INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the original text directly from the copy submitted. Thus, some dissertation copies are in typewriter face, while others may be from a computer printer.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyrighted material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is available as one exposure on a standard 35 mm slide or as a 17" × 23" black and white photographic print for an additional charge.

Photographs included in the original manuscript have been reproduced xerographically in this copy. 35 mm slides or 6" × 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Synthesis, biochemical and in vivo studies of aromatase inhibitors

Li, Pui-Kai, Ph.D.

The Ohio State University, 1988
SYNTHESIS, BIOCHEMICAL AND IN VIVO
STUDIES OF AROMATASE INHIBITORS

Dissertation

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Pui-Kai Li, B.S.

The Ohio State University
1988

Dissertation Committee:
Robert W. Brueggemeier, Ph.D
Duane D. Miller, Ph.D
Robert W. Curley, Jr., Ph.D
Young C. Lin, Ph.D

Approved by:

Robert W. Brueggemeier
Adviser
College of Pharmacy
Dedication

To dear Dao for her caring, patience and love
I would like to thank the following people:

I am forever grateful to my mom and dad for their devotion and guidance.

I wish to thank my advisor, Dr. R.W. Brueggemeier for his guidance throughout the course of my graduate studies. His enthusiasm and concern are deeply appreciated.

Special thanks to Mr. and Mrs. Thai and their family for their constant encouragement and support during the later stage of my studies in Medicinal Chemistry.
VITA

August 30, 1957..................... Born - Hong Kong

1983............................... B.S. Pharmacy, University of Wyoming, School of Pharmacy, Laramie, Wyoming

1983-1984........................... University fellowship
The Ohio State University
Columbus, Ohio

1984-1986........................... Graduate Teaching Associate
The Ohio State University
Columbus, Ohio

1986-present....................... Graduate Research Associate
The Ohio State University
Columbus, Ohio

PUBLICATIONS

Articles:

R.W. Brueggemeier, P.K. Li, M.V Darby and N.E. Katlic, "7α-Substituted Androstenediones as effective in vitro and in vivo inhibitors of aromatase", Steroid, in press.

R.W. Brueggemeier, P.K. Li, "Inhibition of rat mammary tumor growth by the aromatase inhibitor 7α-APTA". Cancer Res., submitted (1987)

Abstracts and Presentations:


P.K. Li, J. Sunyecz, and R.W. Brueggemeier, "Effects of the aromatase inhibitor 7α-APTA on hormone-dependent mammary tumors in rats", Nineteenth Annual Graduate Student Meeting in Medicinal Chemistry, Columbus, Ohio, July, 1986.


P.K. Li, J. Sunyecz, and R.W. Brueggemeier, "Effects of the aromatase inhibitor 7α-APTA on hormone-dependent mammary tumors in rats", 13th Annual Bio-Medical Graduate Student Forum, ICSABER SOCIETY, Ohio State University, Columbus, Ohio, May 1987, Abstract No. 16.

P.K. Li, and R.W. Brueggemeier, "Synthesis and Biochemical studies on 7-substituted 4,6-androstadiene-3,17-diones and 1,4,6-androstatriene-3,17-diones", Nineteenth Central Regional Meeting of the American Chemical Society, Ohio State University, Columbus, Ohio, June 24-26, 1987, Abstract No.98.

FIELDS OF STUDY

Major Field: Steroid Chemistry and Biochemistry
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEDICATION</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>VITA</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xii</td>
</tr>
<tr>
<td>CHAPTER I. GENERAL INTRODUCTION.</td>
<td>1</td>
</tr>
<tr>
<td>1.1. Role of estrogens in various physiological and disease states</td>
<td>1</td>
</tr>
<tr>
<td>1.2. Estrogen Biosynthesis</td>
<td>4</td>
</tr>
<tr>
<td>1.2.1. Characterization and classification of aromatase</td>
<td>4</td>
</tr>
<tr>
<td>1.2.2. Mechanism of action of aromatase</td>
<td>5</td>
</tr>
<tr>
<td>1.2.3. Purification of aromatase</td>
<td>13</td>
</tr>
<tr>
<td>1.2.4. Cloning of aromatase gene and its applications</td>
<td>14</td>
</tr>
<tr>
<td>1.3. Aromatase Inhibitors</td>
<td>16</td>
</tr>
<tr>
<td>1.3.1. Pharmacological and Biochemical Implications of Aromatase Inhibitors</td>
<td>16</td>
</tr>
<tr>
<td>1.3.2. Classification of Aromatase Inhibitors</td>
<td>17</td>
</tr>
<tr>
<td>1.3.2.1. Reversible Inhibitors of Aromatase</td>
<td>18</td>
</tr>
<tr>
<td>1.3.2.2. Irreversible Inhibitors of aromatase</td>
<td>24</td>
</tr>
<tr>
<td>CHAPTER II. EFFECT OF AN AROMATASE INHIBITOR, 7α-APTA, ON DMBA INDUCED RAT MAMMARY TUMORS</td>
<td>37</td>
</tr>
<tr>
<td>2.1. Introduction</td>
<td>37</td>
</tr>
<tr>
<td>2.1.1. Estrogen and Cancer</td>
<td>37</td>
</tr>
<tr>
<td>2.1.2. Clinical Studies of Aromatase Inhibitors in Breast Cancer Patients</td>
<td>39</td>
</tr>
<tr>
<td>2.1.3. Antitumor Activity of Aromatase Inhibitors in Dependent Rat Tumors</td>
<td>40</td>
</tr>
<tr>
<td>2.2. Statement of Objectives</td>
<td>41</td>
</tr>
<tr>
<td>FIGURE</td>
<td>PAGE</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
<tr>
<td>1. Fate of Oxygen Insertion and Hydrogen Loss in C_{19} Methyl during Aromatization</td>
<td>7</td>
</tr>
<tr>
<td>2. Fishman's Proposed Aromatization Reaction in Which The Third Hydroxylation is at 2β position</td>
<td>10</td>
</tr>
<tr>
<td>3. Akhtar's Proposed Alternate Pathway of Aromatization</td>
<td>12</td>
</tr>
<tr>
<td>4. Proposed Mechanisms of Inactivation of Aromatase by PED</td>
<td>30</td>
</tr>
<tr>
<td>5. Effect of 7α-APTA and 4,6-ADD on Tumor Regression</td>
<td>53</td>
</tr>
<tr>
<td>6. Effects of Co-Administration of Estradiol and 7α-APTA on Tumor Regression</td>
<td>55</td>
</tr>
<tr>
<td>7. Effects of 7α-APTA in Silastic Implants on Tumor Regression</td>
<td>57</td>
</tr>
<tr>
<td>8. Rate of Release of 7α-APTA from Silastic Implants</td>
<td>59</td>
</tr>
<tr>
<td>9. Plasma Estradiol Concentration in Tumored Rats</td>
<td>61</td>
</tr>
<tr>
<td>10. New Proposed Aromatase Inhibitors</td>
<td>66</td>
</tr>
<tr>
<td>11. Synthetic Scheme of New Aromatase Inhibitors</td>
<td>68</td>
</tr>
<tr>
<td>12. Double Reciprocal Plots of Aromatase Inhibition by Inhibitor 34</td>
<td>99</td>
</tr>
<tr>
<td>13. Double Reciprocal Plots of Aromatase Inhibition by Inhibitor 35</td>
<td>101</td>
</tr>
<tr>
<td>14. Double Reciprocal Plots of Aromatase Inhibition by Inhibitor 36</td>
<td>103</td>
</tr>
</tbody>
</table>
15. Double Reciprocal Plots of Aromatase Inhibition by Inhibitor 37 ........................................... 105
16. Double Reciprocal Plots of Aromatase Inhibition by Inhibitor 38 ........................................... 107
17. Double Reciprocal Plots of Aromatase Inhibition by Inhibitor 39 ........................................... 109
18. Double Reciprocal Plots of Aromatase Inhibition by Inhibitor 40 ........................................... 111
19. Double Reciprocal Plots of Aromatase Inhibition by Inhibitor 41 ........................................... 113
20. Double Reciprocal Plots of Aromatase Inhibition by Inhibitor 42 ........................................... 115
21. Double Reciprocal Plots of Aromatase Inhibition by Inhibitor 43 ........................................... 117
22. Overlapped Energy Minimized Structure of 7α-APTA and Inhibitor 34 ...................................... 122
23. Overlapped Energy Minimized Structure of 7α-APTA and Inhibitor 35 ...................................... 124
24. Overlapped Energy Minimized Structure of 7α-APTA and Inhibitor 36 ...................................... 126
25. Overlapped Energy Minimized Structure of 7α-APTA and Inhibitor 37 ...................................... 128
26. Overlapped Energy Minimized Structure of 7α-APTA and Inhibitor 38 ...................................... 130
27. Overlapped Energy Minimized Structure of 7α-APTA and Inhibitor 39 ...................................... 132
28. Overlapped Energy Minimized Structure of 7α-APTA and Inhibitor 40 ...................................... 134
29. Overlapped Energy Minimized Structure of 7α-APTA and Inhibitor 41 ...................................... 136
30. Overlapped Energy Minimized Structure of 7α-APTA and Inhibitor 42 ...................................... 138
31. Overlapped Energy Minimized Structure of 7α-APTA and Inhibitor 43 .......................... 140
32. Overlapped Energy Minimized Structure of Inhibitor 34 and Inhibitor 36 ....................... 142
33. Overlapped Energy Minimized Structure of Inhibitor 34 and Inhibitor 38 ....................... 144
34. Overlapped Energy Minimized Structure of Inhibitor 36 and Inhibitor 38 ....................... 146
35. Overlapped Energy Minimized Structure of Inhibitor 38 and Inhibitor 39 ....................... 148
36. Graphical Representation of Radial and Angular parameter ........................................... 150
37. Plotting of Log K_1 vs θ ........................................ 154
38. Plotting of Log K_1 vs d ........................................ 156
39. Inactivation of aromatase by Inhibitor 35 in the Presence of NADPH ............................. 165
40. Inactivation of aromatase by Inhibitor 35 in the Absence of NADPH ............................. 167
41. Protection of Inhibitor 35 from Inactivation of Aromatase by Substrate .......................... 169
42. Inactivation of aromatase by Inhibitor 35 in the Presence of a Nucleophilic Trapping Agent .......................... 171
43. Irreversibility Studies of Inhibitor 35 .................................................. 173
44. Plot of the Inactivation Half-Time (min) vs 1/[I]μM⁻¹ for inhibitor 35 .......................... 175
45. Inactivation of aromatase by Inhibitor 37 in the Presence of NADPH ......................... 177
46. Inactivation of aromatase by Inhibitor 37 in the Absence of NADPH .............................. 179
47. Protection of Inhibitor 37 from Inactivation of Aromatase by Substrate ....................... 181
48. Inactivation of aromatase by Inhibitor 37 in the Presence of a Nucleophilic Trapping Agent....183
49. Irreversibility Studies of Inhibitor 37..................185
50. Plot of the Inactivation Half-Time (min)
    vs 1/[I]μM⁻¹ for inhibitor 37.............................187
51. Inactivation of aromatase by Inhibitor 39 in the Presence of NADPH..............................189
52. Inactivation of aromatase by Inhibitor 39 in the Absence of NADPH......................191
53. Protection of Inhibitor 39 Inactivation of Aromatase by Substrate.........................193
54. Inactivation of aromatase by Inhibitor 39 in the Presence of a Nucleophilic Trapping Agent....195
55. Irreversibility Studies of Inhibitor 39.................197
56. Plot of the Inactivation Half-Time (min)
    vs 1/[I]μM⁻¹ for inhibitor 39.............................199
57. Scheme for a Mechanism-Based Inactivation Process......201
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Effects of 7α-APTA on Rat Mammary Tumors</td>
<td>51</td>
</tr>
<tr>
<td>2. The $K_m$ and $K_i$ of Inhibitors 34-43</td>
<td>97</td>
</tr>
<tr>
<td>3. Angular and Radial Parameter of Inhibitors 34-43</td>
<td>152</td>
</tr>
<tr>
<td>4. Comparison of Inactivation Kinetics For Irreversible Inhibitors of Aromatase</td>
<td>163</td>
</tr>
</tbody>
</table>
CHAPTER I

GENERAL INTRODUCTION

1.1. The role of estrogens in various physiological process.

One of the two major classes of sex steroids produced in the female are estrogens. Estrogens play an important role in the development of female sexual organs important for reproduction and sexual characteristic. Female reproductive function occurs in a cyclic fashion. This cycle is termed the sexual or menstrual cycle. One of the primary role of estrogens is the maintenance of the menstrual cycle. In the human female, the most obvious manifestation of the sexual cycle is the monthly discharge of blood known as menstruation. Just after menstruation, the anterior lobe of the pituitary gland increases secretion of follicle stimulating hormone (FSH). FSH has two effects: (1) it causes a Graafian follicle to develop in the ovary and (2) it stimulates the granulosa cells in the follicle to produce estrogens (1, 2), mainly estradiol, through the induction of the enzyme, aromatase. The immediate effect of estrogen is to induce healing and repair of the uterine wall following menstruation. In the course of the next two weeks the amount of estrogen in the body builds up until it reaches a critical concentration which results in stimulation of the secretion of a second hormone from the anterior pituitary. This is called luteinising hormone (LH). LH brings about ovulation and causes the Graafian follicle to change into a corpus luteum. It then stimulates the corpus
luteum to secrete yet another hormone, progesterone, whose principal function is to cause proliferation of the uterine wall in preparation for implantation. Progesterone also inhibits FSH and LH production by the anterior pituitary, so no further follicles develop and estrogen production is reduced. As the corpus luteum degenerates, progesterone production ceases, at which point menstruation takes place. With the sudden drop in the level of progesterone, the anterior pituitary starts secreting FSH again, and the cycle is repeated.

If fertilization takes place, the corpus luteum, instead of degenerating, persists and continues to secrete progesterone. This, coupled with a small but steady secretion of estrogen from the ovary, continues the development of the uterus. By inhibiting the anterior pituitary from producing FSH, progesterone also prevents follicles from developing during pregnancy. After the first trimester of pregnancy, the corpus luteum begins to regress and the placenta takes over the job of secreting progesterone and estrogens. In the course of pregnancy, the level of estrogens in the blood rise and progesterone falls, and it has been suggested that this plays some part in bringing about parturition (3). Certainly, estrogen promotes uterine contraction and progesterone inhibits it. However, a more direct cause of parturition is another hormone, oxytocin (4,5), secreted by the posterior lobe of the pituitary. This causes the uterine muscle to contract. Estrogen achieves its effect by making the uterine muscle more sensitive to oxytocin.
Estrogen and progesterone are also responsible for the growth of the mammary glands in readiness for milk production. After parturition, the flow of milk is initiated by yet another pituitary hormone, prolactin. Prolactin secretion is inhibited by estrogen and progesterone. The sudden drop in these two hormones at the end of pregnancy permits the onset of lactation.

Estrogens affect many other organs as well. In the liver, estrogens stimulate the synthesis of CBG, TeBG and TBG. They also increase blood coagulation rate by increasing the level of some coagulation factors. Moreover, estrogens not only induce secretion of renin substrate, but also contribute to water retention which results in increasing the risk of hypertension. Estrogens are also indicated in the formation and maintenance of bone. In the juvenile, estrogens accelerate linear growth and epiphyseal closure. In addition, estrogens inhibit somatomedin (6).

The discovery of the presence of estrogen receptors in brain cells (7, 8, 9) opened up a new research area studying estrogen action in the brain. The most thorough studies of estrogen in the brain were of their involvement in sex differentiation in rats. In male rats, estradiol, synthesized from testosterone by aromatase in the brain, altered permanently certain neural circuits during 'critical' periods of brain development. This process is called defeminization. After defeminization, male rats are able to exhibit male patterns of behavior and noncyclical gonadotropin release. The defeminizing effect of
estradiol can be antagonized by the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD) (10, 11). In the late fetal and neonatal period of female rats, an important serum protein called α-fetoprotein is present and binds estradiol (12). This fetoprotein prevents estradiol from defeminizing female rats.

1.2. Estrogen biosynthesis.

1.2.1 Characterization and classification of aromatase

Aromatase (estrogen synthetase) is the enzyme responsible for the conversion of androgens to estrogens. The name aromatase was coined because the A ring of androgens is aromatized with the loss of the C$_{19}$ methyl group via C$_{10}$-C$_{19}$ of the androgens.

Ryan (13) first demonstrated that the aromatization reaction requires both oxygen and NADPH. In addition, the reaction could be inhibited by KCN and aminogluthethimide (14). The above findings supported the suggestion that aromatase is a cytochrome P-450 enzyme. However, crude aromatase preparations did not exhibit a CO-difference spectrum with maximum absorption at 450nm (14, 15). This had led to the debate as to whether aromatase was truly a cytochrome P-450 isozyme. Recently, aromatase has been purified to homogeneity by Nakajin, et al (16) and the purified aromatase exhibits a CO-difference spectrum with an absorption maximum at 450nm.
1.2.2. Mechanism of action of aromatase

Aromatase is the enzyme responsible for the conversion of androstenedione or testosterone to estrogens through the loss of the C$_{19}$ methyl group.

The complete mechanism of aromatization has not been fully elucidated and is still the subject of intensive studies. The stoichiometry of aromatization is such that 3 moles each of O$_2$ and NADPH are required for each mole of estrogen formed (17), implicating the participation of three enzymatic hydroxylations. The first two of these hydroxylations have been identified and involve the successive hydroxylation at the C$_{19}$ methyl group to first form 19-hydroxy derivative and then form gem-diol. The gem-diol can dehydrate to form a C-19 aldehyde (18, 19, 20). The C$_{19}$ methyl group is subsequently lost as formic acid (21). Also, the hydrogen atoms at the 1β (22, 23) and 2β (24, 25) positions are lost stereospecifically during the last step of aromatization. Since only two oxygen atoms are present in formic acid, Akhtar and coworkers, using $^{18}$O$_2$ and $^3$H intermediates, were able to show that the oxygens of formic acid were derived from the first and the third hydroxylations (26, 27). The same research group was also able to show that the pro-R hydrogen atom from C-19 was removed as a proton and the pro-S hydrogen atom was incorporated into formic acid (figure 1) (28, 29, 30).

Efforts have concentrated on investigating the location of the third hydroxylation and subsequent release of formic acid to form estrogen.
Figure 1. Fate of oxygen insertion and hydrogen loss in C₁₉ methyl during aromatization.
Figure 1.
Fishman proposed the third hydroxylation was at the 2β position and results in the formation of 2β-hydroxy-19-oxo-androstenedione which aromatized to estrone (figure 2) (31, 32, 33). This proposal was strengthened when the compound 2β-hydroxy-19-oxo-[1α-3H]-androstenedione was synthesized (34). Additional evidence was provided by using immunological probe in which an antibody was obtained that recognized 2β-hydroxy-19-oxoandrostenedione (35). The rate of aromatization of androstenedione was decreased when incubated with placental microsomes in the presence of the antibody. Based on the experimental evidence described above, Fishman hypothesized the mechanism of aromatization as shown in Figure 2. He proposed the rate-determining hydroxylation is at the 2β position and this pathway is the dominant route of estrogen biosynthesis. Recently, Caspi et al (36) synthesized [2β-18O,19-3H]2β-hydroxy-10β-formylandrost-4-ene-3,17-dione. They were unable to show the incorporation of 2β-oxygen into formic acid under enzymatic or nonenzymatic condition. This may suggest that Fishman's proposed pathway is not an obligatory pathway of estrogen biosynthesis by placental aromatase.

Akhtar proposed an alternate pathway of aromatization in which the third mole of oxygen is involved in the formation of FeIII-peroxide species which attacks the C-19 aldehyde (figure 3) (27, 37). In this model, aromatization results directly through a cyclic mechanism. The FeIII-peroxide species was also a proposed intermediate in the catalytic cycle of other cytochrome P-450 isozymes (38).
Figure 2. Fishman's proposed aromatization reaction in which the third hydroxylation is at 2β position.
Figure 2.
Figure 3. Akhtar's proposed alternate pathway of aromatization.
Figure 3.
1.2.3. Purification of aromatase

Since the study of the mechanism of action and active site elucidation of aromatase requires highly purified enzyme, recent efforts have focused on the purification of aromatase. Due to its membrane bound nature and instability after solubilization, aromatase has proven extremely difficult to purify. Pusanen et al. (39) partially purified aromatase from human placental microsome to the extent that a specific content of 3-7 nmol/mg protein was obtained. Osawa and coworkers reported the isolation of multiple forms of aromatase from human placenta (40), one converting androstenedione to estrone and the other from 16α-hydroxytestosterone to estriol. Tan et al. (41) described the purification of aromatase using Aminohexyl-Sepharose and hydroxyapatite column. With the combination of the detergent CHAPS during solubilization and stabilization of the enzyme with the substrate androstenedione, they were able to purify the enzyme with specific content of 4.2 nmol/mg protein. Nakajin and coworkers (42) purified aromatase to homogeneity. However, the preparation only had a specific content of 2.7 nmol of P-450/mg of protein. Moreover, the enzyme was extremely unstable and had a half life of only 2.5 days. The most recent report of aromatase purification described was published by Kellis and Vickery (43). They were able to purify the enzyme with the highest specific content ever reported - 11.2 nmol of P-450/mg protein. In addition, the preparations were stable for weeks when stored at -20°C. With a different approach, Mendelson and Simpson (44) partially
purified aromatase using phenylsepharose and DEAE-cellulose chromatography to 2 nmol of P-450/mg of protein. The protein was subjected to SDS-polyacrylamide gel electrophoresis and the excised band from the gel with M.W. 55,000 was injected into rabbits to raise polyclonal and monoclonal antibodies. The monoclonal antibody was used in immunoaffinity chromatography for further purification of aromatase.

1.2.4. Cloning of the aromatase gene and its application.

The success in purification of aromatase has led to the cloning of aromatase gene. Recently, two groups have claimed to successfully identify a cDNA clone for aromatase by screening a human placental λgt11 cDNA expression library. Simpson et al (45) et al (45) used polyclonal antibodies against human placental aromatase cytochrome P-450 as a probe and obtained a cDNA clone which was found to contain 1.8 kb in length. The cDNA was able to hybridize several mRNA's in both placental and adipose stromal cells. Further characterization of the cDNA by restriction endonuclease digestion obtained several fragments. Some fragments were sequenced to determine the amino acid sequence of cytochrome P-450arom protein. Part of the amino acid sequence of one of the segments was identical to the cysteine-containing peptide T-16 reported by Chen (46).

Hall and coworkers (47) used a synthetic oligonucleotide, which was based on the amino acid sequence of a segment of aromatase, as a probe to identify a cDNA for aromatase. A cDNA insert of 2.4 kb was identified.
Simpson et al utilized the cDNA they identified to study the regulation of estrogen formation in both human adipose stromal cells (48) and human ovarian granulosa cells (49). By using the characteristic that the cDNA contained the base sequence complementary to human cytochrome P-450<sub>arom</sub> mRNA, cDNA was used as hybridization probe to detect the extent of mRNA production. Simpson was able to show that the action of FSH and forskolin to increase aromatase activity in human granulosa cells, and the ability of dibutyl cyclic AMP (Bt<sub>2</sub>cAMP) and glucocorticoid to increase aromatase activity in human adipose stromal cells, was the result of an increase in the level of mRNA cytochrome P-450<sub>arom</sub>.
1.3. Aromatase inhibitors

1.3.1. Pharmacological and biochemical implications of aromatase inhibitors.

Aromatase is the enzyme complex involved in the conversion of androgen to estrogen, the last in the series of steps in steroidogenesis. Therefore, blockade of the enzyme will not deprive the body from the formation of other essential steroids. Aromatase inhibitors are potential agents in treating estrogen related disease such as gynecomastia, endometriosis, premature labor, endometrial and breast disease through inhibition of estrogen formation in estrogen synthesizing tissues. Inhibition of aromatase has also been shown to block preovulatory estrogen surge in rats without inhibiting basal estrogen secretion (50). Aromatase inhibitors may therefore be useful in contraception.

Aromatase inhibitors also have several biochemical implications. They have been used to investigate the role of estrogens in the development of the ovarian follicle (51). In addition, radiolabeled enzyme activated irreversible inhibitors can be used in evaluation of the mechanism of action of aromatase and purification of the enzyme.

Currently, the main clinical use of aromatase inhibitors has been devoted to the treatment of estrogen-dependent breast cancer. For estrogen-dependent breast cancer patients, hormonal therapy is chosen over other treatments such as surgical ablation, cancer chemotherapy,
and radiation treatment because of its low toxicity. Current hormonal therapy includes using antiestrogens which have proven effective in promoting tumor regression in estrogen receptor positive (ER+) patients. Aminoglutethimide was the first aromatase inhibitor used in clinical trials for estrogen-dependent breast cancer patients. It is effective in 40% of postmenopausal breast cancer patients and is active in some patients who have relapsed from antiestrogen tamoxifen (52, 53). However, toxicities of aminoglutethimide, such as blockade of corticosteroid biosynthesis and CNS effects, prompted the investigation of new aromatase inhibitors. 4-Hydroxyandrostenedione (4-OH-A) is currently in clinical trial in London (54, 55). Preliminary results indicate that 4-OH-A has a longer duration of action and is devoid of toxicities experienced by using aminoglutethimide.

1.3.2. Classification of aromatase inhibitors

Aromatase inhibitors can be divided into two categories: 1) reversible or competitive inhibitors and 2) irreversible inhibitors which bind to the enzyme and subsequently inactivate the enzyme.

Reversible inhibitors achieve their inhibition in one of two ways. First, the inhibitor interferes with aromatization by competing with the substrate for the substrate binding site. Inhibitors of this type produce type I high-spin binding spectra (14). Inhibition of aromatase may also be achieved by interfering with steroid hydroxylation through binding to the heme iron of cytochrome P-450. Such inhibitors produce
type II low spin binding spectra and usually contain a heteroatom such as nitrogen.

1.3.2.1. Reversible inhibitors of aromatase

Competitive aromatase inhibitors containing the steroid moiety usually give type I difference spectra. This indicates that the inhibitors act on the enzyme by interacting with the substrate binding site. Schwarzel and coworkers (56) systemically studied the structural features of over 100 steroids as inhibitors of aromatase. The major characteristic of an inhibitor with high affinity to the enzyme was that it contained the 4-ene-3-one system or a trans A/B steroid ring junction. The most effective C17 substituent appeared to be 17-keto (57). In addition, 19-nor steroids were less inhibitory than the corresponding C-19 steroids. Increasing the linear conjugation by inserting C1-C2 or C4-C6 double bonds enhanced the binding affinity to the enzyme. Substitution at C-9 or C-11 decreased affinity.

C19 steroids with either 16α or 7α substituents proved to be effective aromatase inhibitors. 16α-Bromoandrostenedione bound competitively to aromatase (58). In 1977, Brueggemeier and coworkers reported the synthesis and biochemical studies of some 1α and 7α-thio substituted 4-androstene-3,17-diones (59, 60). The 7α-thio inhibitors were found to be superior to the 1α-thio substituted inhibitors. Among those compounds reported, 7α-(4'-amino)phenylthio-4-androstene-
3,17-dione (7α-APTA) \(_1\), was the most potent inhibitor identified with an apparent \(K_i\) of 18 nM. Later, further studies were carried out among the 7α-(phenylthio)-4-androstene-3,17-dione inhibitors to obtain more information on the structure-activity relationship (61). There was no direct correlation between substituent electronic effect and inhibitory potency. Substitution of the p-amino functional group by halogens resulted in inhibitors with similar potency as 7α-APTA. Moreover, the more strongly electron withdrawing acetyl group also gave rise to an effective inhibitor.

Solo and coworkers synthesized some 7α-alkyl testosterone derivatives as inhibitors of aromatase (62). Inhibition studies indicated the enzyme could tolerate considerable bulk at the 7α position of 4-androstene-3,17-dione or testosterone. In addition, there was no correlation between the hydrophilicity of the substituent and the affinity of the inhibitors for the enzyme. The most potent inhibitor of
the series was 7α-(3′-acetoxypropyl)-4-androstene-3,17-dione which was as effective as 4,6-androstadiene-3,17-dione as an inhibitor of aromatase.

Recently, potent reversible inhibitors (63, 64) were reported which not only bound to the steroid binding site, but also produced a type II binding spectra by coordinating with the heme-iron. Inhibitors of this type have either sulfur 2, 3, nitrogen 4 or oxygen 5 attached to C-19.

Aminoglutethimide (AG) 6 was first used as an anticonvulsant, but was observed later to cause adrenal insufficiency which led to its with-
drawal from the market. The drug inhibits several enzymes in the pathway of steroidogenesis, of which the cholesterol side chain cleavage enzyme ($P_{-450}^{SCC}$) (65) and aromatase (66) are the principal ones. AG was found to be a more potent inhibitor of aromatase than $P_{450}^{SCC}$. The L-isomer of AG is 30 times less potent than the D-isomer in aromatase inhibition, whereas the D-form is 15 times less potent than the L-form in inhibiting $P_{450}^{SCC}$ (67). Aminoglutethimide is believed to inhibit aromatase through the coordination of the arylamine to the P-450 heme-iron. This is supported by the spectral evidence that AG produces type II difference spectra characteristic of nitrogen coordination to the heme-iron. While the arylamine played an important role in aromatase inhibition, the piperidinedione ring appeared to have no specific role. On the other hand, it was thought to be responsible for side effects such as CNS depression and sedation (15).

Analogs of aminoglutethimide were synthesized to elucidate the structure activity relationship of the drug and in order to design inhibitors with higher inhibitory potency toward either aromatase or $P_{-450}^{SCC}$. Foster (68), showed that relocation of the p-amino functional group of AG to other positions resulted in either decrease or loss of inhibitory activity toward both enzymes. Thus the position of the amine functional group is important for enzyme inhibition. Foster (69) later was able to separate the aromatase inhibitory activity from $P_{-450}^{SCC}$ by substituting the aminophenyl group of AG with a pyridyl group 7. This analog is a strong competitive inhibitor of aromatase but is
noninhibitory toward P-450_{scc}. Vickery, in order to prove that the arylamine is the sole functionality necessary for the inhibition of aromatase, tested the compound 4-cyclohexylaniline 8, which lacked the piperidinedione as an inhibitor of aromatase (70). The compound exhibited a type II difference spectrum proving that the arylamine is the moiety responsible for aromatase inhibition. Other modifications included replacing the piperidinedione of AG with pyrrolidinedione 9. Compound 9 and its analogs were more selective in inhibiting aromatase than P450_{scc}, however, they were weaker inhibitors compared with AG toward aromatase inhibition (71).
Other nonsteroidal aromatase inhibitors include 7,8-benzoflavone \textsuperscript{10} (72) and miconazole \textsuperscript{11} (73). 7,8-Benzoflavone is a competitive inhibitor and produced a reverse type I difference spectrum (low spin). The exact nature of inhibition of aromatase is unknown; however, the low spin nature of the aromatase inhibitor complex suggests the presence of the sixth ligand to the heme-iron which could be contributed by an oxygen of the 7,8-benzoflavone (72). Miconazole is an antimycotic shown to be a potent inhibitor of aromatase (73). Under the assay conditions employed, miconazole was nearly seventy times more effective an inhibitor than aminogluthethimide. Miconazole produced a type II difference spectrum indicating the coordination of imidazole nitrogen with the heme-iron. Recently, Ciba-Geigy published the study of a nonsteroidal aromatase inhibitor containing an imidazole moiety (74). The inhibitor (CG16949A) \textsuperscript{12} had a marked activity against estrogen-dependent DMBA-induced carcinomas in intact female Sprague-Dawley rats with an ED\textsubscript{50} of about 0.1mg/kg.
1.3.2.2. Irreversible aromatase inhibitors

Irreversible aromatase inhibitors inactivate the enzyme by forming a covalent bond with a functional group on the enzyme. They can be divided into two categories: i) the active-site directed irreversible inhibitors and ii) the mechanism-based or $K_{cat}$ inhibitors.

Active-site directed irreversible inhibitors, also called affinity labels, are structural analogs of the substrate with a built-in reactive functional group. These inhibitors take advantage of their binding specificity and they subsequently inactivate the enzyme by forming a
covalent bond at or near the active site of the enzyme. In 1976, Bellino reported the synthesis of several potential active-site directed irreversible inhibitors of aromatase. Of all the inhibitors synthesized, 6α-bromoandrostenedione (13) and 7α-(3'-bromoacetoxypropyl)-androstenedione (75) displayed time-dependent inactivation of aromatase. Only 6α-Bromoandrostenedione underwent further extensive study since the latter compound was unstable when incubated with human placental microsome. 6α-Bromoandrostenedione also acted as a competitive inhibitor of androstenedione aromatization. In addition, it exhibited a type I cytochrome P450 binding spectrum, providing evidence that the inhibitor inactivates the enzyme at the active site. Later 6β-bromoandrostenedione was also shown to exhibit time-dependent inactivation when incubated with microsomal aromatase with dithiothreitol containing buffer (75).
Brueggemeier and Snider reported the synthesis of 7α-thio substituted compounds that contained alkylating moieties 14, 15, and 16 as active site directed irreversible inhibitors of aromatase (72, 76). Inhibitors 14 and 15 inactivated aromatase for a short time, but they were degraded by hydrolysis at longer incubation times. Inhibitor 16 produced a time-dependent, first-order inactivation of aromatase. The inactivation decreased when the substrate androstenedione was included in the incubation mixture. In addition, incubation of $^{14}$C labelled inhibitor with partially purified aromatase demonstrated that a protein with MW of 50-55 kD was covalently labelled by the inhibitor (76).
The second generation of irreversible inhibitors of aromatase received several terminology, including mechanism-based inhibitors, $K_{\text{cat}}$ inhibitors or suicide substrate (77, 78, 79). They are different from affinity labels in that a latent functional reactive group of the inhibitor has to be unmasked by the enzyme. Subsequently, the catalytic activity of the enzyme is destroyed through a covalent bond formation between the enzyme and the reactive functional group.

Mechanism-based inhibitors are analogs of $C_{19}$ androgens. The structural features responsible for the inactivation of aromatase are the presence of either $C_1-C_2$ double bond or the replacement of the $C_{19}$ methyl with various substituents on androstenedione or testosterone.

The first C-10 substituted analog of the natural substrate androstenedione, evaluated as a mechanism based inhibitor was 10-2'-propynyl-4-estrene-3,17-dione (PED) 17. It was synthesized independently by three different groups (80-82). The inhibitor caused a time-dependent inactivation of aromatase in the presence but not the absence of NADPH. Thus, PED required activation by the enzyme before inactivation occurred. The inhibitor bound to aromatase with an apparent $K_i$ of 23nM and $k_{\text{app}}$ of $1.11 \times 10^{-3}\text{sec}^{-1}$ (80).
Covey proposed the mechanism of inhibition of PED was through the formation of a Michael acceptor generated by the enzyme's normal mode of action (Figure 4, path a) in a similar manner as the substrate androstenedione. The propargylic alcohol 18 and propargylic ketone 19 were synthesized and both showed time-dependent inactivation of aromatase in the presence of NADPH (80). Inactivation was also observed for propargylic ketone in the absence of NADPH.

Metcalf proposed an alternate mechanism (Figure 4, path b) of inactivation (81). Instead of forming a Michael acceptor, the reactive functional group generated by the enzyme was suggested to be an oxirene species which involved oxygen insertion into the carbon-carbon triple bond. The oxirene species could bind to the prosthetic heme via its α-keto carbene analog. The proposed mechanism was based on the fact that the rate of inactivation by propargylic alcohol and propargylic ketone reported by Covey had a 1000-fold difference. In addition, there was no isotope effect on the rate of inactivation induced by 19,19-dideuterio-PED.
Figure 4. Proposed mechanisms of inactivation of aromatase by PED.
Figure 4.
Marcotte et al synthesized both the 19,19-difluoro-4-androstene-3,17-dione 20 and 19-fluoro-4-androstene-3,17-dione 21 as mechanism-based inhibitors of aromatase (83, 84). 19,19-Difluoro-4-androstene-3,17-dione was found to cause time-dependent inactivation of aromatase in the presence of NADPH, while 19-fluoro-4-androstene-3,17-dione, did not cause time-dependent inactivation, but rather was aromatized by the enzyme to estrone. The mechanism of inactivation by 20 proposed by the authors was via formation of an acyl fluoride by sequential 19-hydroxylation and subsequent dehydration. Authentic acyl fluoride was synthesized to prove the proposed mechanism; however, the result was inconclusive because of its intrinsic reactivity.

Some C_{19} thiol-substituted androstenediones have also been synthesized as mechanism-based inhibitors. Flynn et al reported the synthesis of 17β-hydroxy-10-methylthio-1,4-estradien-3-one 22 (85). The inhibitor exhibited two time-dependent pathways of aromatase.
inactivation. An NADPH dependent pathway in which time dependent loss of enzyme activity was observed in the presence of NADPH. Simultaneously, a slower inactivation was also observed in the absence of NADPH. It was proposed that the mechanism of inactivation in NADPH dependent pathway was through the formation of sulfenyl ester while direct active-site alkylation involving the methythio group occurred in time-dependent inactivation in the absence of NADPH.
Bednarski et al synthesized 17β-hydroxy-10β-mercapto-4-estren-3-one 23 and 19-mercaptoandrost-4-ene-3,17-dione 24 as mechanism-based inhibitors of aromatase (86). Both inhibitors exhibited time-dependent inactivation of aromatase in the presence of NADPH. No loss of enzyme activity was seen in the absence of NADPH.

\[ \text{OH} \]

\[
\text{SH} \quad \text{SH}_2C
\]

1,4,6-androstatriene-3,17-dione (ATD) 25, 4-hydroxyandrostenedione (4-OH-A) 26 and \( \Delta^1 \)-testololactone 27. Initially, they were thought to be competitive inhibitors of aromatase. Later, they were found to cause a slow time-dependent loss of aromatase activity in both placental and ovarian microsome in the presence of NADPH (87-89). There was no loss of enzyme activity without the addition of cofactors.
The other mechanism-based inhibitors of aromatase containing C₁-C₂ double bond are 1-methyl-1,4-androstadiene-3,17-dione (SH 489) \textsuperscript{28} \cite{90} and 7α-(p-amino)-1,4-androstatriene-3,17-dione (7α-APTADD) \textsuperscript{29} \cite{91}. Both compounds are potent mechanism-based inhibitors of aromatase producing a rapid and irreversible inactivation of aromatase.
Recently, irreversible inhibitors were synthesized which were neither an affinity label nor mechanism-based inhibitor. The inhibitors, containing a hydroperoxy group, were believed to achieve inactivation through oxidation. Tan et al synthesized both the 6α- and 6β-hydroperoxyandrostenedione 30a and 30b (92, 93). Both inhibitors caused a time-dependent inactivation of aromatase. The inactivation was not dependent on the presence of NADPH. Both the substrate, androstenedione, and an antioxidant, dithiothreitol, protected the enzyme from inactivation. On the other hand, p-hydroxymercuribenzoate decreased the protective effect. It was suggested that the inhibitors inactivated the enzyme through oxidation of a cysteine residue at or near the active site to cause an irreversible, chemical or conformational change of the enzyme.

Covey synthesized 10β-hydroperoxy-4-estrene-3,17-dione 31 as an inactivator of aromatase (94). In the presence of NADPH, the inhibitor was only a competitive inhibitor. The inactivation could be partially reversed by addition of dithiothreitol. Therefore, it was suggested that the cause of inactivation was through the oxidation of sulfhydryl group to sulfenic acid at the active site.
\[30a \quad \alpha - OOH\]

\[30b \quad \beta - OOH\]
CHAPTER II

EFFECT OF AN AROMASE INHIBITOR,

\( \gamma\alpha-(4'-\text{AMINO})-\text{PHENYLTHIO-4-ANDROSTENE-3,17-DIONE, } \gamma\alpha-\text{APTA,} \)

ON DMBA INDUCED RAT MAMMARY TUMORS.

2.1 Introduction

2.1.1. Estrogen and cancer

Breast and endometrial cancers are the two most common cancers found in females. The role of estrogens in these cancers still remains uncertain. However, the ratio of the incidence of breast cancer between male and female is the ratio of 1:100 and leads scientists to postulate that estrogens may participate in the etiology of the disease.

Breast cancer is the most common malignancy in women. In the United States 1 out of every 11 women is likely to develop this disease, and 25% of female cancer patients will have breast cancer (95). Approximately 114,000 new cases are diagnosed and 37,000 deaths caused annually by this neoplasm. The risk factors for breast cancer include high total fat, high saturated fat intake (96) and obesity (97). In addition, hormonal regulation in the breast is also important in the development of the disease, e.g., early menarche and late menopause are associated with increased incidence of breast cancer (98).
Approximately one third of the cases of breast cancer in women are hormone dependent (99) and the lesions regress upon endocrine treatment. Jensen and coworkers (99, 100) were the first to demonstrate that estrogen dependent tissues contained estrogen receptors and that this component was present in some cases of breast cancer. They also showed that there was a clear relationship between the presence or absence of estrogen receptors in breast cancer tissues and the likelihood of patients to respond to endocrine therapy.

Efforts were undertaken to demonstrate whether estrogens were directly tumorigenic. Unfortunately, these efforts were mostly without reward. However, experimental evidence suggested that estrogens might played a role as tumor promotors following initiation by agents such as chemical carcinogens or oncogenic viruses. Sirbasku proposed that estrogens stimulated neoplastic growth through autocrine, paracrine or endocrine mechanisms by producing growth factors (100).

In premenopausal women, the major source of estrogens is the ovary. Androstenedione and testosterone are converted to estrone and estradiol via the enzyme aromatase. After menopause, the ovaries cease to function and the adrenal gland becomes the major source of circulating steroids. The adrenal gland produces estrogen an estrogen precursor, androstenedione, which is then aromatized to estrone in peripheral tissue. Extraglandular aromatase is present in adipose tissue, muscle, liver, and brain (101, 102, 103). Recent data suggested that estrogens
might also be formed in breast cancer cells, either from androstenedione via aromatase pathway, or from estrone sulfate via estrone sulfatase pathway (104-107).

The initial treatments of estrogen-dependent breast cancer concentrated on removal of estrogen sources involving ovariectomy in premenopausal women or adrenalectomy/hypophysectomy in postmenopausal women. The response rate was usually 30%, but the rate was higher if tumor cells were estrogen receptor positive. Other types of treatment involved interfering with estrogen production by using androgen or antagonizing estrogen action by using progesterone (108, 109). Recently, tamoxifen is being used to block estrogen receptor. Even after adrenalectomy and hypophysectomy, estrogens were still produced in significant amounts in breast cancer patients (110). Blockade of estrogen production with aromatase inhibitors should be an effective therapeutic regimen.

2.1.2. Clinical studies of aromatase inhibitors in breast cancer patients

Δ1-Testololactone had been used for over 20 years for the treatment of breast cancer. It was thought to be an active intermediate of testosterone metabolism responsible for producing objective regression in advance breast cancer. However, recently the compound was found to be an aromatase inhibitor which could also slowly inactivate the enzyme (111). A multiinstitutional study on Δ1-testololactone was undertaken
in Europe in 362 women with advance breast cancer (112). The maximum response rate was 15% in patients given 1000 mg/day orally with minimal toxicity. Aminogluthethimide has been the other drug used for treatment of advance breast cancer since 1967. Its effectiveness is equal to that of surgical adrenalectomy with a response rate of about 30%. The standard dose of aminogluthethimide for advance breast cancer patients is 1.0 g/day with hydrocortisone 20-40 mg/day replacement therapy. However, because of the toxicities e.g, nausea, drowsiness and ataxia, 5% of patients cannot tolerate the full dose. A lower dose (125 mg twice daily plus hydrocortisone 20 mg twice daily) was used in these cases and found to have lower CNS toxicities.

Because of the low potency of α1-testololactone and the CNS toxicities of aminogluthethimide, an aromatase inhibitor with high potency and less side effects was desired. 4-Hydroxyandrostenedione (4-OH-A), with its high potency in vitro and ease of synthesis, was chosen for clinical trial in England (54, 55). In the latest phase II study, patients were given 500-1000 mg intramuscularly, 14 out of 52 patients responded (27%), and in 10 (19%) the disease stabilized. The sites of responses were mostly in soft tissue and lymph nodes. Of the 14 patients responding, 10 remained in remission between 2 to 18 months.
2.1.3. Antitumor activity of aromatase inhibitors in estrogen dependent rat tumors.

Administration of the carcinogen 7,12-dimethylbenzanthracene (DMBA) to female rats of 50-55 days old results in the formation of estrogen-dependent mammary tumors (113). This tumor model has been used extensively to study estrogen-dependent mammary tumors. The in vivo effects of 4-OH-A, 4-acetoxy-A and ATD were studied by using this tumor model (112-115). Treatment of tumored rats with 4-OH-A, 4-acetoxy-A, or ATD as daily injection (50mg/kg/day) caused marked tumor regression. Tumor volume decreased by 80% after 4 weeks of treatment. Tumor regression could be maintained by once a week injection for up to 20 weeks. This effect might be due to their property of inactivation of aromatase. The blood estradiol level decreased by about 70% compared with the controls. Furthermore, co-administration of estradiol (0.3 µg/kg) with aromatase inhibitors caused significant increase in tumor volume. In addition, 4-OH-A consistently suppressed LH and FSH secretion in both ovariectomized rats and tumor rats. In summary, the mechanism of 4-OH-A and related aromatase inhibitors are through inhibition of estrogen production, and these compounds act at the pituitary level by suppression of gonadotropins (FSH and LH) secretion.

2.2. Statement of objectives

Aromatase inhibitors have high potential in treatment of estrogen-dependent conditions such as breast cancer has been described.
Potential inhibitors should have high affinity and specificity for the enzyme; thus, therapeutic response will be achieved with low dose of inhibitors. The therapeutic efficacies of aromatase inhibitors such as 4-hydroxyandrostenedione (4-OH-A) and aminogluthethimide (AG) are currently in clinical trial and have been shown to cause regression of hormone-dependent breast tumors in rats (114-116) and humans (54, 55, 117, 118).

Several 7α-thiosubstituted androstenediones were prepared in our laboratory and were found to be potential aromatase inhibitors (71-73, 76, 119), with 7α-(4'-amino)phenylthio-4-androstene-3,17-dione (7α-APTA) being one of the most potent competitive inhibitor with an apparent $K_i$ of 18nM. Inhibitory activity studies by Abul-Hajj (120, 121) in microsomes from mammary carcinoma showed that 7α-APTA was the best inhibitor with a 10-fold lower $K_i$ than aminogluthethimide (AG) and 4-hydroxyandrostenedione (4-OH-A). Because of its high affinity for the enzyme aromatase, 7α-APTA is a strong candidate for further in vivo study.

DMBA induced estrogen-dependent mammary tumors has been one of the primary animal tumor models for studies of the biology and therapy of hormone dependent tumors (113). The aromatase inhibitor 7α-APTA is examined for its effectiveness in reducing the number and size of the DMBA induced mammary tumor in rats.
2.3 Experimental

2.3.1 Chemicals.

Steroids were obtained from Steraloids (Wilton, NH) and checked for purity by melting point and thin layer chromatography. 7α-(4'-Amino)phenylthio-4-androstene-3,17-dione (7α-APTA) was prepared following the procedure of Brueggemeier et al. (71).

7,12-Dimethylbenz(a)anthracene was purchased from Aldrich Chemical Co., Milwaukee, WI. Estradiol radioimmunoassays were performed by the Department of Obstetrics and Gynecology, College of Medicine, Ohio State University. Silastic tubing (Silastic 0.062 in. ID x 0.125 in. OD) was purchased from Dow Corning Corporation, Midland, MI.

2.3.2 Animals.

Female Sprague-Dawley rats (50 - 60 days old) were purchased from Harlan Industries, Inc., Cumberland, IN. Animals were housed in metal cages containing ground corn cob (Anderson's, Maumee, OH), provided Purina Lab Chow and water ad libitum, and maintained in an AAALAC-accredited animal facility with a 12 hour alternating light/dark cycle.

2.3.3 Induction of tumors

Female rats (Sprague-Dawley, 50 days old) are gavaged with 20 mg of DMBA in 2 ml of sesame oil per rat (113). Each week the animals were examined for the appearance of tumors and any tumors present measured with calipers. Rats were selected for the study when at least one tumor
has a diameter of 2 cm., which was about 4 months after the administration of DMBA. The tumor volume is calculated using the equation \( v = \frac{4}{3}\pi r_1^2 r_2 \), where \( r_1 \) is the minor radius.

2.3.4. Treatment with Aromatase Inhibitor.

The rats were divided into groups consisting of 7 animals with approximately the same number of tumors per rat and the same tumor volume per rat. 7α-(4'-Amino)phenylthio-4-androstene-3,17-dione (7α-APTA) was examined at a dose of 25 and 50 mg/kg rat/day. The compound was dissolved in sesame oil (0.5 ml/injection) and each rat was injected subcutaneously daily. The rats were weighed and the number and volume of the tumors present determined twice a week. Animals were treated over a six week period. Another group of tumored rats received 4,6-androstadienedione (4,6-ADD) at a dose of 50 mg/kg rat/day. Rats in the control group received only sesame oil (0.5 ml daily).

2.3.5 Co-Treatment with Aromatase Inhibitor and Estradiol.

7α-APTA (50 mg/kg) was dissolved in sesame oil (0.5 ml/injection) and each rat was injected subcutaneously daily for three weeks. Beginning at week four, 7α-APTA (50 mg/kg) and estradiol (0.3 µg/kg) were dissolved in sesame oil (0.5 ml/injection) and each rat was injected subcutaneously daily for three more weeks. The rats were weighed and the number and volume of the tumors present determined twice a week.
2.3.6 Treatment with Aromatase Inhibitor in Silastic Implants.

Each rat was implanted with 2 silastic tubings (3 cm in length each) with 50 mg of 7α-APTA per tubing inserted under the dorsal skin. The implants were replaced once a week. The rats were weighed twice a week and the number and volume of the tumors present determined over a six week period. Used implants were excised and the amount of 7α-APTA left was weighed.

2.4. RESULTS & DISCUSSION

The initial 7α-substituted C₁₉ steroidal aromatase inhibitor examined was 7α-(4'-amino)phenylthio-4-androstene-3,17-dione (7α-APTA). This competitive inhibitor was dissolved in sesame oil and each rat was injected subcutaneously daily for six weeks at dosages of 25 or 50 mg per kg per day over a six week period. The rats were weighed and the number and volume of the tumors present determined twice a week. Rats in the control group received only sesame oil (0.5 ml daily).

The tumors of the control group grew steadily during the study, reaching an increase in total tumor volume of approximately 550% of the original volume (Figure 5). On the other hand, the 7α-APTA treated groups demonstrated a reduction in tumor volumes during the first week (Figure 5). Furthermore, tumor volumes continued to decrease to less than 20% of the original volumes (80% reduction) during the last two
weeks of the treatment with 50 mg per kg per day. The group receiving 25 mg per kg per day responded with approximately a 50% reduction in tumor volume by the second week of treatment and maintained a 30% to 40% reduction of total tumor volume throughout the rest of the six-week study. Thus, effective reduction of tumor volume was observed with 7α-APTA at doses of 25 and 50 mg/kg/day (Figure 5 and Table I). Approximately 80% of tumors responded either completely or partially to 7α-APTA at the two doses examined.

Tumored animals receiving 4,6-ADD at 50 mg/kg/day demonstrated only a weak response of 10% reduction of tumor volume during the first two weeks of treatment (Figure 5). The tumors slowly began to increase in tumor volume at three weeks of treatment and were approximately 50% larger at the end of the six-week study. Thus, 4,6-ADD, a possible degradative product of 7α-APTA via a retro-Michael reaction, is much less effective in reducing tumor volumes. These results suggest that the effects of 7α-APTA on tumor reduction is not due to degradation of the inhibitor to 4,6-ADD.

Since 7α-APTA was effective at a dose of 50 mg/kg rat/day, experiments were performed to determine if this tumor reduction is due to inhibition of estrogen biosynthesis. Tumor-bearing rats were administered only 7α-APTA at 50 mg per kg per day for the first three weeks, followed by co-administration of 7α-APTA at 50 mg per kg per day and estradiol at 0.3 µg per kg per day for the last three weeks. Again,
the tumors responded to 7α-APTA treatment with tumor reduction of 70% of the original volume during the first three weeks. Beginning at week 3, the co-administration of estradiol resulted in tumor growth (Figure 6). Thus, the reversal of tumor reduction was observed in 10 out of 12 tumors, resulting in 6 tumors having a greater volume than their original volumes at the end of the treatment period. Interestingly, 2 tumors that completely regressed during the first three weeks did not reappear upon estradiol co-administration.

Silicone rubber in the form of silastic tubing has been proven to be useful in chronic implants because it does not cause foreign body reaction even after prolonged period (122, 123). In addition, certain steroids were found to pass through the capsule wall at a constant rate (124). Capsules of such material might provide a means of chronic administration of constant rate for a long period of time. The implants were used as sustain release dosage forms for 7α-APTA. Each rat was implanted with two pieces of silastic tubing with 50 mg of 7α-APTA per tubing inserted under the dorsal skin. The implants were replaced once a week over a six week period. The rats were weighed and the number and volume of the tumors present determined. Used implants were excised and the amount of 7α-APTA remaining was weighed.

There was no change in tumor volume in the group receiving 7α-APTA in silastic implants during the first week. However, from the beginning of week two to the end of week four, the tumor volumes increased.
approximately 300%. The tumor volume decreased during the fifth week (Figure 7).

The amount of \(7\alpha\)-APTA released was examined (Figure 8). The rate of release of \(7\alpha\)-APTA was the highest during the first week (4 mg/rat/day). From week two to four, the amount of release decreased significantly which corresponded to the increase in tumor volume. The amount of steroid released increase during the fifth week (3.5 mg/rat/day) after the implants were imbedded at different site. It was believed that scar tissue formed around the implant site hindered the release of steroid.

A summary of the results are presented in Table I. Tumor response to \(7\alpha\)-APTA was classified as to the number of tumors that have completely regressed, tumors that have regressed to less than 0.02 cc, tumors that have regressed to one-half original volume, tumors that have regressed less than one-half original volume, and tumors that have grown. The overall percentage of tumors responding completely or partially to \(7\alpha\)-APTA at both the 25 and 50 mg per kg per day doses was 75% to 80% (9 out of 12 and 8 out of 10, respectively). Co-administration of estradiol and \(7\alpha\)-APTA resulted at the end of the six-week study with 50% of the tumors increasing in size (6 out of 12). In the group receiving silastic implants, 7 out of 9 tumors grew. In the control group, 9 out of 10 tumors (90%) grew.

Finally, the plasma estradiol concentrations were determined at the end of the six-week study in the treated animals. The results are shown
in Figure 9. 7α-APTA dramatically lowered plasma estradiol concentrations at both the 25 and 50 mg/kg/day doses to concentrations of 981 pg/ml and 777 pg/ml, respectively. Elevated plasma estradiol concentrations were observed in the animals receiving the co-administration of estradiol with 7α-APTA (1446 pg/ml) and in animals receiving 4,6-ADD (1634 pg/ml). After 5 weeks, the surviving control animals that received only vehicle had large tumors, diminished weight, and widely varied plasma estradiol levels ranging from 640 pg/ml to 2800 pg/ml. Normal female adult rats have plasma estradiol levels of approximately 1400 pg/ml (114).
Table 1. Effects of 7-α-APTA on Rat Mammary Tumors.
Table 1.

<table>
<thead>
<tr>
<th>Tumors</th>
<th>25 mg/kg/d</th>
<th>50 mg/kg/d</th>
<th>50 mg/kg/d + E₂ (0.3 μg/kg/d)*</th>
<th>Silastic implants</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>completely regressed</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>regressed to less than 0.02 cc</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>regressed greater than 1/2 vol.</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>regressed less than 1/2 vol.</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>have grown</td>
<td>3</td>
<td>2</td>
<td>6</td>
<td>7</td>
<td>9</td>
</tr>
</tbody>
</table>

* Estradiol (0.3 μg/kg/day) was co-injected with 7α-APTA beginning at Week 3.
Figure 5. Effects of 7α-APTA and 4,6-ADD on Tumor Regression. Tumor-bearing rats were treated subcutaneously with 7α-APTA at 25 mg/kg/day — (no. of rats = 6, no. of tumors = 12); 7α-APTA at 50 mg/kg/day — (no. of rats = 6, no. of tumors = 10) or 4,6-ADD at 50 mg/kg/day — (no. of rats = 5, no. of tumors = 7) in sesame oil suspension. Control group of rats — (no. of rats = 4, no. of tumors = 10) received only vehicle.
Change in Tumor Size
(n = 10–12 tumors)

Figure 5.
Figure 6. Effects of Co-Administration of Estradiol and 7α-APTA on Tumor Regression. Tumor bearing rats were treated subcutaneously with 7α-APTA at 50 mg/kg/day—●— (no. of rats = 6, no. of tumors = 10) in sesame oil suspension for three weeks. The rats then received both 7α-APTA at 50 mg/kg/day and estradiol at 0.3 μg/kg/day—●— (no. of rats = 6, no. of tumors = 12) from week 3 through 6. Control group of rats —▲— (no. of rats = 4 and no. of tumors = 10) received only vehicle.
Figure 6.

Change in Tumor Size
(n = 10–12 tumors)
Figure 7. Effects of 7α-APTA in Silastic Implants on Tumor Regression. Tumor-bearing rats were implanted with 2 silastic tubings with 50 mg of 7α-APTA per tubing — ■ — (no. of rats = 6, no. of tumors = 9). The tubings were replaced weekly. The results were compared with rats receiving daily injection of 7α-APTA 50 mg/kg/day + (no. of rats = 6 and no. of tumors = 10). Rats in control group received only sesame oil — ◊ — no. of rats = 4, no. of tumors = 10).
Change in Tumor Size

Figure 7.
Figure 8. Rate of Release of 7α-APTA from Silastic Implants. The silastic tubings were replaced weekly and the amount released were measured, week 1 ( □ ), week 2 ( ■ ), week 3 ( □ ), week 4 ( ■ ) and week 5 ( ■ ).

* silastic was implanted at different site.
Figure 8.

Amount of 7-APTA (in mg/week)
Figure 9. Plasma Estradiol Concentrations in Tumored Rats. At the end of the six-week treatment, plasma samples were obtained from the tumor-bearing rats and estradiol concentrations were determined by radioimmunoassays. Plasma estradiol levels were determined in rats treated subcutaneously with 7α-APTA at 50 mg/kg/day (A), 7α-APTA at 25 mg/kg/day (B), 4,6-ADD at 50 mg/kg/day (C), or 7α-APTA at 50 mg/kg/day for the first three weeks, followed by both 7α-APTA at 50 mg/kg/day and estradiol at 0.3 μg/kg/day for the last three weeks (D).
estradiol (pg/ml)

Figure 9.
CHAPTER III

SYNTHESIS AND BIOCHEMICAL STUDIES OF 7-SUBSTITUTED
4-6-ANDROSTADIENE-3,17-DIONE AND 1,4,6-ANDROSTATRIENE-3,17-DIONE

3.1. Introduction

Aromatase inhibitors have been proven to have therapeutic usefulness such as treating estrogen-dependent breast cancer in post-menopausal women.

Schwarzel et al studied the structural features of over 100 steroids as inhibitors of aromatase (68). The most effective inhibitors sterically and electronically resemble the substrate 4-androstene-3,17-dione. Two of the most effective inhibitors were androgens containing Δ⁴,⁶-3-one or Δ¹,⁴,⁶-3-one moieties. Concerning the structure-activity relationship of the D-ring of steroids, the C-17 keto group was the most effective. Thus, 4,6-androstadiene-3,17-dione (4,6-ADD) and 1,4,6-androstatriene-3,17-dione (1,4,6-ATD) were effective inhibitors.

Previous work from our laboratory has illustrated that several 7α-thio substituted derivatives of androstenedione were effective inhibitors of aromatase (71-73, 76). Among the compounds synthesized, 7α-(4'-amino)phenylthio-4-androstene-3,17-dione (7α-APTA) was found to
be the most potent inhibitor with an apparent $K_i$ of 18 nM. In the initial study, the inhibitory activity of the inhibitors progressively decreased when the p-amino substituent was replaced with p-methoxy or hydrogen. It was postulated that increasing the electron-donating capacity of the para substituents might increase the potency of the inhibitors (71). However, further examination of the effect of electron-donating and electron-withdrawing ring substituents upon inhibitory activity toward aromatase concluded that there was no linear correlation between the electronic character of the substituents and the inhibitory activity since inhibitors with electron-withdrawing groups (Cl, Br, I) are as potent as $7\alpha$-APTA (73).

Solo and coworkers synthesized some $7\alpha$-alkyltestosterone derivatives as inhibitors of aromatase (74). The most active inhibitors were found to be the acetate and bromoacetate of $7\alpha$-(3'-hydroxypropyl)-4-androstene-3,17-dione which further confirmed that the enzyme aromatase could tolerate significant bulk at the $7\alpha$-position of 4-androstene-3,17-dione. In addition, the affinity of the inhibitors did not depend on the hydrophilicity of the side chain since the $7\alpha$-hydroxypropyl and the isosteric $7\alpha$-butyl derivative of testosterone have similar affinity for the enzyme.
3.2. Statement of objectives

Inhibitors with a high degree of specificity and high affinity for the enzyme would produce therapeutic response at low doses.

The $\Delta^4$-ene-3-one and C-17 keto group of the substrate 4-androstene-3,17-dione provide two points of attachment to the enzyme aromatase. Effective inhibitors of aromatase (4,6-ADD) and (1,4,6-ATD) with extended linear conjugation in the A and/or B ring retain the two points of attachment to the enzyme.

Aromatase inhibitors, which are derivatives of the substrate androstenedione containing 7α-thio substituents, have high affinity for the enzyme. It was postulated that the 7α-thio substituent provided a third point of attachment to the enzyme. Inhibitors with 7α-aryl thio-substituents are more potent than those with 7α-alkyl substituents. However, the 7α-thioether linkage is susceptible to metabolic cleavage when incubated with placental microsome (125). In addition, as in the case of spironolactone, the 7α-thioester could undergo retro-Michael elimination to form a C₆ double bond or oxidation to give sulfoxide or oxygenated sulfur metabolite (126,127). Inhibitors containing 7-aryl substituents with increased metabolic stability are desired.

Androstenedione derivatives with extended linear conjugation in ring A and/or B result in effective inhibitors. In addition, 7α-substituted androstenedione have high affinity to aromatase. Thus, compounds with both features should be potent inhibitors. New proposed inhibitors are
shown in figure 10 and the synthetic scheme of 7-substituted 4,6-androstadiene-3,17-diones and 1,4,6-androstatriene-3,17-diones is shown in figure 11.

In the synthesis of 7-substituted 4,6-androstadiene-3,17-diones and 1,4,6-androstatriene-3,17-diones, the known compound 32 was converted to the key intermediate 3,3,17,17-bisethylenedioxy-5-androstene-7-one 33, with tert-butyl hydroperoxide (t-BuOOH) and chromium hexacarbonyl Cr(CO)₆ as catalyst. Compound 33 was then reacted with either Grignard or organolithium reagents followed by dehydration to obtain the 7-substituted 4,6-androstadiene-3,17-diones 34, 36 and 38. The 7-substituted 1,4,6-androstatriene-3,17-diones 35, 37 and 39 were obtained by oxidation of the corresponding 7-substituted 4,6-androstadiene-3,17-diones with DDQ. Para-substitution with a nitro group of 36 and 38 was achieved by reacting with nitric and sulfuric acids to obtain 40 and 42. The nitro analogs were then reduced to the amines 41 and 43 by using stannous chloride and hydrochloric acid.
Figure 10. New proposed aromatase inhibitors.
Figure 10.
Figure 11. Synthetic scheme of 7-substituted 4,6-androstadiene-3,17-dione and 1,4,6-androstatriene-3,17-dione.
Figure 11.
3.3. Experimental

3.3.1. Chemistry

Steroids were purchase from Searle Laboratories (Skokie, IL) or Steraloids (Wilton, NH) and checked for purity by thin layer chromatography or melting point. Chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and solvents were obtained from campus. Dioxane was dried with calcium hydride and distilled from sodium. Silica gel was purchased from E. Merck (Darmstadt, Germany) and aluminum oxide (basic) from Fischer Scientific (Fair Lawn, NJ). TLC plates were purchased from Analtech Inc. (Newark, NE). Biochemicals were obtained from Sigma Chemical Co. [1β-3H]4-Androstene-3,17-dione was purchased from New England Nuclear (Boston, MA). Melting points were obtained on a Thomas-Hoover capillary melting point apparatus and were uncorrected. IR spectral data was recorded on a Beckman IR 4230 spectrophotometer. NMR spectra were obtained with either a Bruker HX-90E NMR spectrometer (90 MHz), a Bruker WP-80DS NMR spectrometer (80 MHz) or an IBM AF/250 spectrometer in the pulse mode. Mass spectra were obtained at The Ohio State University Chemical Instrumentation Center using a Kratos MS-30 mass spectrometer. Elemental analyses were performed by Galbraith Lab. Inc., Knoxville, TN. Centrifugation was performed on a Sorvall RC2-B centrifuge and a Beckman L5-50B ultracentrifuge was used for ultracentrifugation. Radioactive samples were detected with a Beckman LS 6800 scintillation counter using Formula 963 (New England Nuclear) as the counting solution.
A mixture of 4-androstene-3,17-dione (20 g, 70 mmol), ethylene glycol (100 ml), p-toluenesulfonic acid (0.5 g) and benzene (400 ml) was heated under reflux for 48 h in a 1 L round bottom flask equipped with a Dean-Stark trap. Saturated sodium bicarbonate solution (50 ml) was added to the cooled mixture and the benzene layer was separated, washed with water (2x100 ml), dried (Na₂SO₄) and concentrated to dryness under reduced pressure. Recrystallization from ethanol gave pure 32 (18.1 g, 70.3%). mp 169-170°C (lit: 171-173.5°C). IR (KBr) 2940, 1440, 1380, 1310, 1100 cm⁻¹; ¹H NMR δ 0.88 (s, 3H, C₁₈), 1.05 (s, 3H, C₁₉), 3.82-3.96 (m, 8H, -OCH₂-CH₂O⁻), 5.27-5.41 (m, 1H, 6-vinyl).
To a solution of 3,3,17,17-bisethylenedioxy-5-androstene 32 (1 g, 2.7 mmol) in acetonitrile (CH$_3$CN) (30 ml) were added t-butylhydroperoxide (t-BuOOH) (1 ml, 9.23 mmol) and chromium hexacarbonyl (Cr(CO)$_6$) (0.135 g, 0.16 mmol). The mixture was refluxed for 24 h and then cooled to room temperature. Water (20 ml) was added, and the product extracted with ethyl acetate. The ethyl acetate layer was dried (Na$_2$SO$_4$) and concentrated to dryness under reduced pressure to give a yellowish solid (0.89 g) which was purified by column chromatography using silica gel. Elution with ethyl acetate/hexane and recrystallization with ethanol/water obtained a slight yellowish solid. mp 206-207°C. IR (KBr) 3030, 2950, 2890, 1670, 1295, 1100 cm$^{-1}$; $^1$H NMR $\delta$ 0.88 (s, 3H, C$_{18}$), 1.22 (s, 3H, C$_{19}$), 3.75-4.05 (m, 8H, -OCH$_2$-CH$_2$O-), 5.65 (d, 1H, vinyl); ms m/e (rel. intensity) 388 (M$^+$, 0.040), 373 (0.094), 100 (0.363), 99 (1.00), 86 (0.116). Analysis. Calculated for C$_{23}$H$_{32}$O$_5$$*_{0.25}$H$_2$O. C, 70.29; H, 8.34. Found C, 70.26; H, 8.46.
To a solution of 3,3,17,17-bisethylenedioxy-5-androstene-7-cne 33 (2.0 g, 7.1 mmol) in THF (50 ml) was added phenylmagnesium chloride (0.5 M, 30 ml, 15 mmol) dropwise in 30 min under argon. The solution was allowed to stir at room temperature for 2 h and then poured into saturated aq NH₄Cl solution and extracted with ether (2x100 ml). The ether layer was washed with saturated NaCl solution, dried (Na₂SO₄) and solvent removed to furnish brown gummy crystals (2.2 g). The crystals were dissolved in dioxane (30 ml) containing 5% H₂SO₄ (5 ml) and the resulting solution was stirred at room temperature for 24 h. The solution was then neutralized with saturated NaHCO₃ and extracted with CHCl₃. The CHCl₃ layer was separated, dried (Na₂SO₄) and solvent evaporated to give a yellowish solid (1 g). Recrystallization from hexane yield yellowish crystals 33 (0.8 g, 42.5%). mp 187-188°C. IR (KBr) 3030, 2960, 2930, 2850, 1735, 1640, 1605, 1365, 1230, 770 cm⁻¹; ¹H NMR δ 1.02 (s, 3H, C₁₈), 1.21 (s, 3H, C₁₉), 5.77 (s, 1H, C₄), 6.18 (d,
7-Phenyl-4,6-androstadiene-3,17-dione 35

To a solution of 7-phenyl-4,6-androstadiene-3,17-dione 34 (0.4 g, 1.11 mmol) in benzene (20 ml) was added 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ, 0.302 g, 1.33 mmol). The mixture was heated under reflux for 12 h. The solution was then cooled to room temperature and solvent evaporated under reduced pressure to leave a dark green sludgy material. The residue was taken up in CHCl₃ and placed on a basic alumina column (5 g). Elution with CHCl₃ afforded 35 which was recrystallized from acetone/hexane to give pure 35 (0.21 g, 52.8%). mp 118-120°C IR (KBr) 3050, 2960, 2940, 2870, 1738, 1655, 1600, 1380 800 cm⁻¹; ¹H NMR δ 1.06 (s, 3H, C₁₈), 1.20 (s, 3H, C₁₉), 6.07 (s, 1H, C₄),
6.23-6.41 (m, 2H, C2 and C6), 7.22 (d, 1H, C1), 7.35-7.50 (m, 5H, aromatic); ms m/e (rel. intensity) 358 (M+, 1.00), 219 (0.152), 210 (0.467), 149 (0.127), 91 (0.100). Analysis. Calculated for C_{25}H_{26}O_{2} C, 83.76; H, 7.31. Found C, 83.92; H, 7.67.

7-Benzyl-4,6-androstdiene-3,17-dione 36

Compound 33 (10 g, 26 mmol) was treated with benzyl magnesium bromide in hexane (2.5 M, 50 ml, 125 mmol) in a similar manner as in 34. Compound 36 was isolated as yellowish solid (4.3 g, 44.6%). mp 80-83°C. IR (KBr) 3020, 2940, 2860, 1740, 1655, 1620, 1375, 750 cm^{-1}; \textsuperscript{1}H NMR \delta 0.92 (s, 3H, C_{18}), 1.13 (s, 3H, C_{19}), 5.43 (s, 1H, C_{4}), 5.89 (s, 1H, C_{6}), 7.15-7.40 (m, 5H, aromatic); ms m/e (rel. intensity) 374 (M+, 0.351), 283 (0.679), 265 (0.100), 173 (0.112), 131 (0.120), 106 (0.200), 91 (1.00). Analysis. Calculated for C_{26}H_{30}O_{2} C, 83.38; H, 8.07. Found C, 83.75; H, 8.39.
To compound 36 (1 g, 2.67 mmol) in benzene (30 ml) was added DDQ (1:2 g, 5.28 mmol). The mixture was treated in a similar manner as in 35. Compound 37 was isolated as yellowish crystals (0.49 g, 49%). mp 87-89°C. IR (KBr) ¹H NMR δ 0.95 (s, 3H, C₁₈), 1.16 (s, 3H, C₁₉), 3.64 s, 2H, benzyl), 5.93 (s, 1H, C₄), 6.04 (s, 1H, C₆); 6.23-6.28 (m, 1H, C₂) 7.03 (d, 1H, C₁), 7.17-7.37 (m, 5H, aromatic); ms m/e (rel. intensity) 372 (M⁺, 0.150), 281 (0.224), 253 (0.500), 219 (0.120), 171 (0.148), 131 (0.132), 106 (0.370), 91 (1.00). Analysis. Calculated for C₂₆H₂₈O₂ C, 83.8; H, 7.58. Found C, 83.8; H, 7.97.
Compound 33 (7 g, 18 mmol) was treated with phenethyl lithium synthesized by the procedure of Screttas (128) in THF (0.5 M, 50 ml, 25 mmol) with a similar manner as in 34. Compound 38 was isolated as yellowish crystals (1.8 g, 25.7%). mp 121-122°C. IR (KBr) 2980, 2875, 1740, 1645, 1615, 1370, 800 cm⁻¹; ¹H NMR δ 0.99 (s, 3H, C₁₈), 1.01 (s, 3H, C₁₉), 5.65 (s, 1H, C₄), 6.08 (s, 1H, C₆), 7.16-7.39 (m, 5H, aromatic); ms m/e (rel. intensity) 388 (M⁺, 0.053), 149 (0.161), 129 (0.173), 111 (0.102), 97 (0.131), 91 (0.532). Analysis. Calculated for C₂₇H₃₂O₂ C, 83.46; H, 8.30. Found C, 83.36; H, 8.34.
Compound 38 (1 g, 2.59 mmol) in benzene (30 ml) was added DDQ (1.2 g, 5.28 mmol). The mixture was treated with similar manner as in 35. Compound 39 was isolated as yellowish crystals (0.56 g, 56%). mp 174.5-176°C IR (KBr) 3020, 2980, 2930, 2870, 1740, 1645, 1615, 1370, 800 cm⁻¹; ¹H NMR δ 1.02 (s, 3H, C₁₈), 1.04 (s, 3H, C₁₉), 5.98 (s, 1H, C₄), 6.23-6.27 (m, 2H, C₂ and C₆), 7.04 (d, 1H, C₁), 7.18-7.38 (m, 5H, aromatic); MS m/e (rel. intensity) 386 (M⁺, 0.015), 185 (0.014), 171 (0.016), 147 (0.020), 115 (0.018), 105 (0.042), 91 (0.024). Analysis. Calculated for C₂₇H₃₀O₂ C, 83.90; H, 7.82. Found C, 83.89; H, 7.81.
7-(p-Nitrobenzyl)-4,6-androstadiene-3,17-dione 40

A 60% aqueous sulfuric acid solution (60 ml) was mixed with concentrated nitric acid (12 ml) at 0°C. 7-benzyl-4,6-androstadiene-3,17-dione 36 (1.29 g, 3.5 mmol) dissolved in acetic acid (5 ml) was added slowly over 20 min at 0°C and stirring was continued for 24h. The mixture was neutralized with saturated NaHCO₃ and extracted with ethyl acetate (EtOAc) (2x150 ml). The EtOAc layer was separated, washed with water (200 ml), dried (Na₂SO₄) and solvent evaporated to give an oil. Column chromatography with ethyl acetate/petroleum ether afforded the product 40 (0.9 g, 62.3%). mp 115-117.5°C. IR (KBr) 2970, 2860, 1740, 1660, 1620, 1600, 1370, 795 cm⁻¹; ¹H NMR δ 0.90 (s, 3H, C₁₈), 1.08 (s, 3H, C₁₉), 3.74 (d, 2H, benzyl), 5.59 (s, 1H, C₄), 5.86 (s, 1H, C₆), 7.28-8.20 (m, 4H, aromatic); ms m/e (rel. intensity) 419 (M⁺, 0.459), 389 (0.242), 283 (1.00), 271 (0.149), 106 (0.400). Analysis. Calculated for C₂₆H₂₉NΟ₄  C, 74.44; H, 6.97; N, 3.34. Found C, 74.37; H, 7.16; N, 3.03.
7-(p-Nitrophenethyl)-4,6-androstadiene-3,17-dione 41

A 60% aqueous sulfuric acid solution (30 ml) was mixed with concentrated nitric acid (6 ml) at 0°C. 7-phenethyl-4,6-androstadiene-3,17-dione 7 (0.6 g, 1.54 mmol), dissolved in acetic acid (5 ml) was added slowly over 20 min at 0°C and stirring was continued for 24 h. The mixture was treated with similar manner as in 40 to afford the product 41 (0.4 g, 59.7%). mp 130-131.5°C. IR (KBr) 2940, 2870, 1735, 1660, 1620, 1350, 805 cm⁻¹. ¹H NMR δ 1.01 (s, 3H, C₁₈), 1.02 (s, 3H, C₁₉), 5.66 (s, 1H, C₅), 6.08 (s, 1H, C₆), 7.27-8.19 (m, 4H, aromatic); ms m/e (rel. intensity) 433 (M⁺, 0.500), 416 (0.433), 398 (0.142), 297 (0.192), 285 (0.205), 269 (0.229), 106 (1.00), 91 (0.159). Analysis. Calculated for C₂₇H₃₁NO₄ C, 74.80; H, 7.21; N, 3.23. Found C, 74.5; H, 7.47; N, 3.06.
7-(p-Aminobenzyl)-4,6-androstadiene-3,17-dione 42 and stannous chloride dihydrate (1.4 g, 6.2 mmol) were dissolved in a solution of ethanol (10 ml) and concentrated hydrochloric acid (1.5 ml). The reaction mixture was stirred under reflux for 30 min, and was then allowed to cooled to room temperature. The mixture was poured into ice water (50 ml), and the solution was extracted with dichloromethane (2 x 30 ml). The dichloromethane solution was washed with saturated NaHCO₃ solution and water, dried (Na₂SO₄) and concentrated to an oil. The oil was chromatographed in silica column eluted with ethyl acetate/petroleum ether (3:1) to afford a yellowish solid which was recrystallized by ethyl acetate/hexane to yield yellowish crystals (0.09 g, 48.4 %). mp 147-149°C. IR (KBr) 3470, 3380, 3030, 2980, 2950, 1740, 1660, 1620, 1530, 1370 cm⁻¹. ¹H NMR δ 0.92 (s, 3H, C₁₈), 1.08 (s, 3H, C₁₉), 3.53 (s, 2H, benzyl), 3.6-3.8 (br, 2H, -NH₂), 5.59 (s, 1H, C₄), 5.92 (s, 1H, C₆), 6.64-6.93 (m, 4H, aromatic); ms m/e (rel. intensity) 389 (M⁺, 0.527),
82

Analysis. Calculated for C\textsubscript{26}H\textsubscript{31}NO\textsubscript{2} C, 80.17; H, 8.30; N, 3.60. Found C, 80.07; H, 8.01; N, 3.53.

7-(p-Aminophenethyl)-4,6-androstadiene-3,17-dione 43

![Chemical Structure](image)

7-(p-Nitrophenethyl)-4,6-androstadiene-3,17-dione 41 (0.2 g, 0.46 mmol) and stannous chloride (1.4 g, 6.2 mmol) were dissolved in a solution of ethanol (10 ml) and concentrated hydrochloric acid (1.5 ml). The reaction mixture was treated with the same manner as in 42 to yield a yellowish crystal (0.12g, 64.5%). mp 160-161.5°C. IR (KBr) 3450, 3360, 2980, 2950, 1740, 1645, 1620, 1530, 1380. \textsuperscript{1}H NMR δ 0.97 (s, 3H, C\textsubscript{18}), 1.00 (s, 3H, C\textsubscript{19}), 3.6 (s, 2H, -NH\textsubscript{2}), 5.62 (s, 1H, C\textsubscript{4}), 6.04 (s, 1H, C\textsubscript{6}), 6.63-6.98 (m, 4H, aromatic); ms m/e (rel. intensity) 403 (M\textsuperscript{+}, 0.082), 279 (0.017), 252 (0.019), 106 (1.00), 91 (0.107). Analysis. Calculated for C\textsubscript{27}H\textsubscript{33}NO\textsubscript{2} C, 80.36; H, 8.24; N, 3.47. Found C, 79.97; H, 8.29; N, 3.25.
3.3.2. Biochemical methods

Placental microsome preparation

Human placenta were obtained immediately upon delivery at the Ohio State University Hospital and stored on ice during transportation to the laboratory. The preparation of microsomes was performed according to the method of Ryan (13). All procedures were carried out at 0-4°C. The placenta was cut free of connective tissue and large blood vessels with scissors. The tissue was then homogenized in a cold Waring blender with two parts of tissues to one part of homogenization buffer. The buffer, pH 7, contained 0.05 M sodium phosphate, 0.25 M sucrose and 0.04 M nicotinamide. The homogenate was centrifuged at 10,000×g for 30 min. The debris was discarded and the supernatant centrifuged at 105,000×g for 1 h. The microsomal pellet obtained was resuspended in 0.1 M sodium phosphate buffer, pH 7 and centrifuged at 105,000×g for 1 h. The procedure was repeated once again and the resulting pellet was stored at -70°C until needed.

Aromatase activity in human placental microsomes was assayed by radiometric method developed by Siiteri (112) in which the tritium from [1β-3H]4-androstene-3,17-dione was released as 3H2O and used as an index of estrogen formation.

Competitive inhibition studies

The procedure is similar to that of Brueggemeier et al. (60). [1β-3H]4-Androstene-3,17-dione (300,000 dpm), various concentration of
4-androstene-3,17-dione (60-500 nM) and a single concentration of inhibitor were preincubated with propylene glycol, (100 µl), NADP (1.8 mM), glucose-6-phosphate (2.85 mM) and glucose-6-phosphate dehydrogenase (5 units) at 37°C for 5 min. Placental microsomes (0.07-0.1 mg), homogenized and diluted to 3.5 ml with 0.1 M sodium phosphate buffer, pH 7, were added to the preincubated mixture in shaking water bath and the solution was incubated at 37°C for 15 min. The reaction was stopped by addition of CHCl₃ (5 ml), followed by vortexing the samples for 20 s. The samples were then centrifuged for 10 min (1,000g). Aliquots of water (200µl) were mixed with scintillation cocktail (5 ml) and counted for radioactivity. Assays were run in duplicate and control samples containing no inhibitor were run simultaneously. Blank samples were obtained by incubating boiled microsomes. Kinetic data was analysed by programs of Cleland (129). Protein concentrations were determined by using Biorad protein Micro-assay (130).

**Time-dependent inactivation studies**

All incubations were carried out in 0.1 M sodium phosphate buffer, pH 7. Incubations at 37°C contained microsomal protein (0.2-0.3 mg/ml), propylene glycol (100 µl), and NADPH (0.2 mM) in 0.1 M sodium phosphate buffer, pH 7, to a total volume of 10 ml. Various concentrations of inhibitor (0.5-15 µM) were added to the incubations. Aliquots (1.5 ml) were removed at various time periods (0-20 min) and immediately diluted 1:10 with cold sodium phosphate buffer (0.1 M). Aromatase activity was assayed by adding the microsomal suspension (3 ml) to a mixture of
[1\beta-^3H] 4-androstene-3,17-dione (300,000 dpm), propylene glycol (100 μl), NADP (1.8 mM), glucose-6-phosphate (2.8 mM) and glucose-6-phosphate dehydrogenase (5 units) to a total of 3.6 ml and incubated for 30 min at 37°C. The reaction was stopped by addition of CHCl₃ (5 ml) followed by vortexing the samples for 20 s. The samples were then centrifuged for 10 min (1,000g). Aliquots of water (1 ml) were mixed with scintillation cocktail (4 ml) and counted for radioactivity. Controls were run simultaneously with the absence of inhibitor.

Inactivation studies in the absence of NADPH were performed in the same manner but without NADPH in the initial incubation. Protection studies were carried out analogous to the inactivation studies with unlabelled 4-androstene-3,17-dione (0.5-0.8 µM) and inhibitor (1.5 µM) included in the initial incubation.

Cysteine protection experiments

The procedure is the same as time-dependent inactivation studies with cysteine included in the initial incubations.

Irreversibility experiments

Inhibitors with various concentrations (4µM to 15µM) were incubated at 37°C with placental microsomes (2-3 mg) in the presence of 1 mM NADPH, 10 mM glucose-6-phosphate, 5 mM MgCl₂, and 0.2 mg of glucose-6-phosphate dehydrogenase in 0.1 M sodium phosphate buffer, pH 7, to a total volume of 3.5 ml. The control incubation lacked NADPH. After 1 h, the incubations were diluted with cold buffer, and the
microsomes were precipitated by ultracentrifugation. The pellets were resuspended in buffer, diluted, recentrifuged, and resuspended a second time. The amount of aromatase activity was then assayed.
3.4. Results and Discussion

3.4.1. Chemistry

As mentioned earlier, the 7α-thiosubstituted group of androstenedione derivatives provided the third point of attachment to the enzyme aromatase and resulted in enhancement of binding affinity of the inhibitor. In addition, androstenedione with extended linear conjugation in either the A and/or B ring provided compounds with good inhibitory activity. 4,6-Androstadiene-3,17-dione (4,6-ADD), 1,4-androstadiene-3,17-dione (1,4-ADD) and 1,4,6-androstatriene-3,17-dione (1,4,6-ATD) have the same affinity as the substrate androstenedione for the enzyme aromatase. It is logical to postulate that addition of the 7-substituent to androstenedione derivatives with extended linear conjugation in A and/or B ring should provide inhibitors of aromatase with high affinity.
The scheme started with the synthesis of 3,3,17,17-bisethylenedioxy-5-androstene 32 (Scheme 1). Briefly, 32 was obtained by refluxing androstenedione with ethylene glycol in benzene overnight with p-toluenesulfonic acid (p-TsOH) as catalyst. Initially, the reaction was run without benzene; however, a high ratio of ethylene glycol to androstenedione had to be used because of the limited solubility of the steroid in ethylene glycol. Benzene provided solubility to the steroid; in addition, water formed during the reaction can be easily removed since benzene and water formed an azeotrope which boiled at lower temperature.

Scheme 1.

3,3,17,17-bisethylenedioxy-5-androstene-7-one 33

One of the most important intermediates in the synthetic scheme is 3,3,17,17-bisethylenedioxy-androst-5-ene-7-one 33. Compound 33 is not readily available, thus it was synthesized from 32 through allylic oxidation. Several oxidizing agents were attempted. The first attempt
was reacting 32 with sodium chromate 4-hydrate in acetic anhydride and acetic acid mixture at 40°C for 24 h. Compound 33 was obtained; however, with very low yield (15%) because of the acidic nature of the reaction medium which resulted in extensive cleavage of the ketal protecting groups. Another attempt using chromium trioxide and dimethylpyrazole as oxidizing agent also afforded 33, but the work up procedure was too tedious and the yield was low (21%). Eventually, satisfactory yield was obtained (55%) by using t-butyl hydroperoxide (t-BuOOH) in the presence of chromium hexacarbonyl Cr(CO)$_6$ catalyst in refluxing acetonitrile (CH$_3$CN) (131) (Scheme 2).
7-Substituted 4,6-androstadiene-3,17-diones 34, 36, 38

Originally, introduction of the aryl groups into C-7 was proposed (132) by reacting 3,3,17,17-bisethylenedioxy-5-androstene-7-one 33 with Wittig Reagents and subsequent hydrolysis of the ketal protecting groups to give the corresponding 7-substituted 4,6-androstadiene-3,17-dione. The first attempt (Scheme 3) was performed by using benzylphosphonium bromide. Addition did not occur when

3,3,17,17-bisethylenedioxy-5-androstene-7-one 33 reacted with benzylphosphonium bromide and sodium hydride (NaH) in benzene at 70°C for 24 h. The failure of the reaction may be due to the steric bulk of the triphenyl group in the phosphonium ylide preventing the initial attack of the ylide to the carbonyl at C-7.

An alternate route of incorporating an aryl group at C-7 position was using a strong nucleophile. 3,3,17,17-Bisethylenedioxy-5-androstene-7-one 33 was reacted with either aryl Grignard or aryl lithium reagents to give the 7-aryl-7-hydroxy-
bisethylenedioxy-5-androstene, which was hydrolyzed without isolation. Methanol-sulfuric acid was used for the hydrolysis of the two ethylene ketal groups with concomitant dehydration of the 7-hydroxy groups to give 7-substituted 4,6-androstadiene-3,17-diones (Scheme 4).

The configuration of the aryl and the hydroxy groups in 7-aryl-7-hydroxy-bisethylenedioxy-5-androstenes were not elucidated; however, attack from the less-hindered α face would favor the assignment of 7α-aryl-7β-hydroxy-bisethylenedioxy-5-androstene. Upon hydrolysis and concomitant dehydration, only one product (7-substituted 4,6-androstadiene-3,17-diones) was obtained. The 7α-aryl-7β-hydroxy-\(\Delta^5\)-ene system however would be expected to yield an exocyclic double bond on dehydration when the aryl groups are benzyl or phenethyl, since the hydroxyl group can achieve a suitable coplanar trans relationship with a hydrogen from the aryl groups. The exocyclic double bond formed may rearrange to form \(\Delta-4,6\)-diene-3-one system which may be thermodynamically more stable products.
7-Substituted 1,4,6-androstatriene-3,17-diones 35, 37, 39 (Mechanism-based inhibitors).

The synthetic goal in obtaining the potential enzyme activated inhibitors was the incorporation of $C_1-C_2$ double bond into 7-substituted 4,6-androstadiene-3,17-dione. The introduction of a double bond preferentially at $C_1-C_2$ can be achieved by oxidation with DDQ under neutral condition. Product isolation was easily achieved by chromatography using basic alumina (Scheme 5).

![Scheme 5.](image)

7-(4'-Substituted)-4,6-androstadiene-3,17-dione 40, 41, 42, 43

Compounds 40 and 41 were synthesized in a one step process as shown in scheme 6. Nitration of 36 and 38 with nitric acid and sulfuric acid in a modification of a procedure of Anselm and Zuckmayer (133) led to the formation of 40 and 41 respectively. Only the para-substituted products were isolated and purification was carried out by recrystallization using ethyl-acetate:hexane.
The amino analogs 42 and 43 were synthesized from their nitro analogs 40 and 41 respectively as shown in scheme 6. Reduction of the nitro group was accomplished using stannous chloride and hydrochloric acid (134).
3.4.2. Biochemistry

3.4.2.1. Competitive binding studies

The newly synthesized 7-substituted 4,6-androstadiene-3,17-diones 34, 36, 38, 40-43 and 7-substituted 1,4,6-androstatriene-3,17-diones 35, 37 and 39 were evaluated in vitro by enzyme kinetic studies using human placental microsomes. Aromatase activity in human placental microsomes were assayed by the radiometric method developed by Siiteri (112) in which the tritium in [1β-3H]4-androstene-3,17-dione was transferred into water during aromatization. The amount of $^3$H$_2$O released was used as an index of estrogen formation. All the inhibitors were evaluated in initial velocity studies performed under limiting enzyme condition in which the substrate concentration was varied while the inhibitor concentration remained constant. Each substrate concentration was run in duplicate and the results of the studies were plotted in a typical Lineweaver-Burk or double-reciprocal plot as 1/velocity vs 1/substrate concentration. The K$_i$'s of the inhibitors is an index of the affinity of the enzyme for the inhibitor and is determined by a weighed regression analysis computer program (129). The apparent K$_i$'s of the compounds 34-43 were determined in the manner described above. The results are shown in the Lineweaver-Burk plots (figures 12-21) and the K$_i$'s of the inhibitors are shown in Table 2.

In all studies, the apparent K$_m$ for androstenedione was found to be 0.051 ± 0.009µM. Also each compound evaluated demonstrated competitive inhibition, as determined from the Lineweaver-Burk plots and V$_{max}$
intercepts (Table 2).

The inhibitors exhibit a wide range of inhibitory activity ($K_i$'s 60.9 nM-2.256 µM). Both the 7-benzyl- and 7-phenethyl-4,6-androstadiene-3,17-dione 36, 38, 40-43 and 1,4,6-androstatriene-3,17-dione 37, 39 are effective inhibitors of aromatase ($K_i$'s 60.9-174 nM). 7-Phenyl- 4,6-androstadiene3,17-dione 34 and 1,4,6-androstatriene-3,17-dione 35, on the other hand, are poor inhibitors ($K_i$'s 1.424 and 2.256 µM respectively).

Previous studies illustrated that aromatase has considerable tolerance to androstenedione and testosterone derivatives with bulky 7α-substituents (71-74). The reason for the low inhibitory activities of inhibitors 34 and 35 may be that the 7-phenyl group of the inhibitors can only adapt themselves at a pseudo β position, whereas the 7-benzyl- and 7-phenethyl- groups of 4,6-androstadiene-3,17-dione and 1,4,6-androstatriene-3,17-dione 36-43 can orient themselves in a way that the phenyl rings can protrude into the 7α pocket.
Table 2. The $K_m$ and $K_i$ of Inhibitors 34-43.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_m$(nM)</th>
<th>$K_i$(nM)</th>
<th>$V_{max}$(nmol/mg/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>61.3±9.7</td>
<td>1424.0±324</td>
<td>0.229±0.0074</td>
</tr>
<tr>
<td>35</td>
<td>40.0±9.1</td>
<td>2256.0±850</td>
<td>0.196±0.0073</td>
</tr>
<tr>
<td>36</td>
<td>42.2±5.0</td>
<td>60.9±6.5</td>
<td>0.178±0.0038</td>
</tr>
<tr>
<td>37</td>
<td>59.3±8.6</td>
<td>73.9±9.5</td>
<td>0.241±0.0076</td>
</tr>
<tr>
<td>38</td>
<td>63.0±7.1</td>
<td>174.0±18.0</td>
<td>0.270±0.0065</td>
</tr>
<tr>
<td>39</td>
<td>63.2±11.0</td>
<td>172.0±30.0</td>
<td>0.233±0.0096</td>
</tr>
<tr>
<td>40</td>
<td>45.3±6.5</td>
<td>93.8±12.6</td>
<td>0.224±0.0060</td>
</tr>
<tr>
<td>41</td>
<td>48.5±5.8</td>
<td>94.6±10.0</td>
<td>0.196±0.0045</td>
</tr>
<tr>
<td>42</td>
<td>46.6±4.7</td>
<td>88.2±8.2</td>
<td>0.190±0.0036</td>
</tr>
<tr>
<td>43</td>
<td>43.8±5.4</td>
<td>88.6±10.4</td>
<td>0.200±0.0045</td>
</tr>
</tbody>
</table>
Figure 12. Double reciprocal plots of aromatase inhibition by inhibitor 34. Varying concentrations of androstenedione were incubated with aromatase at single inhibitor concentrations of 0 µM (*), 1 µM (+), or 2 µM (□). Each point represents the average of two determinations with variation of less than 7%.
Figure 13. Double reciprocal plots of aromatase inhibition by inhibitor 35. Varying concentrations of androstenedione were incubated with aromatase at single inhibitor concentrations of 0 µM (*), 1 µM (+), or 2 µM (□). Each point represents the average of two determinations with variation of less than 7%.
Figure 14. Double reciprocal plots of aromatase inhibition by inhibitor 36. Varying concentrations of androstenedione were incubated with aromatase at single inhibitor concentrations of 0 μM (*), 0.3 μM (+), or 0.6 μM (□). Each point represents the average of two determinations with variation of less than 7%.
Figure 15. Double reciprocal plots of aromatase inhibition by inhibitor 37. Varying concentrations of androstenedione were incubated with aromatase at single inhibitor concentrations of 0 μM (•), 0.3 μM (+), or 0.6 μM (□). Each point represents the average of two determinations with variation of less than 7%.
Figure 15.
Figure 16. Double reciprocal plots of aromatase inhibition by inhibitor 38. Varying concentrations of androstenedione were incubated with aromatase at single inhibitor concentrations of 0 µM (*), 0.3 µM (+), or 0.6 µM (□). Each point represents the average of two determinations.
Figure 17. Double reciprocal plots of aromatase inhibition by inhibitor 39. Varying concentrations of androstenedione were incubated with aromatase at single inhibitor concentrations of 0 µM (•), 0.3 µM (+), or 0.6 µM (□). Each point represents the average of two determinations with variation of less than 7%.
Figure 18. Double reciprocal plots of aromatase inhibition by inhibitor 40. Varying concentrations of androstenedione were incubated with aromatase at single inhibitor concentrations of 0 μM (*), 0.3 μM (+), or 0.6 μM (□). Each point represents the average of two determinations with variation of less than 7%.
Figure 18.
Figure 19. Double reciprocal plots of aromatase inhibition by inhibitor 4. Varying concentrations of androstenedione were incubated with aromatase at single inhibitor concentrations of 0 µM (*), 0.3 µM (+), or 0.6 µM (□). Each point represents the average of two determinations with variation of less than 7%.
Figure 20. Double reciprocal plots of aromatase inhibition by inhibitor 42. Varying concentrations of androstenedione were incubated with aromatase at single inhibitor concentrations of 0 μM (*), 0.3 μM (+), or 0.6 μM (□). Each point represents the average of two determinations with variation of less than 7%.
Figure 21. Double reciprocal plots of aromatase inhibition by inhibitor 43. Varying concentrations of androstenedione were incubated with aromatase at single inhibitor concentrations of 0 μM (*), 0.3 μM (+), or 0.6 μM (□). Each point represents the average of two determinations with variation of less than 7%.
Figure 21.
3.4.2.2. Computer-Assisted Molecular Modeling

Our objectives are to determine the differences in inhibitory ability to the conformational properties of the inhibitors. The overall approach used in these studies was first to derive minimum-energy conformations.

Molecular modeling and molecular graphics were done at the graphic facilities of the Interactive Molecular Center (Image) in the Chemistry Department of the Ohio State University.

X-ray crystallography data of 1,4,6-androstatriene-3,17-dione was obtained from the Cambridge data base. The coordinates of the steroid were used as a structure fragment for building of the three-dimensional structures of the inhibitors. MacroModel (135) was used as a model-builder program and the structures were built from a terminal keyboard employing a mouse as input control device. Appropriate structure fragments were joined together graphically to form the molecules.

The conformational energy calculations and minimizations were performed with MM2 (Molecular Mechanics II) program developed by Allinger and his workers (136). Minimizations of all the molecules were done by at least 12 cycles of derivatizations. After minimization, the molecules were superimposed, displayed and manipulated by using the ChemX programs (137).

After the minimum-energy conformations of the inhibitors were obtained, the orientation of the 7-substituent of the inhibitors were compared. We chose 7α-APTA as the template molecule for superpositions.
because it is one of the most potent inhibitors with a 7-substituent. The overlapped energy minimized-structures of 7α-APTA with inhibitors 34-43 (figures 22-31) as well as the overlapped energy minimized-structures between the inhibitors (figures 32-35) are shown.

Since the overlapped energy minimized-structure of the inhibitors show little difference in conformation of the steroid ring, the difference in inhibitory activities of the inhibitors could be due to the difference in orientation of the 7-substituents. The orientation of the 7-substituents in various inhibitors were compared relative to a reference plane constituted of C9, C12 and C14 on the steroidal template. The reference plane provided the least root mean square deviation when the energy minimized-structure of the inhibitors were overlapped. All calculations were performed on a VAX 11/785 by using ChemX (137). The results are shown in Table 3 and the graphical representation of the parameters are shown in figure 36.

When the results were examined, there was no correlation between D and the K_i of the inhibitors. Interestingly, the inhibitory activity of the inhibitors is related to both θ and d. When log K_i of 7α-APTA and inhibitors 34-39 were plotted against θ, a linear correlation of 0.95 was observed (figure 37). When inhibitors 40-43 were included, the correlation dropped to 0.91. Another interesting observation is that plotting of log K_i of inhibitors 34-39 against d (figure 38) also gives a high linear correlation (0.95), and again the correlation dropped when inhibitors 40-43 were included. The addition of polar substituent in
the para position of the phenyl ring may alter the binding affinity of the inhibitors to the enzyme. In 7-benzyl-4,6-androstadiene-3,17-dione, addition of polar substituent (-NO\textsubscript{2} or -NH\textsubscript{2}) on the phenyl ring result in inhibitors 40, 42 with lower inhibitory activity. However, the opposite result was observed for 7-phenethyl-4,6-androstadiene-3,17-dione. Addition of polar substituents (-NO\textsubscript{2} or -NH\textsubscript{2}) on the phenyl ring of 7-phenethyl-4,6-androstadiene-3,17-dione result in inhibitors 41, 43 with increased inhibitory activity. It is suggested that additional factors other than geometry may be involved in enhancing the affinity of the inhibitors to the enzyme.
Figure 22. Overlapped energy minimized-structure of 7α-APTA and inhibitor 34.
Red=7α-APTA
Green=inhibitor 34
Figure 23. Overlapped energy minimized-structure of 7α-APTA and inhibitor 35.
Red=7α-APTA
Green=inhibitor 35
Figure 24. Overlapped energy minimized-structure of 7α-APTA and inhibitor 36.
Red=7α-APTA
Green=inhibitor 36
Figure 25. Overlapped energy minimized-structure of 7α-APTA and inhibitor 37.
Red=7α-APTA
Green=inhibitor 37
Figure 26. Overlapped energy minimized-structure of 7α-APTA and inhibitor 38.
Red=7α-APTA
Green=inhibitor 38
Figure 27. Overlapped energy minimized-structure of 7α-APTA and inhibitor 39.
Red=7α-APTA
Green=inhibitor 39
Figure 28. Overlapped energy minimized-structure of 7\alpha-APTA and inhibitor 40.
Red=7\alpha-APTA
Green=inhibitor 40
Figure 29. Overlapped energy minimized-structure of 7α-APTA and inhibitor 41.
Red=7α-APTA
Green=inhibitor 41
Figure 30. Overlapped energy minimized-structure of 7α-APTA and inhibitor 42.
Red=7α-APTA
Green=inhibitor 42
Figure 31. Overlapped energy minimized-structure of 7α-APTA and inhibitor 43.  
Red=7α-APTA  
Green=inhibitor 43
Figure 32. Overlapped energy minimized-structure of inhibitor 34 and inhibitor 36
Red= inhibitor 34
Green= inhibitor 36
**Figure 33.** Overlapped energy minimized-structure of inhibitor 34 and inhibitor 38
Red=inhibitor 34
Green=inhibitor 38
Figure 34. Overlapped minimized-stucture of inhibitor 36 and inhibitor 38
Red=inhibitor 38
Green=inhibitor 36
Figure 35. Overlapped energy minimized-structure of inhibitor 38 and inhibitor 39
Red=inhibitor 38
Green=inhibitor 39
Figure 36. Graphical representation of radial and angular parameters.

\(D\) = Distance of the Centroid of the reference plane \((C_9, C_{12}, C_{14})\) to Centroid of the phenyl ring.

\(\Theta\) = Angle between the reference plane and the plane of the phenyl ring.

\(d\) = Perpendicular distance of the Centroid of the phenyl ring to the reference plane.
Figure 36.
Table 3 Angular and radial parameter of inhibitor 34-43.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>D (in Angstrom)</th>
<th>θ (in degree)</th>
<th>d (in Angstrom)</th>
<th>LogK_i</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>5.15</td>
<td>88.14</td>
<td>0.21</td>
<td>3.15</td>
</tr>
<tr>
<td>35</td>
<td>5.10</td>
<td>101.3</td>
<td>0.63</td>
<td>3.35</td>
</tr>
<tr>
<td>36</td>
<td>6.54</td>
<td>64.15</td>
<td>2.62</td>
<td>1.79</td>
</tr>
<tr>
<td>37</td>
<td>6.54</td>
<td>61.42</td>
<td>3.30</td>
<td>1.87</td>
</tr>
<tr>
<td>38</td>
<td>7.35</td>
<td>80.31</td>
<td>2.26</td>
<td>2.24</td>
</tr>
<tr>
<td>39</td>
<td>7.33</td>
<td>80.80</td>
<td>2.30</td>
<td>2.24</td>
</tr>
<tr>
<td>40</td>
<td>6.58</td>
<td>99.83</td>
<td>2.38</td>
<td>1.97</td>
</tr>
<tr>
<td>41</td>
<td>7.34</td>
<td>78.76</td>
<td>2.16</td>
<td>1.98</td>
</tr>
<tr>
<td>42</td>
<td>6.58</td>
<td>81.56</td>
<td>2.16</td>
<td>1.95</td>
</tr>
<tr>
<td>43</td>
<td>7.35</td>
<td>80.36</td>
<td>2.21</td>
<td>1.95</td>
</tr>
<tr>
<td>12</td>
<td>5.37</td>
<td>32.69</td>
<td>2.53</td>
<td>1.25</td>
</tr>
</tbody>
</table>
Figure 37. Plotting of $\log K_1$ vs $\theta$
Figure 37.
Figure 38. Plotting of Log $K_i$ vs $d$. 
Figure 38.
3.4.2.3. Enzyme-activated inactivation studies

Compound 35, 37, 39 were synthesized from the corresponding 7-substituted 4,6-androstadiene-3,17-dione by oxidation with DDQ. These inhibitors were proposed as potential enzyme-activated irreversible inhibitor of aromatase and must meet the following five criteria (138):

1. The inhibitor should exhibit a time-dependent, pseudo-first-order inactivation of the enzyme.

2. The inactivation requires cofactors that are also required for normal catalytic reaction of the enzyme.

3. Reversible substrates should protect the enzyme from inactivation.

4. Nucleophiles should not protect the enzyme from inactivation.

5. The inhibition should be irreversible.

Compounds 35, 37, and 39 were examined in inactivation studies. All the compounds exhibited time-dependent, first order inactivation of placental aromatase when incubated with various concentrations of inhibitor in the presence of NADPH (figures 39, 45, 51). In the absence of NADPH, no inactivation was observed (figures 40, 46, 52). Thus, normal catalytic reaction appears to be necessary for inactivation. In order to determine if inactivation is at the active site, protection studies were performed by adding the substrate 4-androstene-3,17-dione to the incubation mixture of enzyme, inhibitor and NADPH. The enzyme half-life was lengthened when the substrate concentration was increased from 0-0.8 μM which indicated that inhibitors are inactivating aromatase at the active site (figures 41, 47, 53).
Mechanism-based irreversible inhibition requires that once the inhibitor is converted to a reactive intermediate, it immediately forms a covalent bond with the enzyme at the active site without first diffusing out to the incubation medium. Nucleophiles such as cysteine present in the incubation medium which react with the electrophilic intermediate if it diffuses out of the active site, resulting a decrease in the rate of inactivation. Thus, inactivation studies were conducted in which cysteine (0.5 mM) was added to the incubation mixture with the inhibitor, enzyme and NADPH. Cysteine failed to protect aromatase from inactivation by inhibitors 37 and 39 after correcting for its general stabilizing effect on the microsomal enzyme preparation (figures 48 and 54). Thus, covalent bond formation between the enzyme and the reactive inhibitor intermediate appears to occur rapidly at the active site, therefore preventing diffusion of the activated inhibitor into the surrounding media. For inhibitor 35, cysteine did partially protect the enzyme from its inactivation (figure 42). At the inhibitor concentration of 15 μM, the half-time of inactivation increased from 8.77 min to 34.35 min with the presence of cysteine (0.5 mM). Once the inhibitor is activated, it may slowly diffuse out into the incubation medium where it reacts with cysteine. Thus, lower concentrations of activated inhibitor is available to form a covalent bond at the active site. This is conceivable because of the low affinity and high Kᵢ (1.4μM) of the inhibitor for aromatase.

The irreversible nature of the enzyme-inhibitor interaction was further supported by irreversibility studies. In these studies,
placental microsomal protein was incubated with inhibitors (5-15 μM) in the presence of 1 mM NADPH. The control incubation lacked NADPH. After 1 h, the microsomal pellet was precipitated by ultracentrifugation. The placental microsomes did not regain aromatase activity after exhaustive washing following removal of the inhibitor. The studies demonstrate the irreversible nature of the inhibition (figures 43, 49, 55).

The studies described above suggest that inhibitors 35, 37 and 39 inactivate aromatase by an enzyme-catalyzed pathway as shown (figure 57). In this process, the inhibitor itself is chemically unreactive, however, the product of the enzymic conversion is a highly reactive molecule. This product immediately reacts with an active-site moiety, resulting in the irreversible inhibition of the enzyme. The structure of the reactive intermediate formed by inhibitors 35, 37 and 39 are unknown. The normal pathway of aromatization of substrate is consecutive hydroxylation at C₁₉ to give a 19-aldehyde intermediate. If the inhibitors follow normal catalytic pathway as the substrate, the aldehyde of inhibitors would be generated. A current theory of the final hydroxylation in aromatization occurs at C₁₉ to give an enzyme-bound peroxide species (27), which would result in the generation of reactive intermediates of inhibitors 35, 37 and 39. Covalent bond formation and subsequent inactivation of the enzyme will occur as depicted below (Scheme 7).
Scheme 7.
An alternate theory of inactivation of the enzyme may be through the formation of epoxide at C1-C2 double bond. The epoxide generated can then be opened up by a nucleopholic moiety at the active site (Scheme 8).

![Scheme 8.

The kinetics of the inactivation process were evaluated by plotting the half-time of inactivation vs 1/inhibitor concentration. Linear plots were obtained exhibiting saturation kinetics (figures 43, 49 and 55). The apparent $K_{\text{inact}}$ and minimum half-time of inactivation of the inhibitors is shown in Table 4. The minimum half-time of inactivation at saturating concentrations of inhibitors 35, 37 and 39 are 6.1, 11.1 and 5.79 min respectively, which are more rapid than PED and ATD.
Table 4. Comparison of inactivation kinetics for irreversible inhibitors of aromatase.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ (nM)</th>
<th>$K_{inact}$ (µM)</th>
<th>$T_{1/2}$ (min)</th>
<th>$K_{app}$ (sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>2220</td>
<td>3.32</td>
<td>6.1</td>
<td>1.89 x 10$^{-3}$</td>
</tr>
<tr>
<td>37</td>
<td>60.9</td>
<td>2.65</td>
<td>11.1</td>
<td>1.04 x 10$^{-3}$</td>
</tr>
<tr>
<td>39</td>
<td>172</td>
<td>0.84</td>
<td>5.79</td>
<td>1.20 x 10$^{-3}$</td>
</tr>
<tr>
<td>20</td>
<td>9.9</td>
<td>0.16</td>
<td>1.40</td>
<td>8.4 x 10$^{-3}$</td>
</tr>
<tr>
<td>PED</td>
<td>4.5</td>
<td>ND</td>
<td>11.2</td>
<td>1.0 x 10$^{-3}$</td>
</tr>
<tr>
<td>4-OH-A</td>
<td>10.2</td>
<td>ND</td>
<td>2.9</td>
<td>1.0 x 10$^{-3}$</td>
</tr>
<tr>
<td>ATD</td>
<td>110</td>
<td>ND</td>
<td>29.8</td>
<td>3.9 x 10$^{-3}$</td>
</tr>
</tbody>
</table>

ND - not determined.
Figure 39. Inactivation of aromatase by inhibitor 35 in the presence of NADPH. A time dependent, first-order inactivation of aromatase activity is produced at inhibitor concentration of 2.5 μM (o), 5 μM (□), 10 μM (△) and 15 μM (x). Control samples contained no inhibitor (*). Each point represents the average of two determinations with variation of less than 7%.
Figure 39.
Figure 40. Inactivation of aromatase by inhibitor 35 in the absence of NADPH. In the absence of NADPH, the inhibitor at concentration of 10 μM (○) failed to produce an inactivation of aromatase while in the presence of NADPH, a first-order inactivation was observed (□). Control samples contained no inhibitor (*). Each point represents the average of two determinations.
Figure 40.
Figure 41. Protection of inhibitor 35 inactivation of aromatase by substrate. Androstenedione at concentration of 0 µM (○), 0.5 µM (□), 0.8 µM (△) was incubated with microsomal aromatase, inhibitor (10 µM), and NADPH and protected the enzyme from inactivation. Control samples were run simultaneously and contained no inhibitor (*). Each point represents the average of two determinations with variation of less than 7%.
Figure 41.
Figure 42. Inactivation of aromatase by inhibitor 35 in the presence of a nucleophilic trapping agent. A first order inactivation of aromatase by inhibitor 35 (15 μM) was observed in the presence (□) or absence (▲) of 0.5 mM cysteine. Control samples with (*) and without (o) cysteine contained no inhibitor. Each point represents the average of three determinations with variation of less than 7%. 
Figure 42.
Figure 43. Irreversibility studies of inhibitor 35
Figure 43.
Figure 44. Plot of the inactivation half-time (min) vs 1/[I] μM⁻¹ for inhibitor 35
Figure 45. Inactivation of aromatase by inhibitor 37 in the presence of NADPH. A time dependent, first-order inactivation of aromatase activity is produced at inhibitor concentration of 0.5 μM (o), 1 μM (□), 2 μM (△) and 4 μM (x). Control samples contained no inhibitor (*). Each point represents the average of two determinations with variation of less than 7%.
Figure 45.
Figure 46. Inactivation of aromatase by inhibitor 37 in the absence of NADPH. In the absence of NADPH, the inhibitor at concentration of 4 μM (o) failed to produce an inactivation of aromatase while in the presence of NADPH, a first-order inactivation was observed (□). Control samples contained no inhibitor (*). Each point represents the average of two determinations.
Figure 46.
Figure 47. Protection of inhibitor 37 inactivation of aromatase by substrate. Androstenedione at concentration of 0 μM (○), 0.5 μM (□), 0.8 μM (△) was incubated with microsomal aromatase, inhibitor (4 μM), and NADPH and protected the enzyme from inactivation. Control samples were run simultaneously and contained no inhibitor (*). Each point represents the average of two determinations with variation of less than 7%. 

180
Figure 47.
Figure 48. Inactivation of aromatase by inhibitor 37 in the presence of a nucleophilic trapping agent. A first order inactivation of aromatase by inhibitor 37 (4 μM) was observed in the presence (□) or absence (△) of 0.5 mM cysteine. Control samples with (*) and without (o) cysteine contained no inhibitor. Each point represents the average of three determinations with variation of less than 7%.
Figure 48.
Figure 49. Irreversibility studies of inhibitor 37
Figure 49.
Figure 50. Plot of the inactivation half-time (min) vs $1/|I| \mu M^{-1}$ for inhibitor 37
Figure 5. Inactivation of aromatase by inhibitor 39 in the presence of NADPH. A time dependent, first-order inactivation of aromatase activity is produced at inhibitor concentration of 0.5 μM (o), 1 μM (□), 2 μM (△) and 4 μM (x). Control samples contained no inhibitor (*). Each point represents the average of two determinations with variation of less than 7%.
Figure 51.
Figure 52. Inactivation of aromatase by inhibitor 39 in the absence of NADPH. In the absence of NADPH, the inhibitor at concentration of 1.5 μM (o) failed to produce an inactivation of aromatase while in the presence of NADPH, a first-order inactivation was observed (□). Control samples contained no inhibitor (*). Each point represents the average of two determinations.
Figure 52.
Figure 53. Protection of inhibitor 39 inactivation of aromatase by substrate. Androstenedione at concentration of 0 μM (○), 0.5 μM (□), 0.8 μM (Δ) was incubated with microsomal aromatase, inhibitor (1.5 μM), and NADPH and protected the enzyme from inactivation. Control samples were run simultaneously and contained no inhibitor (*). Each point represents the average of two determinations with variation of less than 7%. 192
Figure 53.
Figure 54. Inactivation of aromatase by inhibitor 39 in the presence of a nucleophilic trapping agent. A first order inactivation of aromatase by inhibitor 39 (1.5μM) was observed in the presence (□) or absence (△) of 0.5 mM cysteine. Control samples with (*) and without (o) cysteine contained no inhibitor. Each point represents the average of three determinations with variation of less than 7%.
Figure 54.
Figure 55. Irreversibility studies of inhibitor 39
Figure 55.
Figure 56. Plot of the inactivation half-time (min) vs 1/[I] μM\(^{-1}\) for inhibitor 39.
Figure 56.

INACTIVATION HALF-TIME

$\frac{1}{I/I_0}$ vs. time (seconds)
Figure 57. Scheme for a enzyme-activated inactivation process.
Figure 57.
CHAPTER IV

CONCLUSIONS

The aromatase inhibitor, 7α-APTA, is effective in reducing tumor volumes in the estrogen dependent DMBA-induced mammary carcinoma rat model. Administration of 7α-APTA to tumored animals at a dose of 50 mg/kg/day reduced tumor volumes by 80% during a six-week treatment schedule. In addition, plasma estradiol levels in these animals were decreased by approximately 50%. Further evidence of tumor reduction by aromatase inhibition is reversal by co-administration of estradiol with 7α-APTA. These results indicate that 7α-APTA is effective in vivo and encourage further development of these steroids as potential medicinal agents for the treatment of estrogen-dependent disease states such as breast and endometrial cancer.

The 7-substituted 4,6-androstadiene-3,17-diones and 1,4,6-androstatriene-3,17-diones were shown to be potent inhibitors of aromatase. The 7-benzyl- and 7-phenethyl- analogs are more effective inhibitors than the 7-phenyl- analogs. 7-Substituted 1,4,6-androstatriene-3,17-diones 35, 37, and 39 produced a time-dependent, first-order inactivation of aromatase, which was decreased when the substrate, androstenedione, was included in the incubation mixture. The enzyme kinetic studies indicate that the inhibitors
are interacting in an irreversible manner at the active site of the enzyme.

Computer assisted molecular modeling studies showed a correlation between difference in orientation of the 7-substituents and the difference in the affinity of the inhibitors to the enzyme. Comparison of energy minimized structures of the inhibitors should provide information on the design of more potent inhibitors. Further studies of the energy barrier involve in various possible spatial orientation of the 7-substituents should provide more information on the topography of the 7α-pocket.

Thus effective inhibitors with 7-substituted 4,6-androstadiene-3,17-dione and 1,4,6-androstatriene-3,17-dione were prepared and examined biochemically in vitro. These may offer advantages of metabolic stability in vivo over 7α-thio analogs.

Future studies should include the examination of these inhibitors in MCF-7 cell culture, which may provide information on hormonal or antihormonal effects of these compounds. In addition, covalent binding studies of the enzyme-activated inhibitors using a reconstituted preparation of purified aromatase should provide information on the exact chemical nature of the active site.
BIBLIOGRAPHY


23. Townsley J. D. and Brodie H. J., Biochemistry 7, 33 (1968)


47. Chen S., Besman M. J., Shively J. E. and Hall P. F., Aromatase, Future perspective, Miami, Florida, March 4-7 (1987) abstr#1


77. Walsh C., Molecular Biology, Biochemistry and Biophysics vol.32 p.62 Springer-Verlag, Heidelberg (1980)


87. Covey D. F. and Hood W. F., Cancer Res. (Suppl.) 42, 3327s (1982)


95. Silverberg E., CA 33, 9 (1983)

96. Miller A. B. Cancer 39, 2704 (1977)

97. Donegan W. L. Cancer 41, 1590 (1978)

98. MacMahon B. Cancer Inst. 50, 21 (1973)


105. Varela R. M. and Dao T. L., Cancer Res. 38, 2429 (1978)


133. Anselm F. and Zuckmayer F., *Ber.* 32, 3283 (1899)

135. The 1986 Version of MacroModel was provided by Professor Clark Hill, Department of Chemistry, Columbia University.


137. Created by Davies E. K., Chemical Crystallography Laboratory, Oxford University and distributed by Chemical Design Ltd. Oxford, U.K.