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COVALENT MODIFICATION OF AROMATASE BY A RADIOLABELED IRREVERSIBLE INHIBITOR

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Summary—7α-Substituted 4-androstene-3,17-diones are effective inhibitors of aromatase. The microsomal enzyme complex has a greater affinity for several of these inhibitors than for the substrate androstenedione, with 7α-(4′amino)phenylthio-4-androstene-3,17-dione being the most potent competitive inhibitor of the series. A potential affinity analog, the bromoacetamide derivative of the amino compound, has been synthesized in both unlabeled and ^{14}C-labeled forms via a condensation of bromoacetic acid with the amino compound using DCC. Inactivation studies with the unlabeled inhibitor showed a time-dependent, first-order inactivation of aromatase enzymatic activity. Androstenedione, when incubated in varying concentrations with the irreversible inhibitor, provided protection from inactivation. Binding studies with radiolabeled inhibitor and microsomal aromatase preparations showed that irreversible binding had occurred. SDS-electrophoresis, followed by fluorography, identified four major microsomal proteins that were radiolabeled, with the protein band at 52,000 mol. wt predominating. Similar studies with a solubilized aromatase preparation decreased the amount of nonspecific binding. Thus, covalent bonds between the irreversible inhibitor and the aromatase cytochrome P450 molecule were formed.

INTRODUCTION

Pharmacological inhibitors of estrogen biosynthesis have potential applications in the treatment of advanced estrogen-dependent mammary carcinoma and in the modulation of reproductive processes [1]. The 7α-substituted androgens [2–4] are especially interesting because of the high affinity of the analogs for the aromatase enzyme complex, particularly 7α-(4′-amino)phenylthio-4-androstene-3,17-dione (**1**) with an apparent K_i of 18 nM [2, 3]. Other aromatase inhibitors have been examined over the past few years and include numerous available steroids [5–7], 4-hydroxy- and 4-acetoxy-4-androstene-3,17-diones [8–11], various bromoandrogens [12, 13], enzyme-activated irreversible inhibitors of aromatase [14–17], and the nonsteroidal compound aminoglutethimide [18, 19]. Further studies with the irreversible and photoaffinity inhibitors [3, 4] have been initiated in order to elucidate the interactions of the 7α-substituent with aromatase. The studies described in this paper focus on the preparation and biochemical evaluation of both a stable and a radiolabeled bromoacetamide analog.

EXPERIMENTAL

Reagents

Steroids were obtained from Steraloids, Wilton, NH, or Searle Laboratories, Skokie, IL and purified by recrystallizations. 7α-(4′-Amino)phenylthio-4-androstene-3,17-dione (**1**) was prepared as previously described [2]. Chemical reagents were purchased from Aldrich Chemical Co., Milwaukee, WI and purity determined by melting point and/or thin layer chromatography. Radiolabeled steroids from New England Nuclear, Boston, MA were purified prior to use by thin layer chromatography. Radioactive samples were dissolved in NEN Formula 963 (5.0 ml) in mini-vials and counted by liquid scintillation in a Beckman LS 8100 scintillation counter. Biochemicals were purchased from Sigma Chemical Co., St Louis, MO.

7α-(4′-Bromoacetamido)phenylthio-4-androstene-3,17-dione (**2**)

A solution of **1** (500 mg, 1.22 mmol) in 30 ml of dry dichloromethane was precooled to 0°C. A solution of bromoacetic acid (500 mg, 3.62 mmol) in 9 ml of dichloromethane was added, followed by a solution of dicyclohexylcarbodiimide (DCC, 1.00 g) in 9 ml of dichloromethane, and the resultant solution stirred for 1 h at 0°C. The reaction was then allowed to stir at room temperature for an additional hour. The reaction mixture was filtered to remove the dicyclohexylurea precipitate and the solvent removed under reduced pressure. The residue was crystallized from acetone–hexane and further recrystallizations yielded analytically pure product (380 mg, 59%). m.p. 196–198 0°C; i.r. (KBr) 3300 (NH), 1730 (C_{17}=O),

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1650–1660 (C_3=O, amide C=O) cm^{-1}; pmr 0.94 (s, 3H, C_{18}), 1.23 (s, 3H, C_{19}), 3.50 (m, 1H, 7), 4.00 (s, 2H, CH_2Br), 5.70 (s, 1H, vinyl), 7.3–7.7 (m, 4H, aromatic), 8.3 (s, 1H, NH); ms *m/e* (relative intensity) 284 (0.356), 247 (0.317), 245 (0.285), 149 (0.282), 136 (0.782), 125 (1.00). Anal. C, H, N for $C_{27}H_{32}BrNO_3S$.

[1″-^{14}C] 7α-(4′-Bromoacetamido)phenylthio-4-androstene-3,17-dione (**3**)

A solution of **1** (2.0 mg, 4.93 μmol) in 1 ml of dry dichloromethane was precooled to 0°C. A solution of [1-^{14}C]bromoacetic acid (125 μCi, 2.4 μmol) in 0.5 ml of dichloromethane and a solution of unlabeled bromoacetic acid (0.33 mg, 2.4 μmol) in 0.5 ml of dichloromethane were added. Dicyclohexylcarbodiimide (DCC, 2.97 mg) in 1 ml of dichloromethane was then added and the resultant solution stirred for one hour at 0°C. The reaction was then allowed to warm to room temperature, a solution of unlabeled bromoacetic acid (0.67 mg, 4.8 μmol) in 1 ml of dichloromethane was added, and the reaction solution allowed to stir for an additional hour. The solvent was then removed under nitrogen and the residue dissolved in chloroform (0.2 ml). The product **3** was purified by preparative thin-layer chromatography on silica gel (10 × 20 cm, 500 μm thick) developed with dichloromethane–ethyl acetate (1:1). Further purification was achieved by reverse-phase HPLC (Ultrasphere ODS, 4.5 mm × 25 cm) using a linear gradient of 50–100% methanol in water over 20 min. Compound **3** was obtained with greater than 98.0% radiochemical purity. The specific activity of the final product was 8.61 mCi/mmol.

Competitive inhibition studies

Placental microsomes were prepared from fresh human term placenta following published procedures [2]. Various concentrations of [1-^{3}H]4-androstene-3,17-dione (60–500 nM; 200,000 dpm) and a single concentration of inhibitor in propylene glycol (100 μl) were incubated at 37°C for 10 min with NADP (1.8 mM), glucose-6-phosphate (2.85 mM), glucose-6-phosphate dehydrogenase (2.5 units), and placental microsomes (0.05 mg microsomal protein) in 3.5 ml of 0.1 M sodium phosphate buffer, pH 7.0. Incubations were stopped by addition of chloroform (5.0 ml) and the samples vortexed for 20 s. The samples were centrifuged (1250 *g* for 5 min) and aliquots of the aqueous solution (200 μl) were mixed with Formula 963 (5.0 ml) and counted for ^{3}H dpm in glass LSC mini-vials. The assays were run in triplicate, control samples were run simultaneously with no inhibitor, and blank samples were incubated with boiled microsomes. Data was analyzed by the kinetic programs of Cleland [20].

Aromatase inactivation studies

Various concentrations of the bromoacetamide **2** (5.0–20 μM) were incubated at 37°C with placental microsomes (0.2–0.5 mg microsomal protein) and propylene glycol (125 μl) in 0.1 M sodium phosphate buffer, pH 7.0, to a total volume of 1.5 ml. Aliquots of 200 μl were removed at various time periods (0–50 min) and centrifuged at 105,000 *g* for 15 min in a Beckman Airfuge to precipitate the microsomal proteins. The pellet was then resuspended in 20 μl buffer per tube. The remaining aromatase activity was assayed by addition of the microsomal suspension to a solution of [1,2-^{3}H]4-androstene-3,17-dione (0.5 μM, 200,000 dpm) propylene glycol (50 μl), NADP (1.8 mM), glucose-6-phosphate (2.85 mM), and glucose-6-phosphate dehydrogenase (1.0 units) in 0.1 M sodium phosphate buffer, pH 7.0 (total volume 1.5 ml) and incubated at 37°C for 30 min. The assay was stopped by the addition of 3 ml of chloroform, vortexed for 20 s, centrifuged for 5 min at 1250 *g*, and aliquots (200 μl) of the aqueous solution counted for radioactivity. Control samples with no inhibitor present were run simultaneously. Protection studies were carried out in an identical fashion with unlabeled 4-androstene-3,17-dione (7.5 and 15.0 μM) included in the initial incubations.

Covalent binding studies

Compound **3** (20 μM, 172.2 μCi), propylene glycol (300 μl), and placental microsomes (2.49 mg) in 2.2 ml of 0.1 M sodium phosphate buffer, pH 7.0, were incubated at 37°C. Aliquots were removed at 0, 30 and 60 min, diluted with cold buffer to 10 ml, and immediately centrifuged at 105,000 *g* for 60 min. The microsomal pellet was resuspended in 2 ml of buffer and the proteins precipitated by the addition of 2 ml of acetone. Following centrifugation at 1250 *g* for 5 min, the protein pellet was washed twice with 2 ml of acetone. The pellet was then solubilized overnight in 400 μl of an SDS solution consisting of 2% SDS, 2% 2-mercaptoethanol, and 8 M urea in 0.02 M sodium phosphate buffer, pH 7.4. An aliquot (100 μl) was applied to a 5–15% gradient SDS-polyacrylamide slab gel (15 × 20 cm, 1.5 mm thick) and electrophoresis performed overnight at room temperature. The gel was then stained for proteins with Coomassie Blue G250. The gel was scanned for dye density with a Beckman DU-8 equipped with a slab gel scanning feature. The gel was then soaked in a solution of 1 M sodium salicylate and dried. Fluorography was performed on Kodak XAR-5 X-ray film at −70°C [21]. Molecular weight standards consisting of bovine serum albumin, ovalbumin, pepsin, trypsin and cytochrome *c* were run simultaneously. Analogous studies were performed on a solubilized aromatase preparation. This preparation was obtained following the procedures of Osawa and co-workers [22, 23]. Briefly, the lyophilized placental tissue was treated at 0°C with 0.5% deoxycholate in 67 mM sodium–potassium buffer (pH 7.4) containing 20% glycerol and 0.5 mM DTT (GPD buffer). Following centrifugation at 105,000 *g* for 60 min, the supernatant was passed through a Sephadex G-25 column and the solubilized

proteins applied to a Whatman DE52 chromatography column. The column was washed with GPD buffer and the aromatase fraction was eluted from the column with GPD buffer containing 0.25 M Tris (pH 7.4).

RESULTS

An affinity inhibitor, 7α-(4′-bromoacetamido)-phenylthio-4-androstene-3,17-dione was synthesized via a condensation reaction of the aryl amine **1** and bromoacetic acid using DCC as the condensing agent [24, 25]. The ^{14}C-labeled analog was synthesized in the same manner as the unlabeled compound with minor modifications. As the reaction was started, equimolar amounts of labeled and unlabeled bromoacetic acid were added and allowed to react with the amine for 2 h. An excess of unlabeled bromoacetic acid was then added and the reaction allowed to continue for an additional hour. This procedure provided a higher specific activity as well as a maximum chemical yield.

Inhibition studies were performed under initial velocity conditions to determine the affinity of aromatase for the inhibitor. The bromoacetamide **3** exhibits competitive inhibition (Fig. 1), with an apparent K_i of 93.3 nM ($\pm$25.4) and an apparent K_m for androstenedione of the microsomal aromatase being 73.3 nM ($\pm$17.3). When the bromoacetamide was incubated with the enzyme preparation and remaining aromatase activity measured, the inhibitor caused a time-dependent first-order inactivation of aromatase (Fig. 2). This inactivation can be protected by the addition of various amounts of substrate androstenedione to the incubation assays (Fig. 3).

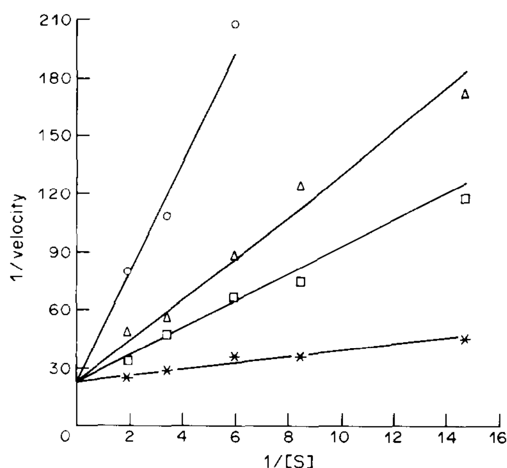


Fig. 1. Double reciprocal plots for the aromatase inhibition by bromoacetamide **2**. Varying concentrations of 4-androstene-3,17-dione were incubated with bromoacetamide at 0 μM (*), 0.3 μM (□), 0.5 μM (△), or 1.0 μM (○). Each point represents the average of three determinations with variation of less than 5%. Competitive inhibition with an apparent K_i of 93.3 nM for bromoacetamide **2** was observed.

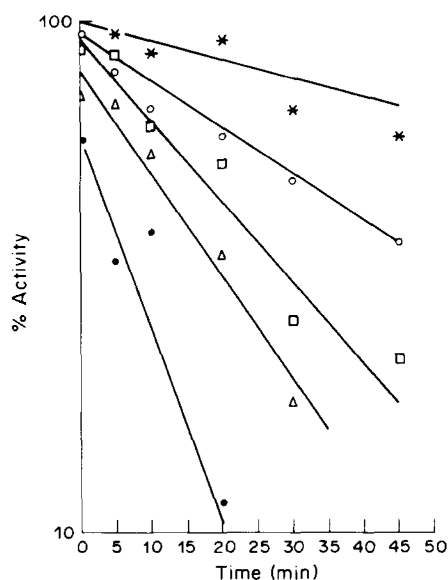


Fig. 2. Inactivation of aromatase by bromoacetamide **2**. Inhibitor **2** produced a time-dependent first-order inactivation of aromatase activity at concentrations of 5 μM (○), 10 μM (□), 15 μM (△), and 20 μM (●). Control samples contained no inhibitor (*). Each point represents the average of three determinations with standard deviations of less than 7%. The value for 100% aromatase activity is 0.0236 ± 0.0018 nmol mg^{-1} min^{-1}.

The apparent K_{inact} for the bromoacetamide **2** (analogous to the K_m for a substrate) can be determined from examining the inactivation of aromatase over time. Figure 4 is a plot of the half-time of

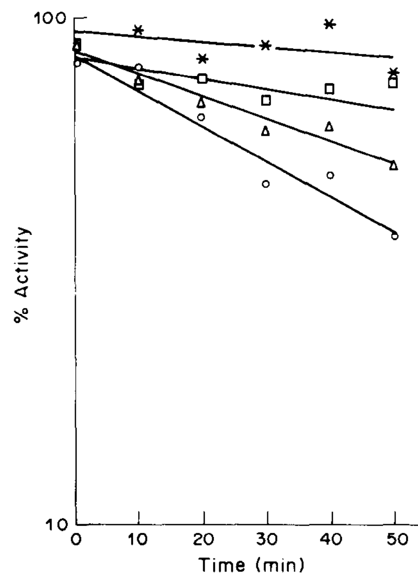
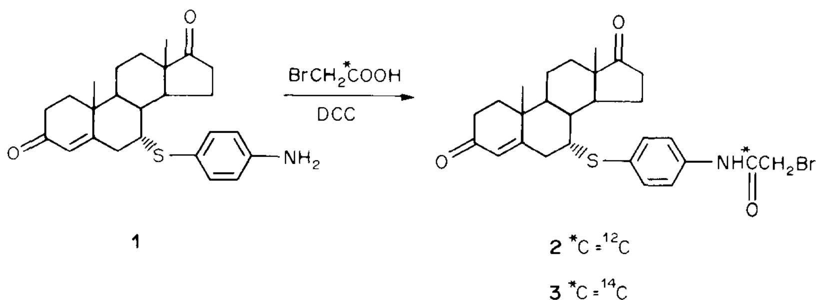


Fig. 3. Protection of the bromoacetamide **2** inactivation of aromatase by substrate. Androstenedione at concentrations of 0 μM (○), 7.5 μM (△), and 15 μM (□) was incubated with bromoacetamide (15 μM) and protected the inactivation of aromatase. Control samples contained no inhibitor (*). Each point represents the average of three determinations with standard deviations of less than 8%. The value for 100% aromatase activity is 0.0114 ± 0.00087 nmol mg^{-1} min^{-1}.



inactivation, i.e. the time required to decrease the enzymatic activity by 50%, vs the reciprocal of the inhibitor concentration. The y intercept of the resulting line is the half-time of inactivation at infinite inhibitor concentration and the slope is equal to the product of the y intercept and K_{inact} [26, 27]. Thus, the apparent K_{inact} for bromoacetamide **2** is 32.9 ($\pm$6.59) μM. Also, since the half-time of inactivation is equal to $0.693/k_{app}$, the apparent rate of inactivation by bromoacetamide **2** was calculated to be 0.1481 ($\pm$0.0296) min^{-1}.

Covalent binding studies of the radiolabeled bromoacetamide with microsomal aromatase were performed in order to determine if the bromoacetamide was covalently binding to the enzyme. The acetone-extracted protein pellet was solubilized in an SDS-urea solution. The protein mixture was separated by electrophoresis on a 5–15% gradient SDS-polyacryl-

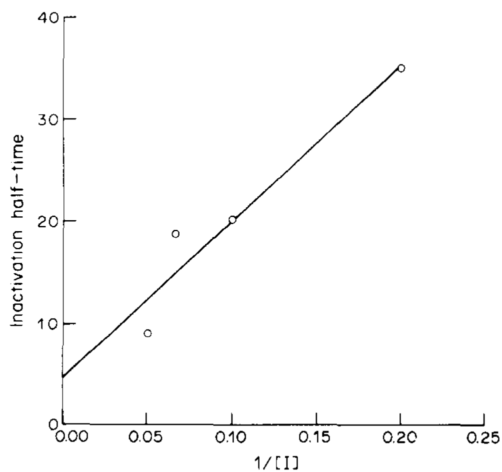


Fig. 4. Plot of the half-time of inactivation vs 1/[I].

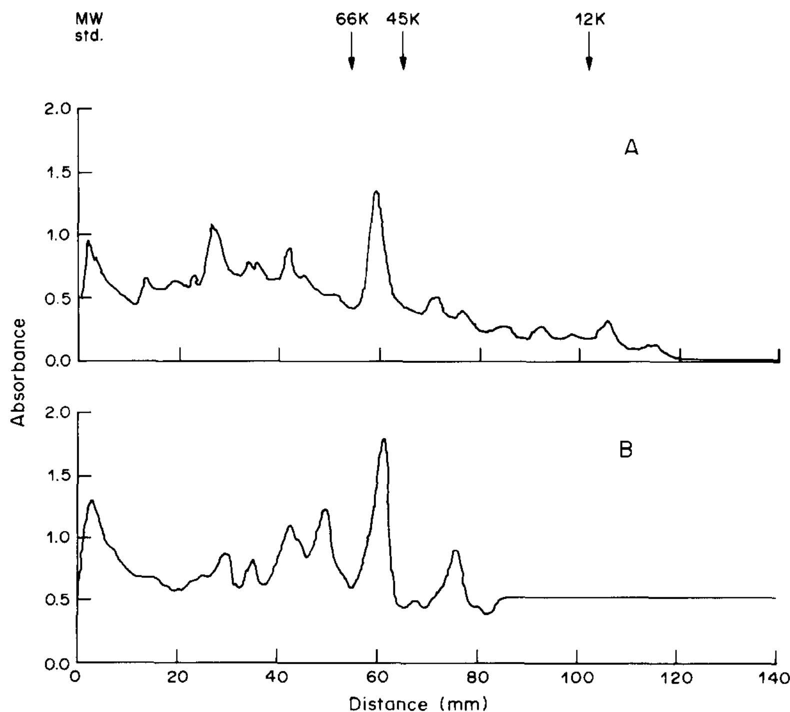


Fig. 5. Densitograms of protein staining (A) and fluorograph (B) from slab SDS gel of [^{14}C]bromoacetamide **3** covalently bound to placental microsomes.

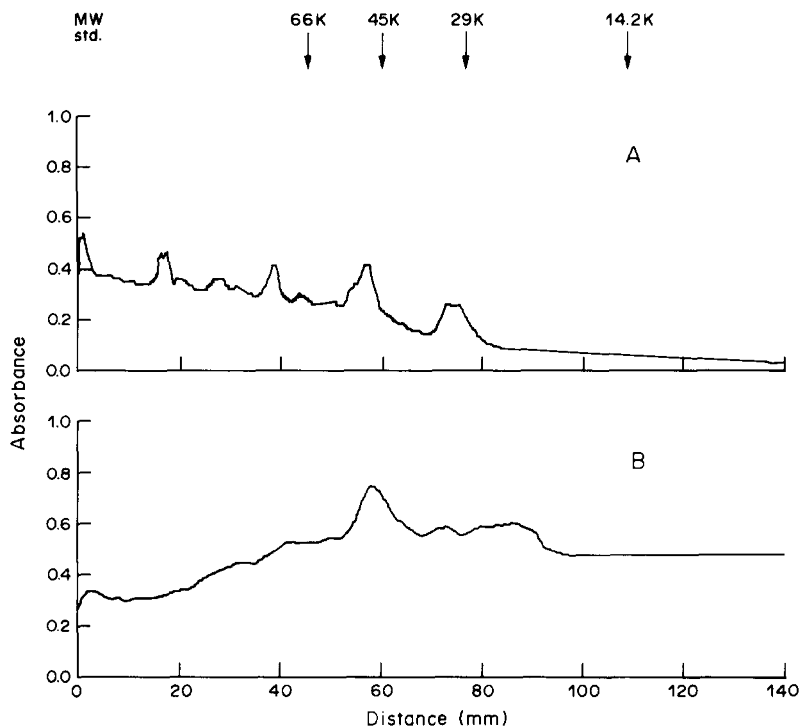


Fig. 6. Densitograms of protein staining (A) and fluorograph (B) from slab SDS gel of [^{14}C]bromoacetamide **3** covalently bound to solubilized aromatase preparation.

amide slab gel, followed by fluorography [21]. The major protein peak to which the radiolabeled inhibitor was bound exhibited a protein(s) with a mol. wt of 52,000 Daltons (Fig. 5). The molecular weight of the cytochrome P-450 component of the aromatase complex has been reported to be 52,000 Daltons [22, 23]. In addition, smaller amounts of radioactivity are associated with several other proteins bands. Covalent binding studies with the solubilized aromatase preparation, followed by electrophoresis, resulted in only the protein at 52,000 Daltons having radioactivity associated with it and a loss of radioactivity associated with other proteins.

DISCUSSION

These studies demonstrate that 7α-(4′-bromoacetamido)phenylthio-4-androstene-3,17-dione competitively inhibits microsomal aromatase, covalently binds to the enzyme complex, and produces an irreversible inactivation of the enzymatic activity. The radiolabeled analog **3** was observed to irreversibly bind to both microsomal and solubilized aromatase, with the radiolabel associated with a protein(s) of 52,000 mol. wt. These results provide evidence that 7α-(4′-bromoacetamido)-phenylthio-4-androstene-3,17-dione (**2**) is interacting at the enzyme site and is inactivating aromatase by a covalent modification of the cytochrome P-450 component of the enzyme. The affinity of aromatase for this 7α-substituted androstenedione is less than those observed for other 7α-substituted analogs [2–4], which suggests that the "pocket" of the enzyme complex that accommodates the 7α-substituent is of a limited size. Nonetheless, irreversible binding is observed with this α-haloketone. Thus, a nucleophilic amino acid may be present in this "pocket" to displace the bromine atom. Finally, other protein bands were identified that irreversibly-bound smaller amounts of radiolabeled **3**. This nonspecific binding to other proteins of the crude microsomal preparation indicates that the reactive α-haloketone can bind with other available proteins. Solubilization and DE52 chromatography eliminated the nonspecific binding while the covalent binding to aromatase P-450 was retained.

The exact interaction(s) of the 7α-substituted androstenedione with the aromatase enzyme remain to be elucidated. These studies suggest that a nucleophilic amino acid is present to lead to the covalent modification of aromatase by 7α-(4′-bromoacetamido)-phenylthio-4-androstene-3,17-dione. However, conclusive evidence must await the examination of this compound and other 7α-substituted C_{19} analogs with highly purified aromatase.

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