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INHIBITION OF AROMATASE CYTOCHROME P-450 (ESTROGEN SYNTHETASE) BY DERIVATIVES OF α-NAPHTHOFLAVONE*

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Abstract—α-Naphthoflavone (ANF; 7,8-benzoflavone) is a potent competitive inhibitor of human aromatase cytochrome P-450 [J. T. Kellis, Jr. and L. E. Vickery, *Science* 225, 1032 (1984)]. We have further investigated inhibition of aromatase by several derivatives of ANF. Using human placental microsomes and 40 nM androstenedione as substrate, the compounds tested and their I_{50} values were: ANF, 0.07 μM; 2-(2-naphthyl)-4*H*-naphtho[1,2b]pyran-4-one, 1.0 μM; 7,8-benzoisoflavone, ≈100 μM; and 2-phenyl-4*H*-naphtho[1,2b]furan, > 100 μM. These findings show the necessity of the keto group of ANF in its binding to the enzyme and the importance of size and position of substitution of the exocyclic phenyl ring. Derivatives of ANF with hydroxyl substitution at positions 5, 6, 7, 8, 9, and 10 were also screened. 9-Hydroxy-ANF, a known metabolite of ANF in liver microsomes, was the most effective (I_{50} = 20 nM). Inhibition by 9-hydroxy-ANF was competitive, and its K_i value of 5 nM indicates a higher affinity for the enzyme than the natural steroid substrates— the K_m values for androstenedione and testosterone under these conditions are 10 and 80 nM respectively. 9-Hydroxy-ANF also induced a change in the absorption spectrum of the aromatase cytochrome P-450 indicative of substrate displacement. Based on these data we propose a model for the binding of 9-hydroxy-ANF in which the 7,8-benzochromone ring system of the ANF derivatives occupies the steroid ring binding site of the enzyme.

Cytochrome P-450 enzymes comprise a class of monooxygenases involved in a wide variety of important physiological roles. These functions include steroid hormone, vitamin D, and bile salt biosynthesis, and the metabolism of xenobiotics, including drugs and carcinogens. Aromatase cytochrome P-450 carries out a crucial step in steroid metabolism, the aromatization of the "A" ring of androgens to yield estrogens [1]. Figure 1 illustrates the reaction which is catalyzed. Estrogens are essential for reproduction and other physiological functions, and they have a pathological role in estrogen-dependent breast cancer.

In our laboratory, part of the research concerning aromatase is focused on studying inhibitors of the enzyme. The studies can yield information about the structure and mechanism of the enzyme and may also reveal aromatase inhibitors of potential therapeutic use in the treatment of estrogen-dependent cancers. We recently reported that α-naphthoflavone (ANF; 7,8-benzoflavone; Fig. 2), an inhibitor of liver microsomal cytochromes P-450 [2–4], is a potent competitive inhibitor of human placental and ovarian aromatase [5]. This compound binds to the enzyme with affinity comparable to that of the natural steroid substrates, androstenedione and testosterone. In this paper we report the results of a structure–activity study of inhibition by derivatives of ANF, which was undertaken to determine which features of the structure of ANF confer its high affinity for aromatase and to discover other potent inhibitors of the enzyme.

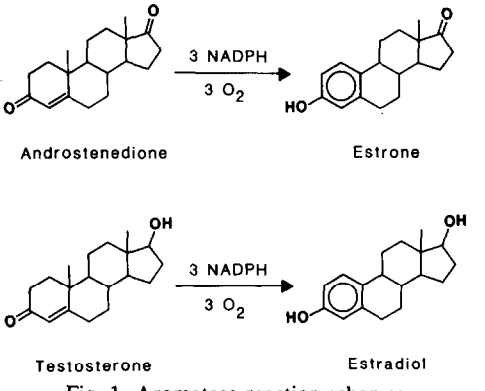

Fig. 1. Aromatase reaction schemes.

For *in vivo* use of ANF, one must consider the effect of its metabolites on aromatase as well. The metabolism of ANF has been studied in liver microsomes from several species, such as rat [6–8], mouse, rabbit, hamster [9], and fish [10], and with purified rat liver microsomal cytochrome P-450 [11]. Among its metabolites are hydroxylated products, and we therefore also examined aromatase inhibition by hydroxylated derivatives of ANF.

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MATERIALS AND METHODS

Human placental microsomes were prepared as previously described and aromatase activity was assayed by tritium release from [1β, 2β-^{3}H]androstenedione or [1β, 2β-^{3}H]testosterone [12]. The spectral studies were also carried out using previously published methods [12]. The ANF derivatives were synthesized and characterized as described in refs. 8 and 13. Test compounds were added to the microsomes from ethanolic stock solutions. Ethanol alone, which did not exceed a concentration of 1%, was added to control incubations. I_{50} values for inhibitors were obtained from theoretical curves fit to the dose–response data [14]. For the computer-generated structures presented in Fig. 6, crystallographic coordinates for ANF and testosterone were obtained from Refs. 15 and 16 respectively. Molecular structures were calculated using a PDP 11/23 computer and were displayed on an Advanced Electronic Design 767 terminal.

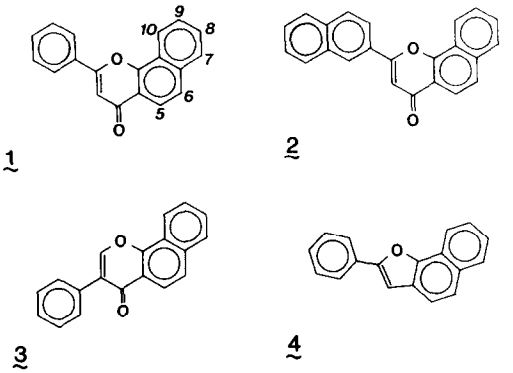

Fig. 2. Structures of ANF and its derivatives which were tested for inhibition of aromatase. Key: (1) ANF and its numbering scheme, (2) 2-(2-naphthyl)-4*H*-naphtho[1,2b]pyran-4-one, (3) 7,8-benzoisoflavone, and (4) 2-phenyl-4*H*-naphtho-[1,2b]furan.

RESULTS

The structures of the compounds tested for aromatase inhibition are presented in Fig. 2. Table 1 gives their I_{50} values obtained from dose-response experiments using 40 nM androstenedione as substrate; in no case was enzyme activation observed. The inhibitory potency relative to that of ANF is also give in Table 1, with ANF assigned an arbitrary index of 100. The results can be summarized as follows. Replacement of the exocyclic phenyl ring of ANF with a naphthyl group (compound 2) decreased inhibitory potency 16-fold, indicating possible steric constraints on the size of the exocyclic group which can be accommodated by the enzyme. This derivative was, nevertheless, 7-fold more potent than flavone (I_{50} = 8 μM [5]), which emphasizes the role played by the 7,8 fused ring of ANF in conferring its high binding affinity. Changing the site of substitution of the exocyclic phenyl ring (compound 3) drastically reduced inhibitory potency; this shows that specific positioning of the exocyclic ring relative to the remainder of the molecule is essential for high-affinity binding to aromatase. Elimination of the carbonyl group by conversion of the pyran-4-one ring to a furan ring (compound 4) caused almost complete loss of inhibitory activity. This suggests that the keto group of ANF plays an important role in its interaction with the enzyme. The presence of the furan ring also slightly altered the position of the exocyclic phenyl ring, and this could also be a factor in reducing binding affinity.

The inhibitory potency of hydroxylated derivatives of ANF is greatly dependent on the position of the hydroxyl group. Addition of a hydroxyl group at the 5 or 6 position decreased affinity several hundred-fold, 7- or 8-hydroxylation decreased affinity slightly, 10-hydroxylation had no effect on inhibitory potency, and 9-hydroxylation *increased* affinity several-fold. The I_{50} value of 20 nM for 9-hydroxy-ANF is one-half of the androstenedione concentration used in the assay. We also tested 9-hydroxy-ANF for inhibition of the aromatization of 0.08 μM testosterone. An I_{50} value of 0.016 μM (one-fifth of the substrate concentration) was observed.

Because of the potency of 9-hydroxy-ANF, we undertook a more detailed study of its mechanism

Table 1. Inhibition of aromatase by α-naphthoflavone derivatives

Number	Compound	I_{50}* (μM)	Relative potency
1	7,8-Benzoflavone (ANF)	0.07, 0.07	100
	Structural analogues		
2	2-(2-Naphthyl)-4*H*-naphtho[1,2b]pyran-4-one	1.0, 1.3	6.1
3	7,8-Benzoisoflavone	≈100	≈0.07
4	2-Phenyl-4*H*-naphtho[1,2b]furan	>100	<0.07
	Hydroxy derivatives		
5	5-Hydroxy-7,8-benzoflavone	15, 10	0.56
6	6-Hydroxy-7,8-benzoflavone	12, 12	0.58
7	7-Hydroxy-7,8-benzoflavone	0.19, 0.20	36
8	8-Hydroxy-7,8-benzoflavone	0.35, 0.30	22
9	9-Hydroxy-7,8-benzoflavone	0.02, 0.02	350
10	10-Hydroxy-7,8-benzoflavone	0.07, 0.09	88

* Values reported represent the averages of duplicate determinations in two separate experiments.

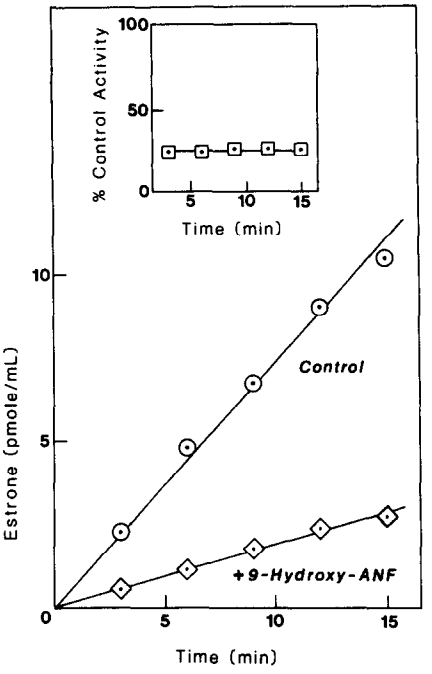

Fig. 3. Time course of androstenedione conversion to estrone by human placental microsomes. The incubations were performed with 40 nM androstenedione as described in Materials and Methods. Key: (⊙) no inhibitor (◇) plus 40 nm 9-hydroxy-ANF. The points plotted represent the mean of duplicate assays; the individual values did not fall outside the plotted symbols. *Inset:* extent of inhibition at each time point (⊡).

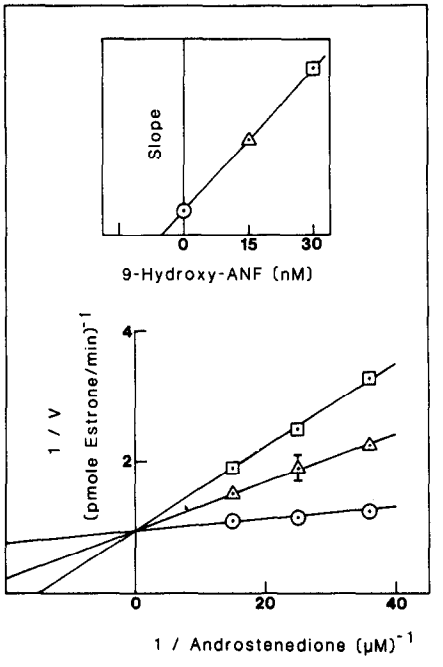

Fig. 4. Kinetic analysis of the mechanism of inhibition of androstenedione aromatization in human placental microsomes by 9-hydroxy-ANF. Standard assay conditions were used. Key: (⊙) no inhibitor; (△) 15 nM 9-hydroxy-ANF; and (⊡) 30 nm 9-hydroxy-ANF. The error bars indicate where duplicate determinations fell outside the plotted point. *Inset:* slope of lines in double-reciprocal plot versus inhibitor concentration.

of inhibition. Figure 3 shows the time course of the aromatization of 40 nM androstenedione by human placental microsomes in the presence and absence of 40 nM 9-hydroxy-ANF. Product formation was linear with respect to time in both cases, and the fractional inhibition by 9-hydroxy-ANF was constant for 15 min (see inset). These results indicate a lack of time-dependent enzyme inactivation and a lack of significant metabolism of the compound to either a more or less potent inhibitor.

Figure 4 shows a Lineweaver–Burk plot of the inhibition of human placental aromatase by 9-hydroxy-ANF. Inhibition was competitive with respect to the substrate, androstenedione, and a replot of the slopes of the lines (shown in the inset) yielded a K_i value of 5 nM. This indicates that 9-hydroxy-ANF binds to aromatase with twice the affinity of androstenedione, which exhibited a K_m value of 10 mM in this experiment. 9-Hydroxy-ANF also had about 10-fold higher affinity for the enzyme than testosterone, which exhibits a K_m of 80 nM under these conditions.

The kinetic analysis suggests that 9-hydroxy-ANF acts by displacement of substrate from the enzyme. Since substrate binding can be monitored by spectral changes in the Soret absorption of cytochrome P-450 [17], we investigated the interaction between 9-hydroxy-ANF and aromatase by difference spectroscopy. When 9-hydroxy-ANF was added to a suspension of human placental microsomes, no spectral change was observed; when the microsomes were pre-equilibrated with androstenedione, however, a "reverse type 1" spectral change was observed (data not shown). We assume that failure to observe a difference spectrum in the absence of androstenedione occurred because the enzyme already existed as the low-spin, substrate-free form. The difference spectrum induced in the presence of androstenedione reflects substrate displacement and conversion of the high-spin aromatase cytochrome P-450 to a low-spin complex (the parent compound, ANF, also induces reverse type 1 spectra [5]).

To obtain adequate absorbance changes without the turbidity introduced by a high concentration of microsomes, we clarified the preparation by cholate extraction and concentrated the aromatase cytochrome P-450 by ammonium sulfate precipitation [12]. Figure 5 (upper panel) shows difference spectra obtained by sequential additions of increasing concentrations of 9-hydroxy-ANF to this preparation in the presence of 1 μM androstenedione. The lower panel of Fig. 5 is a graphical analysis of the spectral data. The double-reciprocal plot indicates that 9-hydroxy-ANF bound to a single high-affinity site with an apparent dissociation constant of approximately 1.4 μM. Since 1 μM androstenedione was present, 9-hydroxy-ANF exhibited binding affinity comparable to that of the steroid under these conditions. Therefore, it is probable that this reverse type 1 spectral change reflects formation of the enzyme–inhibitor complex which is responsible for inhibition of aromatase. In addition, the requirement for pre-equi-

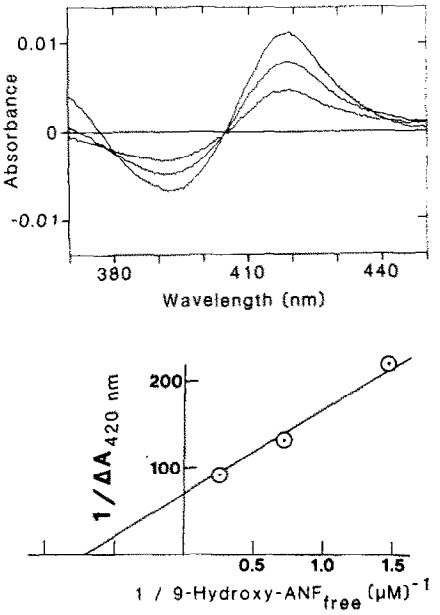

Fig. 5. Spectral titration of a cholate extract of human placental microsomes with 9-hydroxy-ANF in the presence of 1 μM androstenedione. Upper panel: difference spectra obtained by the additions of 0.75, 1.5, and 4 μM 9-hydroxy-ANF. Ethanol alone, which did not exceed 1%, was added to the reference cuvette. The protein concentration was 1.25 mg/ml and the concentration of cytochrome P-450 [18] was 0.26 μM. Lower panel: double-reciprocal plot of the titration data. The concentration of the free ligand was calculated by the equation $L_f = L_{tot} - \Delta A \cdot E/\Delta A_{max}$ where L_f is the free ligand concentration, L_{tot} is the total concentration of ligand added, ΔA is the observed absorbance change at L_{tot}, E is the enzyme concentration, and ΔA_{max} is the absorbance change extrapolated to infinite ligand concentration. $\Delta\varepsilon_{max}$ at 420 nm is assumed to be equal to 0.065 $(\mu M \cdot cm)^{-1}$, which is the value for reverse type 1 difference spectra obtained with cholesterol side chain cleavage cytochrome P-450 [19].

libration with androstenedione in the production of a difference spectrum indicates that the spectral change arises from aromatase and not another form of cytochrome P-450 present in the detergent extract.

DISCUSSION

In an earlier report, we showed that the presence of the 7,8 fused ring of ANF is essential for its high inhibitory potency toward aromatase [5]. The data presented here reveal that the keto group and the size and position of the exocyclic aromatic ring are also important. In addition, we found that substitution of ANF with a hydroxyl group can also greatly influence aromatase inhibition. While 10-hydroxylation had no significant effect, hydroxylation at positions 5, 6, 7, or 8 reduced potency, and hydroxylation at position 9 *enhanced* potency. Consequently, *in vivo* metabolism of ANF to hydroxylated products could profoundly affect its ability to inhibit aromatase. The major monohydroxylated metabolites in rat, mouse, rabbit, and hamster liver microsomes are the 6-, 7-, and 9-hydroxy derivatives; epoxides and dihydrodiols are also produced [9].

The high affinity of 9-hydroxy-ANF for aromatase is very striking. The K_i value of 5 nm which we found in the kinetic analysis of inhibition indicates that it binds to the enzyme more tightly than either androstenedione or testosterone, the natural steroid substrates. It also has a lower I_{50} than two of the most potent steroidal inhibitors of aromatase, 1, 4, 6-androstatriene-3,17-dione [20] and 4-hydroxy-andro-

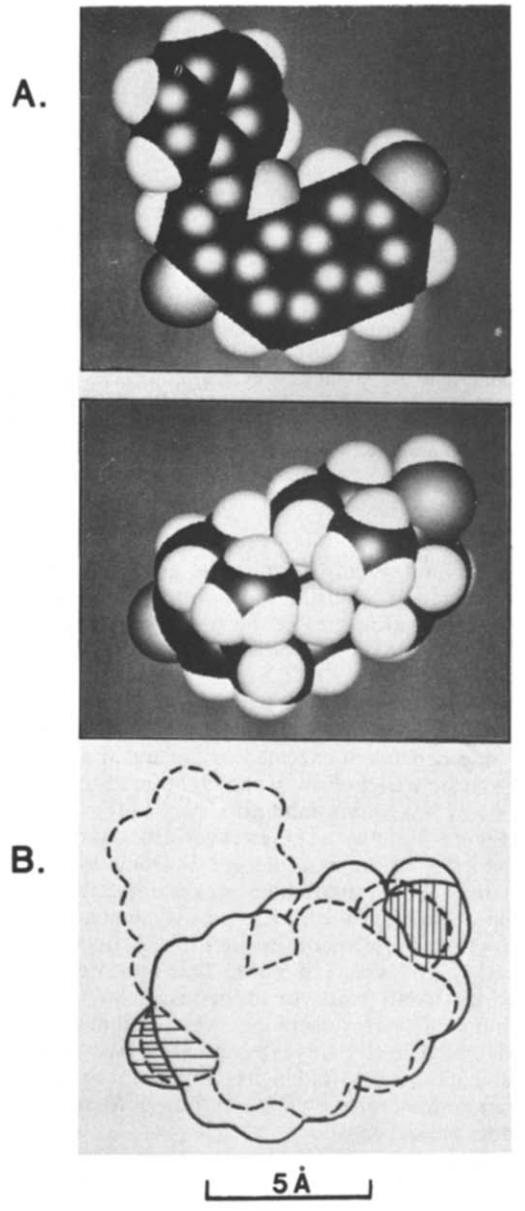

Fig. 6. (A) Computer-generated models of 9-hydroxy-ANF (top) and testosterone (bottom). (B) Schematic diagram of the overlap of the two molecules in the active site of aromatase. Testosterone is indicated by the solid line, and 9-hydroxy-ANF is indicated by the dashed line; the hydroxyl and keto groups of each are shown by vertical and horizontal cross-hatching respectively.

stenedione [21], which under similar conditions yield I_{50} values of 0.08 and 0.05 μM respectively [5].

It is intriguing that non-steroids such as ANF and 9-hydroxy-ANF exhibit such high affinity for aromatase. The spectral and kinetic studies suggest that both ANF [5] and 9-hydroxy-ANF inhibit aromatase by binding to the substrate site. Vyas *et al.* [11] have studied the metabolism of ANF by purified cytochrome P-450c from rat liver and, based on its metabolite profile, they proposed several orientations for the binding of ANF to the substrate site of the enzyme. From the results of our structure–activity findings for inhibition by ANF and its derivatives, we wish to suggest a possible alignment of 9-hydroxy-ANF in the substrate binding site of aromatase cytochrome P-450. Figure 6A shows "top views" of computer-generated space-filling models of 9-hydroxy-ANF and testosterone. The 7,8-benzochromone ring system of 9-hydroxy-ANF is similar in size and shape to the steroid ring system of testosterone, as shown by the tracings of the structures (Fig. 6B). This superposition gives a high degree of overlap of the apolar portions of the molecules; in addition, it allows for the alignment of the keto and hydroxyl groups of the two molecules. The 3- and 17-oxygen functions have been shown to be critical for the binding of androgens to aromatase [22], and in the present study we have shown that the keto group of ANF is essential for binding to the enzyme and that 9-hydroxy-ANF exhibits enhanced affinity relative to ANF. If the heme and substrate sites of aromatase lie in close proximity to one another, as they do in cholesterol side chain cleavage P-450 [19, 23, 24], then the aromatic ring system of 9-hydroxy-ANF may also contribute to its high-affinity binding. It could "stack" with the porphyrin or be involved in a recently described type of aromatic–aromatic interaction in which the dihedral angle of the rings approaches 90° [25]. The exocyclic phenyl ring of 9-hydroxy-ANF, which does not overlap with the steroid pocket in this arrangement, may also be involved in these types of interactions.

The linear time courses for the aromatization reactions that we have observed in the presence of ANF and 9-hydroxy-ANF suggest that a high degree of metabolism of these inhibitors does not occur. Using radiolabeled ANF derivatives, however, it may be possible to detect small quantities of metabolites which would provide additional information about the situation of these molecules in the active site of aromatase.

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