Time-Dependent Inactivation of Human Placental Aromatase by Bromoacetoxy 4-Androsten-3-ones in the Presence of Reduced Nicotinamide Adenine Dinucleotide Phosphate (NADPH)

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6-Bromoacetoxyandrostenediones (1 and 2) and 17β-bromoacetoxy-4-androsten-3-one having a 6β-bromo (3), 6-keto (4), or 6β-methoxyl (5) substituent were evaluated as mechanism-based inactivators of human placental aromatase. All of these compounds except the 6α-bromoacetate 1 showed a time-dependent, pseudo-first-order inactivation of aromatase in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) with apparent K_i's of 40, 50, 30, and 34 μM and k_{inact}'s of 0.048, 0.364, 0.267, and 0.040 min^{-1}, respectively, for compounds 2, 3, 4, and 5. The enzyme inactivation with compounds 3 and 4 was blocked by the addition of the substrate androstenedione to the incubates, and NADPH and oxygen were required for their effective time-dependent inactivation.

Keywords human placental aromatase; time-dependent inhibition; concentration-dependent inhibition; NADPH requirement; oxygen requirement; bromoacetoxy steroid; 4-androsten-3-one derivative; 6β-bromo steroid; 6-keto steroid

The enzyme aromatase is responsible for the conversion of the androgens androstenedione and testosterone into estrone and estradiol. This system involves cytochrome (cyt.) P-450 and reduced nicotinamide adenine dinucleotide phosphate (NADPH)–cyt. P-450 reductase and requires NADPH and oxygen for catalytic activity.[1] A potent selective inhibitor of aromatase activity might be effective in the treatment of advanced estrogen-dependent breast carcinoma and in the modulation of the reproductive process.[2]

Various substrate analogues have been tested in a number of laboratories as inhibitors of aromatase. Irreversible inactivators of estrogen biosynthesis may be more effective agents than competitive inhibitors if they are sufficiently specific. In contrast to chemically reactive agents, *e.g.* affinity labels, mechanism-based or suicide inhibitors of aromatase could be highly useful as therapeutic agents as well as in probing the mechanism of catalysis. We[3] previously synthesized several bromoacetoxy androgen derivatives and characterized them as affinity ligands of aromatase. Osawa *et al.*[4] recently reported that 6β-bromoandrostenedione, a chemically reactive compound, is a mechanism-based irreversible inhibitor of aromatase. We thus have an interest in whether or not bromoacetoxy steroids cause the decrease of aromatase activity in a mechanism-based manner. The present study with the bromoacetates was designed to answer this question. The results are reported here.

Experimental

Materials [1,2-^{3}H]Androstenedione (52 Ci/mmol) was purchased from New England Nuclear Corp. (Boston, U.S.A.). NADPH was obtained from Kohjin Co., Ltd. (Tokyo, Japan). The bromoacetates **1**—**5** were synthesized according to the method[3b] reported previously.

Enzyme Preparation Human term placental microsomes (particles sedimenting at 10500 × *g* for 60 min) were obtained as described by Ryan.[5] They were washed twice with 0.5 mM dithiothreitol solution, lyophilized, and stored at −20 °C. No loss of activity occurred over the period of this study.

Time-Dependent Inactivation Procedure Various concentrations of the bromoactates **1**—**5** were incubated with or without androstenedione at 37 °C with the placental microsomes (1 mg of protein), MeOH (25 μl), and 600 μM NADPH in 67 mM phosphate buffer, pH 7.5, in a total volume of 1 ml under air or N_2. Aliquots (50 μl), in duplicate or triplicate, were removed at various times (0—12 min) and added to a solution of [1,2-^{3}H]androstenedione (2 μM, 3.0 × 10^5 dpm) and NADPH (180 μM) in 67 mM phosphate buffer, pH 7.5 (total volume 0.5 ml). The mixture was incubated at 37 °C for 20 min and the reaction was terminated by the addition of 3 ml of $CHCl_3$, followed by vortexing for 40 s. After centrifugation at 700 × *g* for 10 min, aliquots (0.3 ml) were removed from the water phase and added to scintillation mixture for determination of ^3H_2O production.[6]

Results

When the bromoacetates **1**—**5** were incubated with the enzyme preparation in the presence of NADPH in air and remaining aromatase activity was measured, the steroids **2**—**5** caused a time-dependent pseudo-first-order inactivation of aromatase (Figs. 1A—4A) while the 6α-bromo-

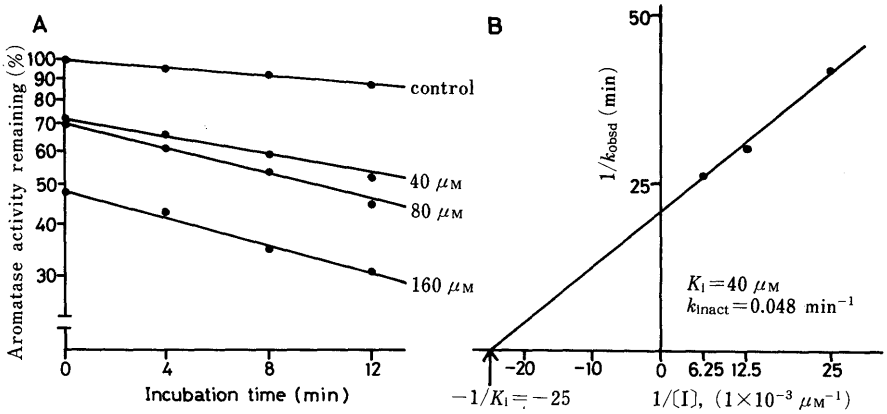

Fig. 1. Time-Dependent Inhibition (A) and Concentration-Dependent Inhibition (B) by the 6β-Bromoacetate **2** in the Presence of NADPH

Each point represents the mean of duplicate determinations.

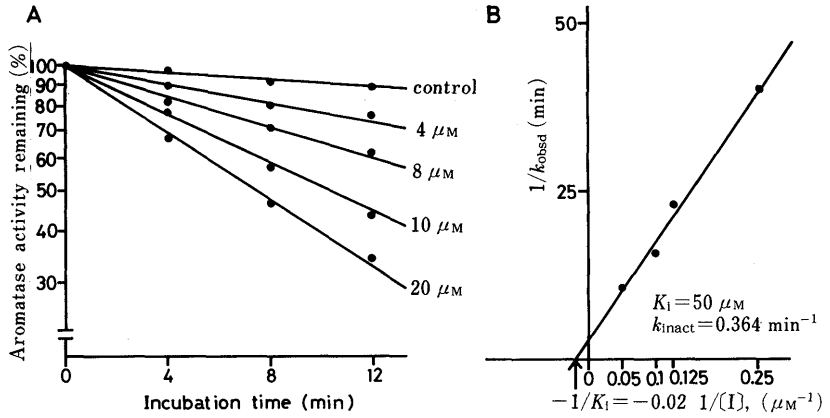

Fig. 2. Time-Dependent Inhibition (A) and Concentration-Dependent Inhibition (B) by the 6β-Bromide **3** in the Presence of NADPH
Each point represents the mean of duplicate determinations.

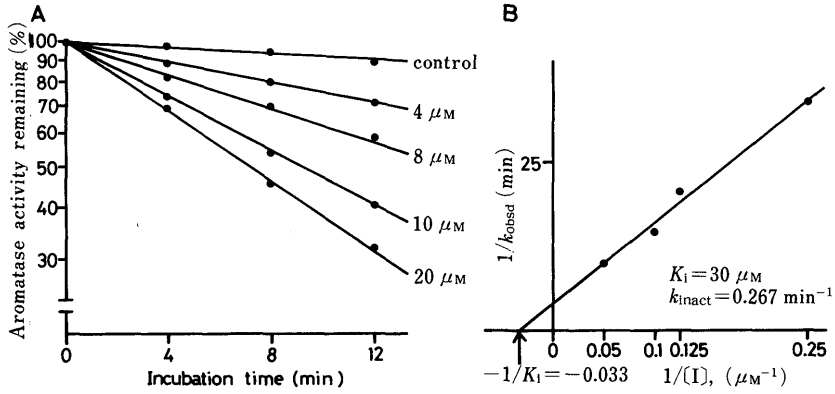

Fig. 3. Time-Dependent Inhibition (A) and Concentration-Dependent Inhibition (B) by the 6-Ketone **4** in the Presence of NADPH
Each point represents the mean of duplicate determinations.

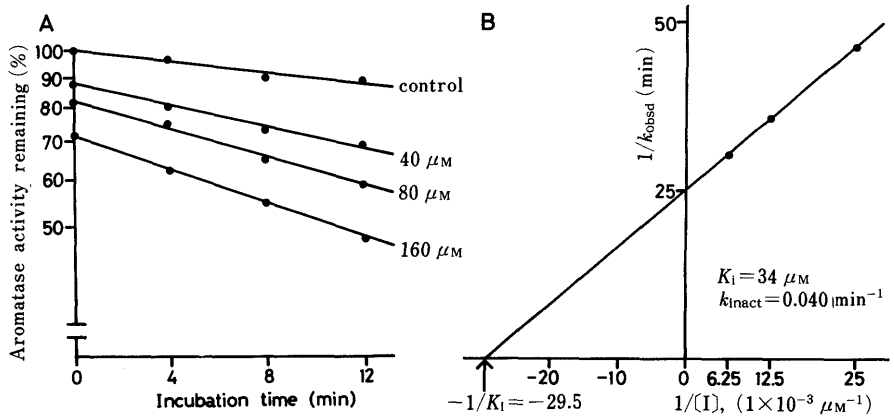

Fig. 4. Time-Dependent Inhibition (A) and Concentration-Dependent Inhibition (B) by the 6β-Methoxide **5** in the Presence of NADPH
Each point represents the mean of duplicate determinations.

acetate **1** exhibited competitive but not time-dependent inhibition (data not shown).

The kinetic parameters, K_i and k_{inact} of the four inhibitors **2**—**5** were determined. With increasing concentrations of inhibitors, increasing k_{obsd} values were obtained for the four inhibitors (Figs. 1B—4B). Double-reciprocal plots[7] of k_{obsd} *vs.* inhibitor concentration yielded K_i's of 40, 50, 30, and 34 μM and k_{inact}'s of 0.048, 0.364, 0.267, and 0.040 min^{-1}, respectively, for compounds **2**, **3**, **4**, and **5**.

The time-dependent loss of aromatase activity caused by the 6β-bromide **3** and the 6-ketone **4** required NADPH and

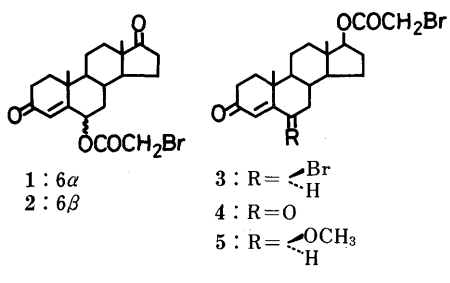

Chart 1

TABLE I. NADPH and Oxygen Dependency and Substrate Protection Experiments for Compounds **3** and **4** (8 μM)

Inhibitor and conditions	Activity[a] remaining (%)	Inhibitor and conditions	Activity[a] remaining (%)
3[b]	69	**3** + N_2 atmosphere	93
4[b]	71	**4** + N_2 atmosphere	94
3 − NADPH	96	**3** + androstenedione[c]	99
4 − NADPH	94	**4** + androstenedione[c]	98

a) Preincubations with inhibitors were carried out for 8 min at 37 °C. The values are the mean of triplicate determinations. *b*) Compounds **3** and **4** were preincubated in the presence of NADPH (600 μM) under aerobic conditions. *c*) Androstenedione (8 μM) was added to the incubate.

oxygen (Table I). Incubations of the two inhibitors in the presence of the substrate androstenedione resulted in a decrease in the inactivation of the enzyme activity, compared to incubations conducted in the presence of inhibitor alone (Table I).

Discussion

Five bromoacetoxy steroids were evaluated to obtain new information on the molecular structure of aromatase suicide substrates. Four steroids among them, but not the 6α-bromoacetate **1**, exhibited the kinetics of a suicide-type inhibitor in causing a time- and concentration-dependent inhibition of aromatase activity by a pseudo-first-order process (Figs. 1—4). Furthermore, the initial inactivation caused by compounds **3** and **4** required NADPH and oxygen and was prevented by the substrate androstenedione (Table I). Accordingly, the two steroids are considered to be aromatase suicide inhibitors and presumably form covalent adducts that inactivate the enzyme irreversibly. Two other steroids **2** and **5** causing slow inactivation are also considered to be the suicide substrates by analogy with the above two steroids.

We[3b] previously reported that the bromoacetoxy steroids caused a time-dependent inactivation of aromatase in the absence of NADPH in an affinity-labeling manner, though much higher concentrations of the inhibitors and a longer preincubation time were required compared to those employed in this study. Indeed, as shown in Table I, compounds **3** and **4** (8 μM) did not cause any significant inactivation in the absence of NADPH or oxygen while the remaining activity was decreased by about 30% in the presence of the cofactor and oxygen. Thus, it is concluded that the time-dependent inactivation observed in this study is principally a mechanism-based inhibition and not an affinity-labeling inhibition. Although we do not yet know the mechanisms of inhibition by the steroids tested, the requirement of both NADPH and oxygen for inhibition implies an oxidized species as the reactive intermediate (a potent electrophile) in each case. The intermediates could bind in a covalent manner with nucleophiles in the aromatase active site and irreversibly inhibit the enzyme.

The 6α-bromoacetate **1** is more potent competitive inhibitor of aromatase than the 6β-isomer **2**.[3b] However, the 6α-isomer **1** did not cause a time-dependent loss of aromatase activity while the 6β-isomer **2** did it. With respect to the stereochemical specificity of aromatase inactivation, analogous results[4,8] have been reported; 6β-bromoandrostenedione, a less potent inhibitor than the 6α-isomer, exhibits suicide enzyme kinetics while the 6α-isomer does not. The bromoacetyl group is a good leaving group as well as bromine. Therefore, it is conceivable that analogous mechanism would be operative in the inactivation with compound **2** and the 6β-bromo steroid. The stereochemical specificity may be related to the process of removal of the axial 6β-substituent. Moreover, the same enzyme inactivation process should be involved in the case of the 6β-bromo-17β-bromoacetate **3** as that with 6β-bromoandrostenedione.

A 4-ene-3,6-dione system is responsible for the mechanism-based aromatase inactivation by 4-androstene-3,6,17-trione.[9] The 6-oxo derivative **4** also has the same system as the trione, suggesting that the 6-ketone **4** inactivates the enzyme in the same manner as the trione. Human placental microsomes contain non-aromatase cyt. P-450 catalyzing benzphetamine demethylation[10] and hydroxysteroid oxidoreductases.[11] Considering this, it is speculated that the time-dependent inactivation observed with the 6β-methoxide **5** would, in part, depend on the 6-oxo steroid **4** produced *in situ* from the methoxide **5** during the preincubation through demethylation of the 6β-methoxyl substituent and subsequent oxidation of the resulting 6β-hydroxyl function.

The bromoacetates **2**—**5** are very interesting compounds, because they act not only as affinity ligands for aromatase but also as suicide inactivator of it, depending on the presence or absence of NADPH. It is expected that they will facilitate further elucidation of the spatial relationship of the substrate molecule to the active site of aromatase and of the aromatization mechanisms of androgens.

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