, allowing elimination of the drugs, and washed microsomes prepared. Protein content was determined by the method of Lowry *et al.* [10]. Estrogen 2-hydroxylase was measured by a product isolation method using high pressure liquid chromatography (HPLC) employing estradiol or estrone as substrate and by a tritiated water method employing [2-^{3}H]estradiol as a substrate.

Product isolation method using HPLC

Incubations containing estradiol or estrone (20 μM), inhibitor (0–750 μM), ascorbic acid (1 mM), NADPH (0.6 mM), microsomal protein (0.5–1.0 mg) and phosphate buffer (1/15 M; pH 7.4) were performed at 37°C (final volume 1.0 ml). The reaction was stopped after 8 min with the addition of HCl (6 M; 100 μl). The incubate was eluted on a C_{18} disposable column with methanol. The eluate was then injected onto a C_{18} reversed-phase HPLC column as described previously [11] with a linear gradient of methanol (65–80%) in aqueous $NH_4H_2PO_4$ buffer solution (0.5%; pH 3.0). The gradient 15%/min was preceded by a 7-min isocratic elution. 2-Hydroxyestradiol and 2-hydroxyestrone were quantified using mestranol as an internal

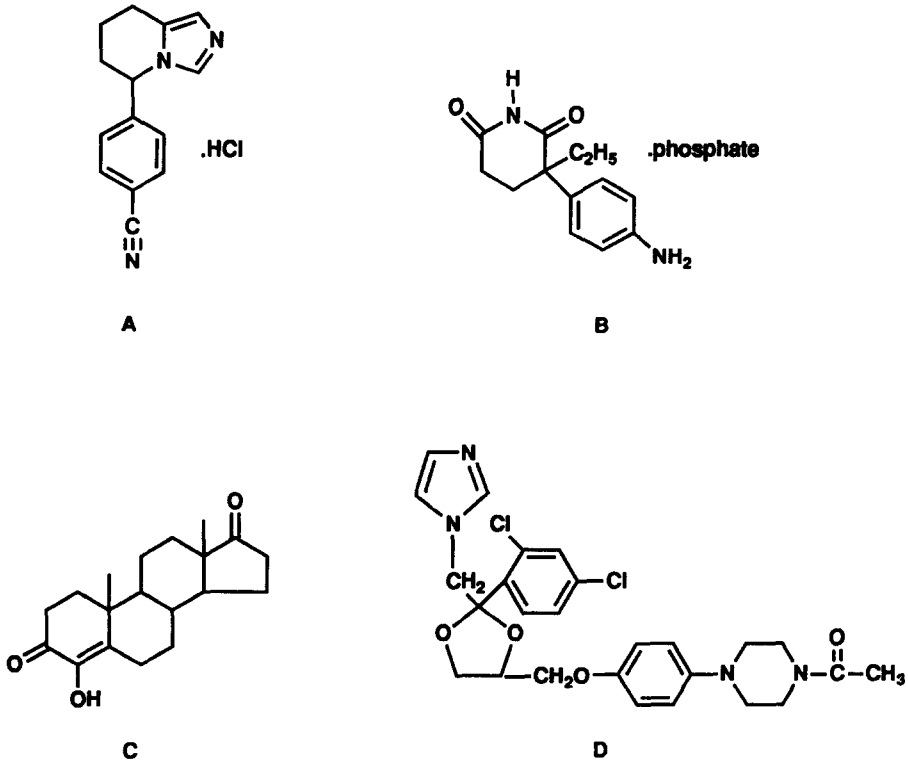

Fig. 1. Structures of (A) CGS 16949A, (B) aminoglutethimide, (C) 4-hydroxyandrostenedione and (D) ketoconazole.

Table 1. Inhibition of estrogen 2-hydroxylase in rat liver microsomes in vitro as determined by HPLC

	IC_{50} (μM)	
Substrate	Estradiol	Estrone
Ketoconazole	2.2	2.6
4-Hydroxyandrostenedione	98	100
CGS 16949A	110	137
Aminoglutethimide	908	814

IC_{50} values are mean of 2–3 individual livers.

standard. The percentage recovery of estrone, estradiol, 2-hydroxyestrone, 2-hydroxyestradiol and mestranol was between 80–95%. The intra- and inter-assay variation was < 10% for both substrates at a concentration of 20 μM.

Tritiated water method using [2-^{3}H]estradiol as a substrate

[2-^{3}H]Estradiol (0.5 μCi) was added to unlabeled estradiol (20 μM) and incubated as above. The reaction was stopped with the addition of dichloromethane (10 ml). Following extraction and centrifugation, an aliquot (0.5 ml) of the aqueous phase was removed and placed in a glass tube to which 1.0 ml of 5% charcoal suspension was added and left to stand for at least 15 min at room temperature. The tubes were centrifuged and two 0.5 ml aliquots were counted in a liquid scintillation counter. For the determination of enzyme kinetic parameters, incubations were performed containing varying concentrations of estradiol and a fixed concentration of the test compound. Kinetic parameters were derived from a Lineweaver–Burk plot. The intra- and inter-assay variation was <5%.

RESULTS

Enzyme inhibition

Estrogen 2-hydroxylase activity was 16–20 nmol/h/mg using estradiol as a substrate as determined by HPLC and a tritiated water assay. The enzyme activity was 6–8 nmol/h/mg when estrone was used as a substrate using HPLC.

Using HPLC and employing estradiol as a substrate, the IC_{50} values (determined graphically) were 2.2, 98, 110 and 908 μM for the reference compound ketoconazole and the aromatase inhibitors, 4-hydroxyandrostenedione, CGS 16949A and aminoglutethimide, respectively (Table 1). When estrone was used as a substrate, the corresponding IC_{50} values were 2.6, 100, 137 and 814 μM. Similar IC_{50} values were obtained using a tritiated water method employing estradiol as a substrate (ketoconazole; 4.1 μM; 4-hydroxyandrostenedione: 89 μM; CGS 16949A: 124 μM; aminoglutethimide: 776 μM; Table 2).

The K_m value for estrogen 2-hydroxylase using estradiol as a substrate in the tritiated water method was 4.3 μM with a V_{max} of 11.89 nmol/h/mg as derived from a Lineweaver–Burk plot using a non-weighted linear regression programme (Fig. 2). Ketoconazole, CGS 16949A and aminoglutethimide exhibited non-competitive inhibition, decreasing the V_{max} from 11.89 to 4.6, 4.59 and 6.63 nmol/h/mg, respectively. 4-Hydroxy androstenedione, on the other hand, exhibited competitive inhibition, increasing the K_m from 4.3 to 10.6 μM. The K_i values were 2.6, 72, 114 and 958 μM for ketoconazole, 4-hydroxyandrostenedione CGS 16949A and aminoglutethimide, respectively (Table 2).

Enzyme induction

Table 3 shows the effect of aminoglutethimide (40 mg/kg/day; i.p.; for 3 days) and CGS 16949A (2 mg/kg/day; p.o.; for 3 days) administered once daily to rats on estrogen 2-hydroxylase activity as determined by HPLC using estradiol and estrone as substrates. Following treatment of rats with aminoglutethimide, estrogen 2-hydroxylase activity was increased by 28 and 30% from 16.28 ± 2.15 to 20.85 ± 1.69 and from 6.64 ± 0.83 to 8.60 ± 0.73 nmol/h/mg microsomal protein using estradiol and estrone as substrates, respectively. Following treatment of rats with CGS 16949A, estrogen 2-hydroxylase activity was increased by 48 and 44% from 19.57 ± 1.26 to 29.01 ± 2.59 ($P < 0.001$) and from 7.58 ± 0.21 to 10.94 ± 1.86 nmol/h/mg microsomal protein ($P < 0.04$) using estradiol and estrone as substrates, respectively.

DISCUSSION

Estrogen 2-hydroxylase activity was 16–20 and 6–8 nmol/h/mg microsomal protein using estradiol and estrone as substrates, respectively. Thus, estrogen 2-hydroxylase has over twice the affinity for estradiol than for estrone. However, the IC_{50} values for the aromatase inhibitors, 4-hydroxyandrostenedione, CGS 16949A and aminoglutethimide were of similar magnitude using both estrogens as substrates. Also, using HPLC and a tritiated water method, similar results were obtained using estradiol as a substrate. Thus, the tritiated water method presents us with a rapid alternative to measure estrogen 2-hydroxylase activity using radiolabeled estradiol as a substrate.

From the IC_{50} and K_i values obtained in this study, it is evident that all three aromatase inhibitors are weak inhibitors of estrogen 2-hydroxylase *in vitro* as compared to the reference drug, ketoconazole. CGS

Table 2. Inhibition of estrogen 2-hydroxylase in rat liver microsomes in vitro using a tritiated water method with estradiol as a substrate

	IC_{50} (μM)	K_i (μM)	Type of inhibition
Ketoconazole	4.1	2.6	Non-competitive
4-Hydroxyandrostenedione	89	72	Competitive
CGS 16949A	124	114	Non-competitive
Aminoglutethimide	776	958	Non-competitive

IC_{50} values are mean of 2–3 individual livers. K_i values were obtained from pooled microsomes from 3 livers. Each value is an average of two determinations.

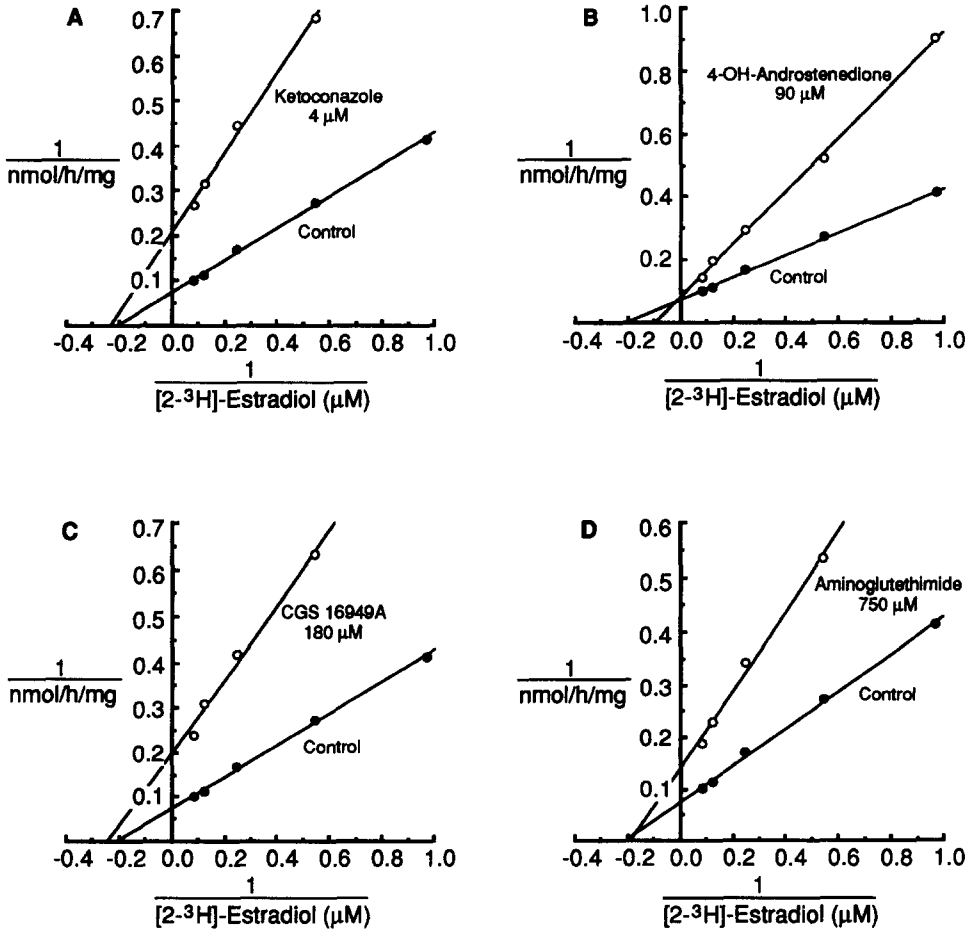

Fig. 2. Typical Lineweaver–Burk plots, showing the effect of (A) ketoconazole, (B) 4-hydroxyandrostenedione, (C) CGS 16949A and (D) aminoglutethimide on estrogen 2-hydroxylase activity in rat liver microsomes.

16949A, aminoglutethimide and ketoconazole exhibited non-competitive inhibition as determined by a Lineweaver–Burk plot. Although 4-hydroxyandrostenedione was a relatively weak inhibitor of estrogen 2-hydroxylase, it appeared to inhibit the enzyme competitively. Substituted androgens are known to inhibit estrogen 2-hydroxylase in a competitive manner [12].

Aminoglutethimide has previously been shown to induce cytochrome *P*-450s [4, 5], and CGS 16949A has already been established as a very potent non-steroidal aromatase inhibitor *in vitro* and *in vivo* [13, 14]. Therefore, these two aromatase inhibitors were selected to study their effects on estrogen 2-hydroxylase activity in the induction study.

Table 3. In vivo induction of estrogen 2-hydroxylase in rat liver

	Estrogen 2-hydroxylase activity (nmol/h/mg)	
Substrate	Estradiol	Estrone
Control	16.28 ± 2.15	6.64 ± 0.83
Aminoglutethimide (40 mg/kg/day i.p. for 3 days)	20.86 ± 1.69	8.60 ± 0.73
% Increase	28	30
Control	19.57 ± 1.26	7.58 ± 0.21
CGS 16949A (2 mg/kg/day p.o. for 3 days)	**29.01 ± 2.59	*10.94 ± 1.86
% Increase	48	44

Mean ± SD; $N = 4$ rats; $^{*}P < 0.04$; $^{**}P < 0.001$.

The product isolation method using HPLC was employed in the *in vivo* study so that the effect of the drugs on the affinity of estrogen 2-hydroxylase for estrone and estradiol could also be observed. Estrogen 2-hydroxylase was found to have a higher affinity for estradiol than estrone under normal and test conditions. The level of induction was higher following CGS 16949A administration (48 and 44%) as compared to aminoglutethimide (28 and 30%) using estradiol and estrone as substrates.

Several compounds have previously been shown to inhibit estrogen 2-hydroxylase activity in human liver microsomes using ethinyloestradiol, the estrogenic component of most contraceptive preparations, as a substrate [15]. However, one of these compounds primaquine, an aminoquinoline antimalarial drug, with a K_i value of 43.1 μM was unable to show any inhibitory effect on the elimination of ethinyloestradiol *in vivo* [16]. Indeed, the aromatase inhibitors under investigation in the present study with relatively high K_i values *in vitro* were unable to inhibit estrogen 2-hydroxylase enzyme *in vivo*. On the contrary, aminoglutethimide and CGS 16949A induced the activity of

aestrogen 2-hydroxylase in liver microsomes of rats following repeated dosing under the present experimental conditions.

CGS 16949A is a relatively weak inducer of cytochrome *P*-450s (unpublished observations) but its almost 2-fold higher induction of estrogen 2-hydroxylase in rat liver microsomes as compared to aminoglutethimide indicates its selective inducing property. The selective induction of estrogen 2-hydroxylase activity *in vivo* reported in this study should enhance the efficacy of this drug in lowering circulating estrogens.

In conclusion, the aromatase inhibitors, 4-hydroxyandrostenedione, CGS 16949A and aminoglutethimide are only weak inhibitors of estrogen 2-hydroxylase *in vitro*. There was no evidence of inhibition *in vivo* with aminoglutethimide or CGS 16949A. On the contrary, CGS 16949A significantly induced estrogen 2-hydroxylase activity *in vivo* in this study, albeit the magnitude of induction was relatively small when compared to the classical inducers of the cytochrome *P*-450 mixed function oxidases. However, combined with a high potency of CGS 16949A as an aromatase inhibitor, the induction of estrogen 2-hydroxylase activity observed in this study would only help to further suppress estrogen levels by inducing the activity of this major enzyme involved in the metabolism of biologically active estrogens.

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