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STUDIES OF THE INACTIVATION OF HUMAN PLACENTAL AROMATASE BY 17α-ETHYNYL-SUBSTITUTED 10β-HYDROPEROXY AND RELATED 19-NOR STEROIDS

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Abstract—The inactivation of human placental aromatase by 17α-ethynyl-10β-hydroperoxy-17β-hydroxy-4-estren-3-one (SCH 10015) was investigated. In either the presence or absence of added NADPH, SCH 10015 ($K_i = 41\ \mu M$) caused a time-dependent loss of aromatase activity (e.g. 50% loss after 20 min with 20 μM SCH 10015). Evidence for the oxidation of an active site sulfhydryl group as the molecular basis for SCH 10015 inactivation is presented. The contraceptive 17α-ethynyl-substituted 19-nor steroids, norethisterone ($K_i = 48\ \mu M$) and norethynodrel ($K_i = 38\ \mu M$), were evaluated and found not to inactivate aromatase, suggesting that the potential conversion of either compound to SCH 10015 did not occur to a significant extent in these microsomal incubations. It is speculated that the previously observed potent contraceptive effects of SCH 10015 may have been the result of irreversible inhibition of estrogen biosynthesis.

There is a great deal of current interest in the development of specific inhibitors of aromatase, the enzyme responsible for estrogen biosynthesis, for use in the treatment of estrogen-dependent tumors and as investigational agents for studies of reproductive endocrinology. In this regard, it was shown recently [1] that 10β-OOH† (**1**, Fig. 1) is a potent active site directed irreversible inhibitor of cytochrome P-450$_{AROM}$ from human placental microsomes. Evidence was presented that supported oxidation of an active site sulfhydryl group as the molecular basis for inactivation of the enzyme. Notably, the observed time-dependent inactivation occurred only when NADPH was not included in the microsomal incubations. Thus, 10β-OOH, although it is of interest for *in vitro* mechanistic studies of cytochrome P-450$_{AROM}$, is unlikely to be of use for *in vivo* studies where inhibition of estrogen biosynthesis is desired.

As noted previously [1], SCH 10015 (**2**) has an obvious structural similarity to 10β-OOH and has been shown to be a potent orally active contraceptive agent in rats and rabbits [2, 3]. It was suggested [1] that aromatase inactivation might play a role in the *in vivo* mechanism of action of this steroid. If this were so, then clearly SCH 10015 would have to inactivate aromatase in the presence of NADPH. The 17α-ethynyl group present in SCH 10015 could make this possible by altering the alignment of the steroid in the active site of aromatase. We report here an evaluation of SCH 10015 as an aromatase inactivator. Norethisterone (**4**) a compound previously shown to inactivate aromatase [4], and norethynodrel (**5**), a compound which is oxidized in solution to SCH 10015 [5, 6], were also examined as aromatase inactivators to evaluate their abilities to serve as precursors for SCH 10015 in the microsomal incubations.

MATERIALS AND METHODS

Chemicals. The 4-[1,2-^{3}H(N)]androstene-3,17-dione (56.0 Ci/mmole) was purchased from the New England Nuclear Corp., Boston, MA. Liquid scintillation counting was done in Budget Solve from

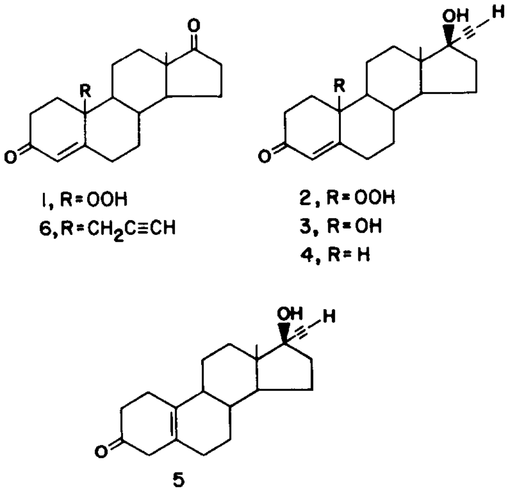
1, R=OOH
6, R=CH$_2$C≡CH
2, R=OOH
3, R=OH
4, R=H
5

Fig. 1. Structures of compounds evaluated as aromatase inhibitors.

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[†] Abbreviations and trivial names: 10β-OOH, 10β-hydroperoxy-4-estrene-3,17-dione; P-450$_{AROM}$, aromatase cytochrome P-450; SCH 10015, 17α-ethynyl-10β-hydroperoxy-17β-hydroxy-4-estren-3-one; compound **3**, 10β,17β-dihydroxy-17α-ethynyl-4-estren-3-one; norethisterone, 17α-ethynyl-17β-hydroxy-4-estren-3-one; norethynodrel, 17α-ethynyl-17β-hydroxy-5(10)-estren-3-one; PED, 10-propynyl-4-estrene-3,17-dione; and testolactone, D-homo-17a-oxaandrosta-1,4-diene-3,17-dione.

Research Products International, Mt. Prospect, IL. Norethynodrel, norethisterone, NADPH, and dithiothreitol were from the Sigma Chemical Co., St. Louis, MO. Protein determinations were done with a Bio-Rad Protein Assay Kit I from Bio-Rad Laboratories, Richmond, CA. Compounds **2**, **3**, and **6** were prepared according to literature methods [5, 7]. The compounds were chromatographically pure and had the expected spectroscopic properties.

Enzyme inhibition studies. Human placental microsomes were prepared by our previously described method [7]. The specific activities of the preparations used were 260 and 333 pmoles/min/mg protein. Standard incubations, done in a shaking water bath at 37° in air, contained 100 mM KCl, 10 mM potassium phosphate buffer, pH 7.5, 1 mM EDTA, 100 μM NADPH (omitted in appropriate controls), propylene glycol (1 drop/0.5 ml), and microsomes (0.046 or 0.036 mg of protein/0.5 ml).

Time course experiments began with the addition of inhibitors dissolved in ethanol (0.01 ml/0.5 ml incubation volume) to standard incubations (3.0 ml). At various times, aliquots (0.5 ml) were removed, added to assay tubes containing 4-[1,2-^{3}H(N)]androstene-3,17-dione (0.5 nmole present as a predried film), and measured for remaining enzyme activity [8]. Five minutes later the assay reactions were stopped by the addition of chloroform (5.0 ml) and vortexing ~40 sec. Then, after centrifuging at 1470 *g* for 5 min, aliquots (0.10 ml) were removed from the water phase and added to scintillation mixture for determination of ^3H_2O production. Further discussions regarding our method of performing this assay can be found elsewhere [9].

Competition experiments performed in standard incubations (0.5 ml) contained 4-[1,2-^{3}H]androstene-3,17-dione (five different concentrations ranging from 0.1 to 1.0 μM) alone or with inhibitors dissolved in ethanol (0.01 ml). Addition of microsomes and NADPH to the incubation initiated the assay reaction. Reactions were terminated, and ^3H_2O was determined as described previously. Compounds evaluated and the concentrations used were as follows: SCH 10015 (10 and 20 μM, higher concentrations caused significant inactivation during the assay), compound **3** (400 μM), norethynodrel (50 and 100 μM), and norethisterone (50, 100, and 200 μM). The K_m for androstenedione was 90 nM. Kinetic results were analyzed by Lineweaver–Burk plots. Regression lines for these kinetic data were drawn according to a least-squares fit.

Experiments done to investigate the reversibility of the inhibition produced by SCH 10015 used initial standard incubations (3.0 ml) containing microsomes without NADPH. The final concentration of SCH 10015 in ethanol (0.06 ml) was 100 μM. Controls received ethanol (0.06 ml) only. Sixty minutes after the addition of SCH 10015, aliquots (0.5 and 0.05 ml) were removed and assayed for remaining activity and protein respectively. The remainder of the incubation was spun at 100,000 *g* for 60 min at 4°. The supernatant fractions were discarded while the pellets were resuspended in standard buffer (2.5 ml). Aliquots were removed and assayed for aromatase activity and protein (0.5 ml and 0.05 ml respectively). The remainder was again spun at 100,000 *g* for 60 min at 4°. Again, the supernatant fractions were discarded, the pellets were resuspended in buffer (2.0 ml), and aliquots were taken and assayed for aromatase activity and protein determination.

Dithiothreitol reactivation experiments used standard incubations (3.0 ml, without added NADPH) containing 100 μM SCH 10015. At 15, 30, or 60 min, an initial aliquot (0.5 ml) was removed and assayed for aromatase activity. Then, 10 mM dithiothreitol was added, and aliquots (0.5 ml) were taken at 0.5, 5, 10, and 20 min and assayed for aromatase activity.

RESULTS AND DISCUSSION

Incubation of SCH 10015 with microsomes in the absence of NADPH resulted in an increased time-dependent inactivation of aromatase relative to control (Fig. 2, lower panel). The observed rate of inactivation did not follow pseudo-first order kinetics for the entire time of incubation, but its magnitude was dependent on the concentration of SCH 10015. The ability of androstenedione, an aromatase substrate, to protect the enzyme against SCH 10015 inactivation shows that the active site of the enzyme is involved in the inactivation event. When 100 μM

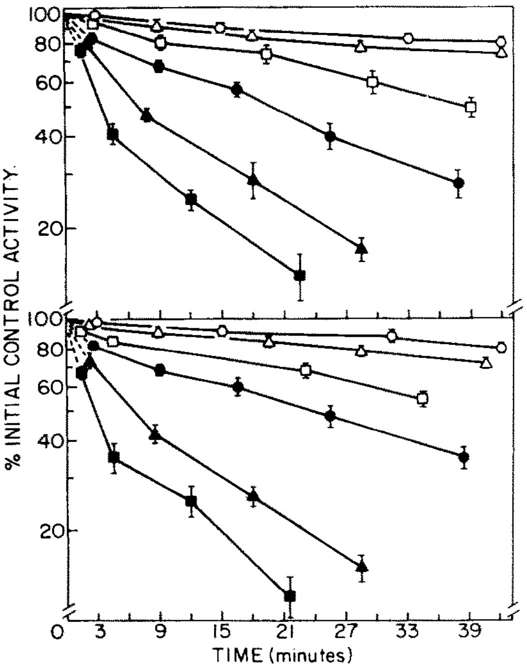

Fig. 2. Time courses for inactivation of aromatase by SCH 10015 in the presence (upper panel) or absence (lower panel) of 100 μM NADPH. Upper panel. Microsomes (open circles). Microsomes and: SCH 10015, 10 μM, and dithiothreitol, 10 mM (open triangles); SCH 10015, 10 μM (open squares), 20 μM (filled circles), 50 μM (filled triangles), and 100 μM (filled squares). Lower panel. Microsomes (open circles). Microsomes and: SCH 10015, 10 μM, and androstenedione, 100 μM (open triangles); SCH 10015, 10 μM (open squares), 20 μM (filled circles), 50 μM (filled triangles), and 100 μM (filled squares). The data shown are the average and the range of duplicate experiments at each inhibitor concentration.

NADPH was included in the incubations, only minimal, if any, protection against inactivation by SCH 10015 was observed (Fig. 2, upper panel). The conversion of SCH 10015 to compound **3** by *in situ* dithiothreitol reduction of the hydroperoxy group prevented inactivation, indicating that this group must be the inactivating moiety. The same result was obtained in inactivation experiments done with 100 μM compound **3** in the absence of dithiothreitol (see Fig. 4, upper panel).

A K_i for SCH 10015 was not calculated from double-reciprocal plots of inactivation rate constants versus steroid concentration because of the marked deviation from pseudo-first order kinetics. Competitive inhibition ($K_i = 41 \pm 4$ μM) was found in competition experiments (see Materials and Methods for details). Compound **3** at concentrations as high as 400 μM had no effect in similar competition experiments.

When aromatase was completely inactivated by a 1-hr incubation of microsomes with 100 μM SCH 10015, no recovery of activity could be obtained by twice pelleting the microsomes and resuspending them in fresh buffer. The second resuspended pellet from the control incubation retained 72% of the activity present at the end of the initial 1-hr incubation. Recovery of microsomal protein was 80 and 82%, respectively, for the two incubations. The results demonstrate the irreversibility of the inactivation caused by SCH 10015.

Since the inactivation caused by 10β-OOH was shown previously [1] to be partially reversed by dithiothreitol, this possibility was also examined for SCH 10015 (Fig. 3). Microsomes were incubated for 15, 30, and 60 min, respectively, with 100 μM SCH 10015; then 10 mM dithiothreitol was added to the incubations and the time course for recovery of aromatase activity was determined. The length of the initial incubation period was found to determine not only how much initial activity was lost (80, 93 and 100%), but also the percent of lost activity that could be restored (72, 41 and 26%) by dithiothreitol. The result is consistent with successive oxidations of an active site sulfhydryl group by 10β-hydroperoxides to a mixture of sulfenic and sulfonic acids. Conversion of the initial sulfenic acid to the sulfonic acid with time would explain the decreasing ability of dithiothreitol to reactivate the enzyme [1, 10].

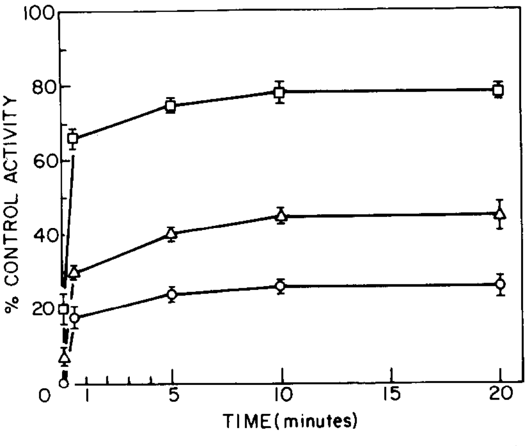

Fig. 3. Reactivation of SCH 10015 treated microsomes by dithiothreitol. Microsomes were incubated with 100 μM SCH 10015 for 15 (open squares), 30 (open triangles), or 60 min (open circles) and then dithiothreitol (10 mM) was added. Aromatase activity was determined at the end of the initial incubation period and 0.5, 5, 10 and 20 min after dithiothreitol addition. The data shown are the average and range of duplicate experiments.

Neither 100 μM norethynodrel (Fig. 4, upper panel) nor 100 μM norethisterone (Fig. 4, lower panel) were found to increase the rate of aromatase inactivation over that observed for control incubations. The time-independent inhibition observed for both compounds is due to their ability to act as competitive inhibitors of androstenedione aromatization. In competition experiments (see Materials and Methods for details), a K_i of 38 ± 5 μM was found for norethynodrel and a K_i of 48 ± 8 μM was found for norethisterone. Since neither compound inactivated aromatase, the results suggest that neither steroid was converted significantly by the microsomes to SCH 10015. That such a conversion could occur was suggested by the isolation of alcohol **3**, which can be formed by reduction of SCH 10015, as a metabolite of both norethynodrel and norethisterone in women [11]. The results further suggest that, if metabolic activation of a 17α-ethynyl group in a manner similar to that observed in inactivation studies of rat liver cytochrome P-450 by norethisterone [12, 13] had occurred, it did not lead to any detectable loss of aromatase activity.

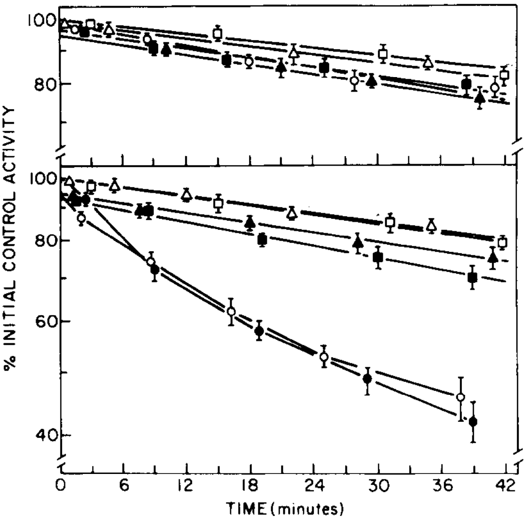

Fig. 4. Time course for inhibition of aromatase by norethynodrel or alcohol **3** (upper panel), and norethisterone or norethisterone and PED (lower panel). Upper panel. Microsomes and NADPH, 100 μM (open squares); microsomes alone (open triangles). Microsomes and: alcohol **3**, 100 μM (open circles); norethynodrel, 100 μM, and NADPH, 100 μM (filled squares); and norethynodrel, 100 μM (filled triangles). Lower panel. Microsomes and NADPH, 100 μM (open squares); microsomes alone (open triangles). Microsomes and: norethisterone, 100 μM (filled triangles); norethisterone, 100 μM, and NADPH, 100 μM (filled squares); PED, 100 nM, and NADPH, 100 μM (filled circles); PED, 100 nM, and NADPH, 100 μM, plus norethisterone, 100 μM (open circles). The data shown are the average and the range of duplicate experiments.

Since the inactivation of aromatase by norethisterone as reported by Osawa *et al.* [4] is frequently discussed in review articles [14, 15] as being possibly relevant to the mechanism of aromatase inactivation caused by 10β-propynyl-substituted steroids [7, 16], the ability of 100 μM norethisterone to influence the inactivation of 100 nM PED (**6**, K_i = 23 nM, [7]) was also investigated (Fig. 4, lower panel). No significant effect of norethisterone on either the rate or the maximal extent of inactivation caused by PED was observed.

Our results with norethisterone are in obvious disagreement with the report by Osawa *et al.* [4] that this compound is an aromatase suicide substrate. We can only speculate that differences in the aromatase preparations may be responsible for the discrepancy. We used a microsomal preparation, while Osawa *et al.* used a deoxycholate solubilized fraction from an initial 900 *g* pellet of homogenized placental tissue. In any event, other investigators [17] using microsomal preparations have also stated (no experimental detail was given) that they do not observe inactivation of aromatase by norethisterone.

A comparison of the results obtained with SCH 10015 and 10β-OOH [1] indicates that oxidation of an active site sulfhydryl group is implicated as the mechanism of inactivation for both compounds. Although NADPH prevented the inactivation caused by 10β-OOH, it had, at best, little effect on the inactivation caused by SCH 10015. We attribute this difference to an altered active site positioning of SCH 10015 caused by the presence of the 17α-ethynyl group. The affinity of SCH 10015 (K_i = 41 μM) relative to that of 10β-OOH (K_i = 330 nM) indicates that 10β-OOH is bound about 100 times more tightly, thus differences in binding orientation for the two compounds are certainly plausible. The recent report [18] of aromatase inactivation (in the absence of NADPH) resultant from oxidation of an active site sulfhydryl group by 6α- and 6β-hydroperoxyandrost-4-ene-3,17-dione further suggests that a unique position for the hydroperoxy group is not required for inactivation by this mechanism.

Additional studies in experimental animals will be required to determine if the contraceptive properties of SCH 10015 are related to irreversible inhibition of estrogen biosynthesis. Since testolactone, another time-dependent inactivator of aromatase with an affinity (K_i = 35 μM) [19] similar to that of SCH 10015, has been shown both to lower circulating estrone levels in humans [20] and to be of use in the treatment of estrogen-dependent breast cancer (for a recent overview, see Ref. 21), we are not discouraged by the reported affinity of SCH 10015 for aromatase. The affinity would be of more concern if reversible rather than irreversible inhibition had been observed in this study.

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