FINAL DETAILED REVIEW PAPER

ON

AROMATASE

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

DETAILED REVIEW PAPER
AROMATASE

1.0 EXECUTIVE SUMMARY

The Food Quality Protection Act of 1996 was enacted by Congress to authorize the EPA to implement a screening program on pesticides and other chemicals found in food or water sources for endocrine effects in humans. Thus, the United States Environmental Protection Agency (U.S. EPA) is implementing an Endocrine Disruptor Screening Program (EDSP). In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial chemicals, and pesticides. The program's aim is to develop a two-tiered approach, e.g., a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. Validation of the individual screens and tests is required, and the Endocrine Disruptor Methods Validation Subcommittee (EDMVS) will provide advice and counsel on the validation assays.

Estrogens are sex steroid hormones that are necessary for female reproduction and affect the development of secondary sex characteristics of females. Estrogens are biosynthesized from cholesterol by a series of enzymatic steps, with the last step involving the conversion of androgens into estrogens by the enzyme aromatase. Estrogen biosynthesis occurs primarily in the ovary in mature, premenopausal women. During pregnancy, the placenta is the main source of estrogen biosynthesis and pathways for production change. Small amounts of these hormones are also synthesized by the testes in the male and by the adrenal cortex, the hypothalamus, and the anterior pituitary in both sexes. The major source of estrogens in both postmenopausal women and men occurs in extraglandular sites, particularly in adipose tissue. One potential endocrine target for environmental chemicals is the enzyme aromatase, which catalyzes the biosynthesis of estrogens. An aromatase assay is proposed as one of the Tier 1 Screening Battery Alternate Methods. A detailed literature review on aromatase was performed and encompassed (1) searching the literature databases, (2) contacting individuals to obtain information on unpublished research, and (3) an evaluation of the literature and personal communications.

Aromatase is a cytochrome P450 enzyme complex responsible for estrogen biosynthesis and converts androgens, such as testosterone and androstenedione, into the estrogens estradiol and estrone. Aromatase is present in the ovary, placenta, uterus, testis, brain, and extraglandular adipose tissues. Two proteins, cytochrome $P450_{arom}$ and NADPH-cytochrome P450 reductase, are necessary for enzymatic activity, and the enzyme complex is localized in the smooth endoplasmic reticulum. The aromatase gene, designated CYP19, encodes the cytochrome $P450_{arom}$ and consists of 10 exons, with the exact size of the gene exceeding 70 kilobases. Aromatase is found in breast tissue, and the importance of intratumoral aromatase and local estrogen production is being unraveled. Effective aromatase inhibitors have been developed as therapeutic agents for estrogen-dependent breast cancer to reduce the growth stimulatory effects

of estrogens in breast cancer. Investigations on the development of aromatase inhibitors began in the 1970's and have expanded greatly in the past three decades.

An *in vitro* aromatase assay could easily be utilized as an alternative screening method in the Tier 1 Screening Battery to assess the potential effects of various environmental toxicants on aromatase activity. Both *in vitro* subcellular (microsomal) assays and cell-based assays are available for measuring aromatase activity. The in vitro subcellular assay using human placental microsomes, is commonly used to evaluate the ability of pharmaceuticals and environmental chemicals to inhibit aromatase activity. In addition, human JEG-3 and JAR choriocarcinoma cell culture lines, originally isolated from cytotrophoblasts of malignant placental tissues, have been used as *in vitro* systems for measuring the effects of compounds on aromatase activity. These cell lines are also utilized for investigations on the effects of agents in placental toxicology.

Numerous flavonoids and related phytoestrogen derivatives have been extensively evaluated for their ability to inhibit aromatase activity for two primary reasons: (1) these plant natural products can serve as possible leads for the development of new nonsteroidal aromatase inhibitors; and (2) humans and other animals are exposed to these agents through the diet. In general, the flavonoids and related analogs demonstrate aromatase inhibition with IC_{50} values in the micromolar range; however, these compounds lack both the potency and specificity of aromatase inhibitors developed for breast cancer therapy. Several pesticides have also demonstrated inhibition of aromatase activity in the human placental microsomal assay system, with IC_{50} values for aromatase inhibition ranging from 0.04 μM to greater than 50 μM.

The human placental microsomal aromatase assay is a straight forward, inexpensive, and rapid *in vitro* assay for measuring the effects of chemicals on aromatase activity. Aromatase inhibition of endocrine disruptors have also been evaluated in intact cell systems such as the JEG-3, JAR choriocarcinoma, the H295R adrenocortical carcinoma or the KGN granulosa cell culture lines. In general, the inhibitory effects on the aromatase activity are similar between the two *in vitro* assays for a wide variety of compounds. Advantages of a cell culture assay are that it can provide information on membrane transport abilities of the test agent, and the assay can provide information on the effect of the test agent on the expression of the aromatase protein, i.e., if the test compound will induce or suppress the production of CYP 19 mRNA and/or new aromatase protein.

The human placental microsomal aromatase assay is recommended as the *in vitro* aromatase screening assay to be included in the Tier 1 Screening Battery Alternate Methods. This assay will detect environmental toxicants that possess the ability to inhibit aromatase activity. The overall protocol for the assay is straightforward and uses a radiometric assay measuring the release of tritiated water from the substrate [1β-^{3}H]-androstenedione. A preliminary screening assay can be performed at concentrations of environmental toxicant at 0.1, 1.0, and 10.0 μM in quadruplicate. For compounds exhibiting 10% inhibitory activity or more, a full dose-response studies will be performed using concentrations ranging from 1.0 nM (1.0×10^{-9} M) to 1.0 mM (1.0×10^{-3} M).

2.0 INTRODUCTION

2.1 DEVELOPING AND IMPLEMENTING THE ENDOCRINE DISRUPTOR SCREENING PROGRAM (EDSP)

In 1996, the passage of the two laws, the Food Quality Protection Act (FQPA) and Amendments to the Safe Drinking Water Act (SDWA) mandated the United States Environmental Protection Agency (U.S. EPA) to screen pesticides and authorized the EPA to screen chemicals found in drinking water to determine whether they possess estrogenic or other endocrine activity (Federal Register, 2001). The U. S. EPA is required to "develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or other such endocrine effect..." (Federal Register, 2001). The U.S. EPA established the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), to provide recommendations regarding a strategy for developing a testing paradigm for compounds that may have activities similar to naturally-occurring hormones. Following the recommendations made by EDSTAC in its final report (EDSTAC, 1998), the EPA established the Endocrine Disruptor Screening Program (EDSP). The program's aim is to develop a two-tiered approach, e.g., a combination of *in vitro* and *in vivo* mammalian and ecotoxicological screens (Tier 1) and a set of *in vivo* tests (Tier 2) for identifying and characterizing endocrine effects of pesticides, industrial chemicals, and environmental contaminants. The Organization for Economic Cooperation and Development (OECD) initiated a high-priority activity in 1997 to develop new test guidelines and revise existing test guidelines for the screening and testing of potential endocrine disruptors. This activity is organized under the Task Force on Endocrine Disrupters Testing and Assessment as part of the OECD test guidelines program and managed by three Validation Management Groups (VMGs) covering mammalian, ecotoxicity, and non-animal methods.

This Detailed Review Paper was prepared for the U.S. EPA in 2002 to review the scientific basis of the aromatase assay and examine assays reported in the literature used to measure the effect of chemical substances on aromatase. It was presented and discussed at the first meeting of the OECD Validation Management Group for Non-Animal methods (VMG-NA) and is being adopted for use by the OECD. As the document has served its original purpose of identifying promising methods for further development and prevalidation, it has not been updated to reflect the literature published after April 2002, but does reflect comments submitted by members of the VMG-NA.

2.2 METHODOLOGY USED IN THE ANALYSIS

2.2.1 Literature Search

The literature review encompassed (1) searching the literature databases, (2) contacting individuals to obtain additional information, and (3) an evaluation of the literature and personal communications.

2.2.2 Databases Searched

The following databases were searched:

Database	*Literature coverage*
MedLine / PubMed	biomedical literature
Biological Abstracts	biological literature
Chemical Abstracts	chemical literature and patents
Toxline	toxicological literature
DART	developmental & reproductive toxicology
CancerLit	cancer literature and abstracts
IPA	pharmaceutical literature

The search strategies involved keyword searching and author searching strategies. The words or phrases selected are those keywords that are indexed by the database, and the search strategies of the databases utilize "expanded" or "exploded" searches with the indexed keywords. Each database was first searched using the keyword aromatase to identify all references for aromatase (for example, 3899 references on aromatase retrieved in MedLine using PubMed). Then, this subset of references on aromatase was searched individually by using the Boolean operator "and" with the other keywords or phrases. The other keywords or phrases utilized were:

> endocrine disruptors, flavonoids, isoflavonoids, phytoestrogens, dioxins, herbicides, pesticides, PAHs (or polycyclic aromatic hydrocarbons), PCBs (or polychlorinated biphenyls)

The retrieved searches were then downloaded electronically either as ASCII files or downloaded directly into Reference Manager for Windows, Version 9 (ISI ResearchSoft, Berkeley, CA). Initially, each database search, e.g., "aromatase and [other keyword]," were stored as individual database files.

Each individual search on a literature database was then combined into a Reference Manager file designated by the name of the particular literature database. For example, each of the individual searches on a PubMed (MedLine) database was first combined into a Reference Manager file designated pubmed search.rmd and pubmed search.rmx. Duplicate references were removed by the software program. The literature databases were then combined into a single, large database designated combined searches.rmd and combined searches.rmx. The software program will not recognize accurately duplicates from different literature databases (due to different formatting of the database providers), so duplicate references appear in this single combined file. These duplicates were sorted manually. The final result was a combined database of over 400 references.

2.2.3 Methods and Sources for Acquiring Additional Information

Additional information can include research presented at recent meetings and research currently on-going in various research laboratories. Information on the research presented at meetings may be available through the abstracts (if published), and some abstracts are available in certain electronic databases. In the latter case, these presentations and abstracts were retrieved in the database searches. Presentations and abstracts not available in public databases can be retrieved electronically by attendees or by members of the organization sponsoring the meeting (e.g., American Association for Cancer Research, Endocrine Society, American Chemical Society) or can be examined manually. Abstracts from recent meetings of American Association for Cancer Research, Endocrine Society, and American Chemical Society were reviewed and abstracts identified.

Research currently on-going in various research laboratories can only be obtained by contacting the researchers or laboratory directors. Such individuals include researchers and/or authors in the field of aromatase pharmacology and toxicology identified from relevant publications. Other scientists involved in similar work can also be identified based upon examination of the participants of relevant symposiums, panels, and committees. At best, clarification of study design, interpretation, and data analysis of studies may be obtained through interviews with these scientists. It is not likely that detailed methodologies and results will be obtained through this interview process, due to confidentiality issues with both scientists in commercial firms and with academic researchers.

Similar methods, sources, and approaches were used to identify and acquire unpublished material and to identify and interview principal investigators in the field. Investigators were selected based upon the number and the quality of recent relevant publications (study design, quality of methods, chemicals tested, data analysis) and the individuals reputation (national/international reputation in area, author of reviews and/or critical documents). The following individuals were contacted and provided responses.

Investigator	***Expertise***
Shiuan Chen, Ph.D. Division of Immunology Beckman Research Institute, City of Hope 1450 E. Duarte Road Duarte, CA 91006, U.S.A. E-mail: schen@smtplink.coh.org	Biochemistry and molecular biology of aromatase; structure-function studies; aromatase inhibition by flavonoids
Rajeshwar R. Tekmal, Ph.D. Department of Ob/Gyn Emory University School of Medicine 4219 WMB, 1639 Pierce Drive Atlanta, GA 30322, U.S.A. E-mail: rtekmal@emory.edu	Molecular biology of aromatase; development of transgenic mice expressing aromatase
Edwin D. Lephart, Ph.D. 633 WIDB, Neuroscience Center Brigham Young University Provo, UT 84602, U.S.A. E-mail: edwin_lephart@byu.edu	Brain aromatase; flavonoids and aromatase

Investigator	*Expertise*
Sari Mäkelä, Ph.D. and Risto Santti, Ph.D. University of Turku Institute of Biomedicine and Medicity Research Laboratory Turku, FINLAND E-mail: sarmak@utu.fi	Biochemistry and pharmacology of aromatase; aromatase inhibition by flavonoids; endocrine disruptors

The individuals were contacted by phone or e-mail. The title and objectives/goals of this particular work assignment and a brief description of the two study designs under consideration (human placental microsomal aromatase assay, human choriocarcinoma cell culture assay) were presented . The interview questions and responses are presented in Appendix B.

2.3 DEFINITIONS

The definitions of terms that will be utilized throughout this DRP are presented here:

placental aromatase	aromatase activity present in the placenta
ovarian aromatase	aromatase activity present in the ovary
brain aromatase	aromatase activity present in the brain
microsomes	aromatase subcellular fraction consisting of the endoplasmic reticulum of the cell; isolated following cell fractionation and differential centrifugation methods
cytochrome $P450_{arom}$	the heme protein in the aromatase complex
NADPH-cytochrome P450 reductase	the protein in the aromatase complex that provides reducing equivalents for the enzyme reaction
aromatase protein complex	two protein complex in the endoplasmic reticulum that consists of the cytochrome $P450_{arom}$ and the NADPH-cytochrome P450 reductase
CYP19	the gene expressing the cytochrome $P450_{arom}$ protein
aromatase radiometric assay (also referred to as ^3H_2O assay)	aromatase enzyme assay that measures the release of ^3H_2O from [1β-^{3}H]-androstenedione
aromatase product isolation assay	aromatase enzyme assay that measures the formation of estrogen products from androgen precursors
JEG-3 cell	a transformed human choriocarcinoma cell line, ATCC no. HTB-36
JAR cell	a transformed human choriocarcinoma cell line, ATCC no. HTB-144

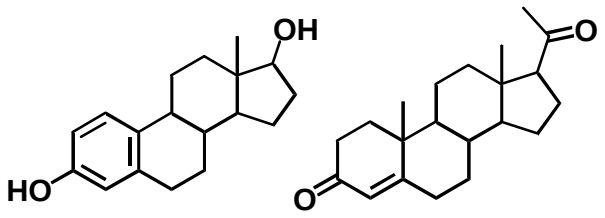
3.0 BACKGROUND ON AROMATASE

3.1 ENDOCRINOLOGY OF ESTROGENS AND ROLE OF AROMATASE

3.1.1 Female Sex Hormones

Estrogens are sex steroid hormones that are necessary for female reproduction and affect the development of secondary sex characteristics of females. The other principal class of female sex steroid hormones is progestins, which are also essential for female reproduction. Chemically, the naturally occurring estrogens are C_{18} steroids and have in common an unsaturated A ring (which is planar) with a resulting phenolic function in the 3 position that aids in separation and purification from nonphenolic substances. The most potent endogenous estrogen is estradiol. The naturally occurring progestins are C_{21} steroids and have in common a 3-keto-4-ene structure in the A ring and a ketone at the C-21 position. The most potent endogenous progestin is progesterone. A brief summary of estrogens, the reproductive cycle, steroidogenesis and hormone action are presented here. More detailed information can be obtained from recent treatise.[1-5]

3.1.2 Female Reproductive Cycle



Estradiol **Progesterone**

Among the key events in the female reproductive process is ovulation, which is regulated by the endocrine and central nervous systems.[3] In humans, sexual maturation, or the period in which cyclic menstrual bleeding begins to occur, is reached between the ages of 10 and 17; the average age is 13. The period of irregular menstrual cycles before the cessation of menses (usually between the ages of 40 and 50) is commonly known as menopause.

The female reproductive cycle in humans and other primates is controlled through an integrated system involving the hypothalamus, pituitary gland, ovary, and reproductive tract. The hypothalamus exerts its action on the pituitary gland through a decapeptide called luteinizing hormone-releasing hormone (LHRH), also referred to as gonadotropin-releasing hormone (GnRH), which is released by the hypothalamus and initiates the release of gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary. The two main gonadotropins, FSH and LH, regulate the ovary and its production of sex hormones. As the name implies, FSH promotes the initial development of the immature graafian follicle in the ovary. This hormone cannot induce ovulation but must work in conjunction with LH. The

combined effect is to promote follicle growth and increase the biosynthesis and secretion of estrogens. Through negative feedback on the brain and pituitary, estrogens inhibit production of FSH and stimulate output of LH. The level of LH rises to a sharp peak at midpoint in the menstrual cycle and acts on the mature follicle to bring about ovulation; LH levels are low during the menses. In contrast, FSH reaches its high level during menses, falls to a low level during and after ovulation, and then increases again toward the onset of menses. Once ovulation has taken place, LH induces luteinization of the ruptured follicle, which leads to corpus luteum formation. After luteinization has been initiated, there is an increase in progesterone from the developing corpus luteum, which in turn suppresses production of LH. Once the corpus luteum is complete, it begins to degenerate toward menses, and the levels of progesterone and estrogen decline.

The endometrium, a component of the genital tract, passes through different phases, which depend on the steroid hormones secreted by the ovary. During the development of the follicle, which takes approximately 10 days and is referred to as the follicular phase, the endometrium undergoes proliferation owing to estrogenic stimulation. The luteal phase follows ovulation, lasts about 14 days, and ends at menses. During the luteal phase, the endometrium shows secretory activity, and cell proliferation declines. In the absence of pregnancy, the levels of estrogen and progesterone decline; this leads to sloughing of the endometrium. This, together with the flow of interstitial blood through the vagina, is called menses and lasts for 4 to 6 days. Because the estrogen and progesterone levels are now low, the hypothalamus releases more LHRH, and the cycle begins again. The reproductive cycle in the female extends from the onset of menses to the next period of menses, with a regular interval varying from 20 to 35 days; the average length is 28 days.

If pregnancy occurs, the menstrual cycle is interrupted because of the release of a fourth gonadotropin. In human pregnancy, the gonadotropin produced by the placenta is referred to as human chorionic gonadotropin (hCG). hCG maintains and prolongs the life of the corpus luteum. The hCG level in the urine rises to the point where it can be detected after 14 days and reaches a maximum around the seventh week of pregnancy. After this peak, the hCG concentration falls to a constant level, which is maintained throughout pregnancy. The corpus luteum, because of HCG stimulation, provides an adequate level of estrogens and progestins to maintain pregnancy during the first nine weeks. After this period, the placenta can secrete the required level of estrogen and progestational hormones to maintain pregnancy. The levels of estrogen and progesterone increase during pregnancy and finally reach their maximal concentrations a few days before parturition. Because the level of HCG in the urine rises rapidly after conception, it has served as the basis for many pregnancy tests.

3.1.3 Steroidogenesis

Estrogens are biosynthesized from cholesterol, primarily in the ovary in mature, premenopausal women.[1] During pregnancy, the placenta is the main source of estrogen biosynthesis and pathways for production change. Small amounts of these hormones are also synthesized by the testes in the male and by the adrenal cortex, the hypothalamus, and the

anterior pituitary in both sexes. The major source of estrogens in both postmenopausal women and men occurs in extraglandular sites, particularly in adipose tissue.

In endocrine tissues, cholesterol is the steroid that is stored and is converted to estrogen, progesterone, or androgen when the tissue is stimulated by a gonadotropic hormone. The major pathways for the biosynthesis of sex steroid hormones are summarized in Figure 1. In the ovary, FSH acts on the preovulatory follicle to stimulate the biosynthesis of estrogens. The thecal cells of the preovulatory follicle convert cholesterol into androgens, whereas the granulosa cells convert androgens to estrogens. After ovulation, LH acts on the corpus luteum to stimulate both estrogen and progesterone biosynthesis and secretion.

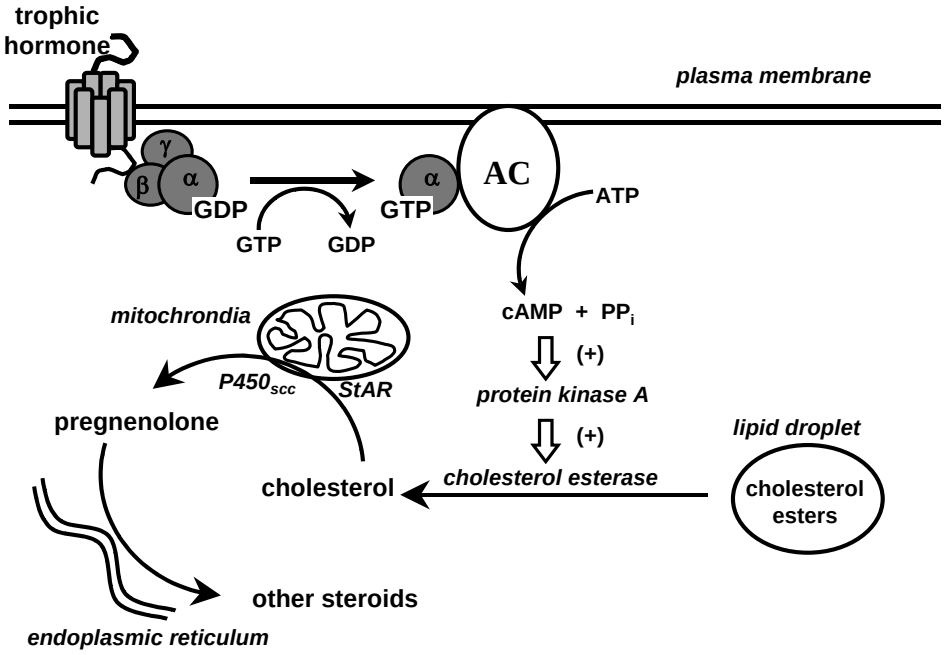


Figure 1. Trophic Hormones and Steroidogenesis

Cholesterol is converted by side chain cleavage into pregnenolone, which can be converted to progesterone or by several steps to the ring A aromatic system found in estrogens (Figure 2). Pregnenolone is converted by 17α-hydroxylase to 17α-hydroxypregnenolone. The next biosynthetic step involves the cleavage of the C_{17}-C_{20} carbon-carbon bond and is referred to as the 17-20-lyase reaction. This step is catalyzed by the same cytochrome P450 enzyme complex, 17α-hydroxylase. The 17α-hydroxylase is found in the endoplasmic reticulum membrane and is comprised of the cytochrome $P450_{17\alpha}$ protein and the ubiquitous NADPH-cytochrome P450 reductase. Cytochrome $P450_{17\alpha}$ is expressed by the gene *CYP17* found on chromosome 10 in humans. Loss of the 17β-acetyl side chain of 17α-hydroxypregnenolone affords the C_{19} intermediate dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one, DHEA). DHEA is converted by 5-ene-3β-hydroxysteroid dehydrogenase/3-oxosteroid-4,5-isomerase to

the 17-ketosteroid, androstenedione (androst-4-ene-3,17-dione). This compound is interconvertible with the reduced 17β-hydroxy intermediate (testosterone) by 17β-hydroxy-steroid dehydrogenase. Loss of the C-19 angular methyl group and aromatization of the A ring of testosterone or androstenedione results in 17β-estradiol or estrone, respectively. 17β-Estradiol and estrone are metabolically interconvertible, catalyzed by estradiol dehydrogenase.

3.1.4 Estrogen Action

The biochemical mechanism of estrogen action is the regulation of gene expression and subsequent induction of protein biosynthesis via specific, high affinity estrogen receptors,[2] as

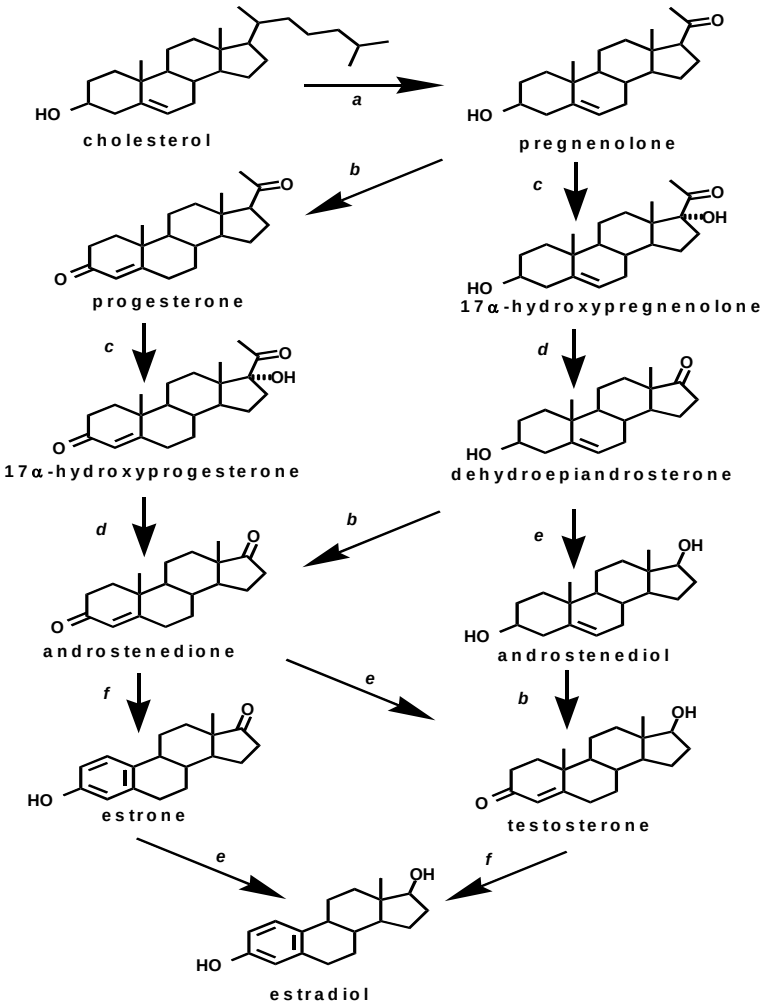


Figure 2. Biosynthesis of Sex Steroid Hormones

Enzyme activities: (a) side chain cleavage, (b) 3β-hydroxysteroid dehydrogenase/4,5-isomerase, (c) 17α-hydroxylase, (d) 17,20-lyase, (e) 17β-hydroxysteroid dehydrogenase, (f) aromatase.

illustrated in Figure 3. These receptors are soluble intracellular proteins that can both bind steroid ligands with high affinity and act as ligand-dependent transcriptional factors via interaction with specific deoxyribonucleic acid (DNA) sites and other proteins associated with the chromatin. Receptor binding sites for estradiol are located in the nucleus of target cells and exhibit both high affinity ($K_D = 10^{-11}$ to 10^{-10} M) and high specificity. When estradiol binds to the estrogen receptor, a conformational change of the estradiol-receptor complex occurs and steroid-receptor complex undergoes homodimerization. The steroid-receptor homodimers interact with particular regions of the cellular DNA, referred to as hormone-responsive elements (HRE) or estrogen responsive elements (EREs) present in the promoter region of responsive genes. Binding of the nuclear steroid-receptor complexes to DNA and interaction with various nuclear transcriptional factors initiate the transcription of the gene to produce messenger ribonucleic acid (mRNA). The elevated mRNA levels result in increased protein synthesis in the endoplasmic reticulum. These proteins include enzymes, receptors, and secreted factors that subsequently result in the steroid hormonal response regulating cell function, growth, differentiation and playing central roles in normal physiological processes, as well as in many important diseases. Initially, a single protein termed the estrogen receptor was believed to be responsible for all the actions of estrogens. Recently, a second estrogen receptor has been identified, and the two estrogen receptors are now referred to as ERα (representing the classical ER, the first ER studied) and ERβ (the second ER identified). The two receptors differ in size and tissue distribution. The predominant estrogen receptor in the female reproductive tract and mammary glands is ERα, whereas ERβ is the primary estrogen receptor in vascular endothelial cells, bone, and male prostate tissues. Estradiol has similar affinities for both ERα and ERβ, whereas certain nonsteroidal estrogenic compounds and antiestrogens have differing affinities between ERα and ERβ, with slightly higher affinities for ERβ.

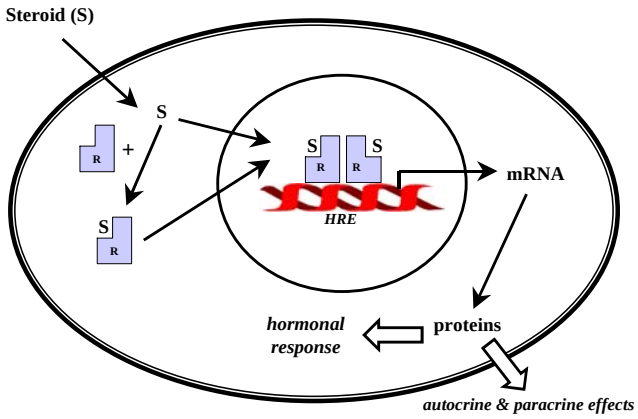


Figure 3. General Mechanism of Steroid Hormone Action

Estrogens act on many tissues in the female, such as those of the reproductive tract, breast, and CNS. In the female, the primary physiological action of estrogens

is to stimulate the development of secondary sex tissues. The growth and development of tissues in the reproductive tract of animals, in terms of actual weight gained, are not seen for as long as 16 hours after administration of the estrogen, although some biochemical processes in the cell are affected immediately. The growth response produced in the uterus by estrogens is temporary, and the maintenance of such growth requires the hormone to be available almost continuously. The initial growth induced by the estrogen is therefore of limited duration, and atrophy of the uterus occurs if the hormone is withdrawn. Another physiologic effect of estrogens, observed one hour after their administration, is edema in the uterus. During this period, vasodilation of the uterine pre- and postcapillary arterioles occurs, and there is an increase in permeability to plasma proteins. These effects appear to occur predominantly in the endometrium and not to any great extent in the myometrium.

Another target of estrogens is breast tissue. Estrogens can stimulate the proliferation of breast cells and promote the growth of hormone-dependent mammary carcinoma. Because the breast is the primary site for cancer in women, considerable research has been focused on understanding breast cancer and the factors that influence its growth. Estradiol will stimulate gene expression and the production of several proteins in breast cancer cells via the estrogen receptor mechanism. These proteins include both intracellular proteins important for breast cell function and growth, and secreted proteins that can influence tumor growth and metastasis.

Finally, estrogens are also important for male sexual development and reproductive function.[6,7] Estrogens, produced locally by aromatase in the Sertoli cells, are important for spermatogenesis in rats, and aromatase has been detected in human testis.[8] Estrogen receptors are present in male reproductive tissues such as Sertoli cells, Leydig cells, epididymis, and accessory sex organs.[7] During gestational development of the rodent, the local aromatization of testosterone to estrogen within the brain, plays a key role in the structural organization of the hypothalamus which can effect sexual differentiation the male brain.[9,10] Although there is currently no definitive evidence to demonstrate analogous effects of estrogen on masculization of the human brain during gestational development, it is likely that estrogen and androgens play a critical role in producing the sexual dimophism of the primate.[11]

3.2 AROMATASE - GENERAL DESCRIPTION

3.2.1 Biochemistry and Molecular Biology

Estradiol is the most potent endogenous estrogen in humans. Estradiol is biosynthesized from androgens by the cytochrome P450 enzyme complex called aromatase. This enzyme was first reported in human placental tissues by K. J. Ryan in 1959.[12] In humans, aromatase is present in the ovaries of premenopausal women, in the placenta of pregnant women, and in the peripheral adipose tissues of women and of men, and these tissues are the major sources for estrogens circulating in the blood stream.[13] Aromatase is also important for the local tissue production of estrogens. Aromatase activity has been demonstrated in breast tissue *in vitro.*[14-17] Furthermore, expression of aromatase is highest in or near breast tumor sites,[15,18] various regions

in the brain of both men and women contain the enzyme aromatase,[10,19] the male testis, and bone in men and women.[20,21]

Aromatase is the enzyme complex responsible for the conversion of androgens to estrogens during steroidogenesis.[22] The enzyme complex is bound in the endoplasmic reticulum of the cell and is comprised of two major proteins.[22,23] One protein is cytochrome $P450_{arom}$, a hemoprotein that converts C_{19} steroids (androgens) into C_{18} steroids (estrogens) containing a phenolic A ring. The second protein is NADPH-cytochrome P450 reductase, which transfers reducing equivalents to cytochrome $P450_{arom}$. Three moles of NADPH and three moles of oxygen are utilized in the conversion of one mole of substrate into one mole of estrogen product (Figure 4). Aromatization of androstenedione, the preferred substrate, proceeds via three successive oxidation steps, with the first two being hydroxylations of the angular C-19 methyl group. The final oxidation step, whose mechanism remains for complete elucidation, proceeds with the aromatization of the A ring and loss of the C-19 carbon atom as formic acid.

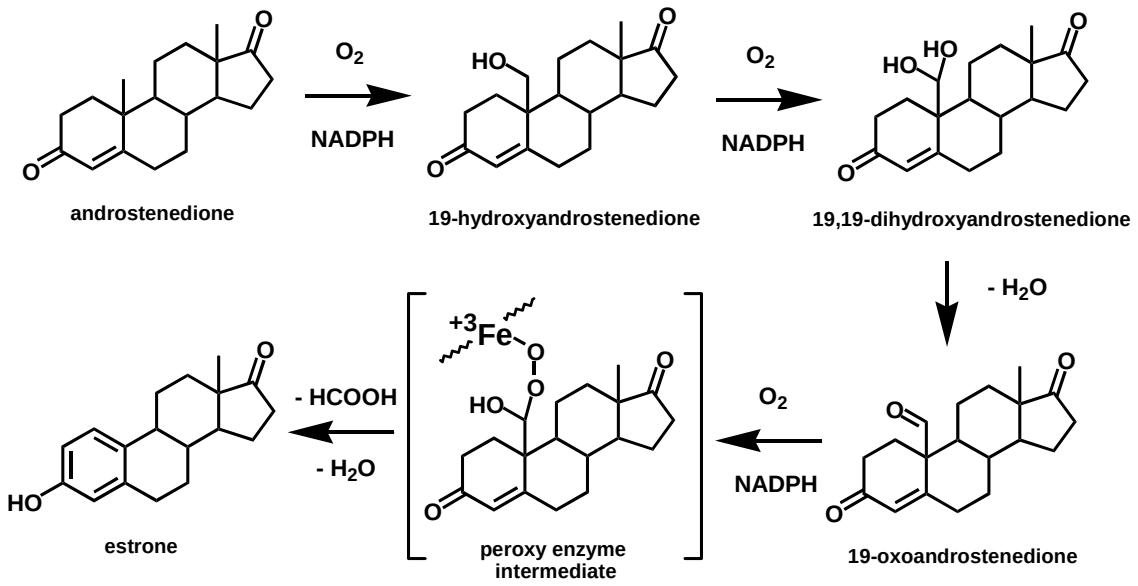


Figure 4. Reaction Mechanism for Estrogen Biosynthesis by Aromatase

Over the past two decades, knowledge of the biochemistry, molecular biology, and regulation of aromatase has increased greatly. The aromatase gene, designated CYP19, encodes the cytochrome $P450_{arom}$ and consists of 10 exons, with the exact size of the gene exceeding 70 kilobases.[22] The gene is located on chromosome 15q21.1. The full length cDNA of 3.4 kilobases encodes for a protein of 503 amino acids with a molecular weight of approximately 55,000 daltons. The regulation of aromatase is complex in various tissues, and several tissue-specific promoter regions have been identified upstream from the CYP19 gene.[24-26] These tissue-specific promoters include promoter P1.1, P1.3, P1.4, and PII. The PII promoter is utilized in the

ovary and in breast cancer tissues, and it contains a cAMP response element. Promoters P1.3 and P1.4 are the primary promoter used in extraglandular sites such as adipose tissue and are responsive to glucocorticoids and cytokines such as IL-1β, IL-6 and TNFα. Promoter P1.1 is the promoter used in placental tissues and its regulation is still under extensive investigations.[27]

Aromatase is found in breast tissue, and the importance of intratumoral aromatase and local estrogen production is being unraveled.[15,28,29] Aromatase has been measured in the stromal cell component of normal breast and breast tumors, but the enzyme has also been detected in the breast epithelial cells *in vitro*. [14,18,29-31] Furthermore, expression of aromatase is highest in or near breast tumor sites.[29] The exact cellular location(s) of aromatase must await more rigorous analysis by several labs with a new monoclonal antibody now being developed and evaluated.[32] The increased expression of aromatase cytochrome $P450_{arom}$ observed in breast cancer tissues was recently associated with a switch in the major promoter region utilized in gene expression, and promoter II is the predominant promoter used in breast cancer tissues.[33,34] As a result of the use of the alternate promoter, the regulation of estrogen biosynthesis switches from one controlled primarily by glucocorticoids and cytokines to a promoter regulated through cAMP-mediated pathways.[33] The prostaglandin PGE_2 increases intracellular cAMP levels and stimulates estrogen biosynthesis, whereas other autocrine factors such as IL-1β do not appear to act via PGE_2.[35]

Local production of PGE_2 via the cyclooxygenase isozymes (constitutive COX-1 isozyme and inducible COX-2 isozyme) can influence estrogen biosynthesis and estrogen-dependent breast cancer. Recent studies suggest a relationship between CYP19 gene expression and the expression of COX genes.[36] Gene expressions of CYP19, COX-1, and COX-2 were performed in 20 human breast cancer specimens and in 5 normal control breast tissue samples. A positive correlation was observed between CYP19 expression and the greater extent of breast cancer cellularity, in agreement with literature reports showing that aromatase levels were higher in tumors than in normal tissue. Furthermore, a positive linear correlation was observed between COX-2 expression and the level of pathology in each sample, and a linear regression analysis using a bivariate model shows a strong linear association between CYP19 expression and the sum of COX-1 and COX-2 expression.[36] This significant relationship between the aromatase and cyclooxygenase enzyme systems suggests that autocrine and paracrine mechanisms may be involved in hormone-dependent breast cancer development via growth stimulation from local estrogen biosynthesis.

Effective aromatase inhibitors have been developed as therapeutic agents for estrogen-dependent breast cancer to reduce the growth stimulatory effects of estrogens in breast cancer. Investigations on the development of aromatase inhibitors began in the 1970's and have expanded greatly in the past three decades. Research summaries of aromatase inhibitors have been presented at international aromatase conferences[32,37-40] and several reviews have also been published.[41-50]

3.2.2 Aromatase in Other Species

In addition to the extensive investigations on the importance of human aromatase in normal physiology and in cancer, this enzyme complex has also been well studied in other mammalian species (rodents, cows, pigs, horses) and in non-mammalian vertebrates as well. In general, these investigations have focused on identifying the presence of aromatase in various tissues, comparative endocrinology of aromatase, and examination of the importance of aromatase in the physiology of various species. In many species, aromatase expression and/or activity is restricted to the gonads and the brain. Placental aromatase is found only in primates, cattle, horses, and pigs. Aromatase in the adult rat ovary is regulated by the gonadotropins, FSH and LH, and by prolactin[51,52] and is induced by elevation in cAMP levels and subsequent activation of protein kinase A.[53] Rat testicular tissues also contain aromatase[54] and convert androgens into estrogens locally for spermatogenesis. The importance of aromatase in the brain was first demonstrated *in vivo* in rodents,[55] and this conversion of androgens to estrogens in the brain is responsible for neural cell differentiation.[56] Aromatase is present in both bovine ovary and bovine placenta[57] and is under complex regulation.[58,59] Pigs express two distinct aromatases, one in gonadal tissues and the other in placental tissues.[60,61] Aromatase has also been identified in gonadal and brain tissues in many non-mammalian vertebrates as well. Numerous species of fish have aromatase activity,[62-64] and higher levels are often found in the brain compared to the gonads.[63] Recently, two distinct aromatases have been reported in fish.[63,65,66] Various reptiles also have aromatase in the gonads and the brain.[67,68]

3.2.3 Significance of Alterations in Aromatase

The levels of estradiol, the most potent endogenous estrogen in humans, are critical for female reproduction and other hormonal effects in females. The levels of estrogens are controlled by the extent of estrogen biosynthesis from androgens by the enzyme aromatase. Alterations in the amount of aromatase present or in the catalytic activity of the enzyme will alter the levels of estrogens in tissues and dramatically disrupt estrogen hormone action. Inhibition of aromatase alters the catalytic activity of the enzyme and results in a rapid decrease in the levels of estrogens. This mechanism of enzyme inhibition is the reason for the therapeutic effectiveness of aromatase inhibitors in the treatment of estrogen-dependent breast cancer and illustrates the importance of estrogen levels to estrogen action. Suppression or induction of the aromatase protein levels also can dramatically influence the subsequent levels of estrogens in tissues and effect hormone action. Environmental agents, toxicants, and various natural products can act via aromatase inhibition and/or alteration in aromatase protein levels to result in altered levels of estrogen and function as endocrine disruptors. Aromatase deficiencies in humans and the aromatase knockout (ArKO) mice illustrate dramatically the importance of aromatase.[27] In females with aromatase deficiency (currently a total of 8 patients worldwide), newborns have psuedohermaphroditism and, at puberty, these patients have primary amenorrhea, lack of breast development, hypogonadism, and cystic ovaries. Two males with aromatase deficiency have been reported, and these individuals exhibit tall stature due to failure of epiphyseal fusion, osteopenia, and infertility. Female ArKO mice exhibit infertility and underdeveloped uteri and mammary glands, and male ArKO mice have impaired spermatogenesis and enlarged

prostates.[69,70] Both male and female ArKO mice have excessive intra-abdominal fat and elevated serum lipid levels,[71] and both have excessive long bone growth and osteopenia.[72] Additional information regarding the significance of aromatase and the effects of aromatase overexpression is obtained from studies of transgenic mice. Tekmal and colleagues generated transgenic mice that overexpress int-5/aromatase under the control of mouse mammary tumor virus enhancer/promoter.[73,74] In this transgenic mouse model, overexpression of aromatase is observed in mammary glands, and this overexpression of the enzyme results in biosynthesis of estrogen locally in the tissues (*in situ* production). The early and continued exposure of mammary epithelial cells to *in situ* estrogen production results in the enlargement of ducts, with hyperplastic, dysplastic, or fibroadenoma lesions.

4.0 METHODS FOR MEASURING AROMATASE ACTIVITY

The method employed for measuring aromatase activity *in vitro* and *in vivo* is critical. This assay endpoint, i.e., measurement of aromatase activity, must be accurate and reproducible. The endpoint is accurate when the assay measurement is in agreement with the accepted reference value. The endpoint is reproducible when the same findings occur under the same conditions within a single laboratory (intra-laboratory) and among other laboratories (inter-laboratory). The two methods that are utilized extensively for the determination of aromatase activity in both *in vitro* and *in vivo* assays are the product isolation method and the radiometric method.

The most rigorous method is the product isolation technique. The method involves administration of a substrate such as androstenedione or testosterone, incubation or treatment for a designated time, isolation of the estrogen products formed, and measurement of the amount of estrogens. One common method uses radiolabeled substrate (either ^{3}H or ^{14}C) in the aqueous incubation medium. At the end of the incubation or treatment period, isolation of the radiolabeled steroids is accomplished by organic solvent extraction techniques. The radiolabeled substrate and products are separated using either thin layer chromatography or high-pressure liquid chromatography (HPLC) and analysis of the quantity of estrogen products formed using liquid scintillation counting. This method is best suited for *in vitro* assays using subcellular enzyme preparations, tissue incubations, or cell culture systems. Variations of the product isolation assay method include unlabeled substrate (or endogenous substrate in an *in vivo* study) and other methods for measurement and quantification. These other measurement methods include mass spectrometry, radioimmunoassays (RIA), or enzyme-linked immunoassays (EIA).

The radiometric method is also utilized for *in vitro* measurements of aromatase activity. This method is also referred to as the $^{3}H_2O$ assay. The level of aromatase activity is determined by measuring the amount of $^{3}H_2O$ released from [1β-^{3}H]-androstenedione substrate. The basis of this assay is that the aromatization of the A-ring catalyzed by aromatase involves the stereospecific cleavage of the covalent bond between the carbon atom at position 1 and the hydrogen on the β face of the steroid ring system. The procedures for this method are similar to the product isolation method but do not involve any chromatography to separate steroid substrate and products. Rather, the amount of $^{3}H_2O$ released is measured in the aqueous phase following rigorous extractions with organic solvents and/or dextran-coated charcoal. This method is very straightforward and more rapid. However, the results of the radiometric method must be confirmed for the specific aromatase assay conditions using the product isolation assay before extensive use. This radiometric method has been confirmed for *in vitro* assays using human placental microsomes, ovarian microsomes and tissues, cell culture systems (including human breast cancer cells and human choriocarcinoma cells), and isolated human breast or ovarian cells. This comparison of the results from the radiometric method with the product isolation assay is critical because other biochemical pathways that involve extensive androgen metabolism without aromatization of the androgen can produce false positive measurements. Examples are determinations of $^{3}H_2O$ released by liver or prostate cells, tissues, or tissue homogenates, which results in much higher activity measurements than is observed with a product isolation method as

a result of androgen metabolism, and presence of a 19-hydroxylase activity in adrenal tissues which does not result in A ring aromatization and formation of estrogens.[75]

Recently, a fluorescence aromatase assay was described using a fluorometric substrate *O*-benzylfluorescein benzyl ester, also referred to as dibenzylfluorescein (DBF).[76] In this report, recombinant human aromatase in microsomal preparations from stably-transfected insect cells was the source of enyzme.[77] DBF was a substrate for aromatase and was converted into the metabolite, fluorescein, which was measured using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The apparent K_m for DBF was reported as 188 nM and the apparent V_{max} was 0.32 min^{-1}. For comparison, apparent K_m for testosterone was 43 nM and the apparent V_{max} was 8.4 min^{-1}. Kinetic data for androstenedione, the preferred substrate for human aromatase, was not reported. Based upon the comparison of enzyme kinetic values, DBF is a poor substrate for aromatase. The IC_{50} values for several agents were reported, and in several cases, the IC_{50} values were quite different than reported literature values using the radiometric method measuring ^3H_2O released.

Aromatase assay activity is typically reported as velocities or rates, examples include descriptions such as nmol product formed per mg microsomal protein per assay minute, pmol product formed per 10^6 cells, or pmol product formed per mg tissue. Also, inhibition data is reported in table or graphical format as % inhibition, with a definition of 100%. For biochemical enzyme assays, the kinetic parameters of V_{max} for the enzyme rate, K_m for the substrate, K_i for an inhibitor should also be reported. Catalytic efficiencies, measured by V_{max}/K_m also provide valuable information. In pharmacological inhibition assays, the IC_{50} values should be reported.

4.1 *IN VITRO* AROMATASE ASSAYS TO DETECT ENDOCRINE DISRUPTORS

This section is subdivided according to aromatase assay method used (i.e., placental microsomal assay, cell line assays, *in vitro* mammalian cell systems, and *in vivo* studies) and includes discussions on the chemical natures of the endocrine disruptors.

4.1.1 Human Placental Microsomal Aromatase Assay

This is one of the most common assays used for measuring the inhibition of aromatase activity. This assay has been utilized to evaluate the ability of various endocrine disruptors to inhibit aromatase. The source of the aromatase is a microsomal preparation isolated from human term placenta. Normal placenta is obtained after delivery at a local hospital, is delivered on ice to the laboratory, and then processed at 4°C following a standard procedure. The microsomal preparation, is isolated by differential centrifugation procedures, with the final centrifugation at 105,000 x g for one hour. This microsomal preparation consists of the endoplasmic reticulum membrane of the cell and contains the membrane-bound cytochrome $P450_{arom}$ and the NADPH-cytochrome P450 reductase. For the enzyme assay, the microsomal preparation is resuspended in phosphate buffer, pH 7.4. Complete enzyme activity requires the addition of either NADPH or an NADPH-generating system, and the activity is measured using either the product isolation

method or the radiometric method. These assay methods have been utilized routinely for the determination of the presence of aromatase in tissues since the first report of aromatase by K.J. Ryan.[12] The highest tissue levels of human aromatase are present in term placenta and the placenta is discarded after birth, thus providing a rich and inexpensive source of the enzyme. Consequently, the human placental microsomal aromatase assay is used extensively in academic labs and pharmaceutical firms as the initial biological evaluation for potential steroidal and nonsteroidal aromatase inhibitors (see discussion in Section 2.2.1).

Numerous flavonoids and related phytoestrogen derivatives have been extensively evaluated for aromatase inhibition for two primary reasons: (1) these plant natural products can serve as possible leads for the development of new nonsteroidal aromatase inhibitors and (2) humans and other animals are exposed to these agents through the diet. The class of flavonoids encompasses the flavones, isoflavones, flavanones, and flavonols, all being derived chemically from the benzopyranone nucleus. These natural products have demonstrated numerous biological activities, interact with various enzymes and receptor systems of pharmacological significance, and have been potential applications in both cancer therapy and in cancer chemoprevention. Considerable interest in flavonoids in breast cancer has been stimulated by the hypothesis that these natural products, present in soy and in rye flour, are dietary factors that may be responsible for the lower incidence of breast cancer in women from certain regions of the world, e.g., Japan and Finland.[78]

The initial reports of aromatase inhibition by flavonoids and flavones appeared in the mid-1980's from the lab of L.E. Vickery,[79,80] and the synthetic benzoflavone analogs were more potent than naturally occurring flavones or flavonoids in the human placental microsomal assay system. Several research groups reported aromatase inhibition by various flavonoids using the human placental microsomal assay system.[78,81-88] In general, the flavonoids and related analogs demonstrate aromatase inhibition with IC_{50} values in the micromolar range; however, these compounds lack both the potency and specificity of aromatase inhibitors developed for breast cancer therapy. Figure 5 provides the chemical structures of selected flavonoids and their IC_{50} values. Flavonoids have also been examined for aromatase inhibitory activity in other biological systems as well. Aromatase inhibition by flavonoids has been observed in human preadipocytes,[89,90] in breast cancer cell systems,[91] in a transformed yeast cell system,[92] and in fish ovarian microsomal assays.[93,94] The relative inhibitory activity is similar to that observed with the human placental microsomal aromatase assay.

Several pesticides have also demonstrated inhibition of aromatase activity in the human placental microsomal assay system.[95] This study reported IC_{50} values for aromatase inhibition ranging from 0.04 μM to greater than 50 μM. Prochloraz, imazalil, propioconazole, fenarimol, triadimenol, and triadimefon inhibited placental aromatase with IC_{50} values of 0.04, 0.34, 6.5, 10, 21 and 32 μM, respectively (Figure 6). Several imidazole antifungal agents, such as econazole, miconazole, clotrimazole and ketoconazole, demonstrated inhibition of aromatase activity in the human placental microsomal assay system, [96-98] with IC_{50} values for aromatase inhibition ranging from 0.03 μM to 60 μM. Further information and comparisons of IC_{50} values is provided later in Tables 1 and 2.

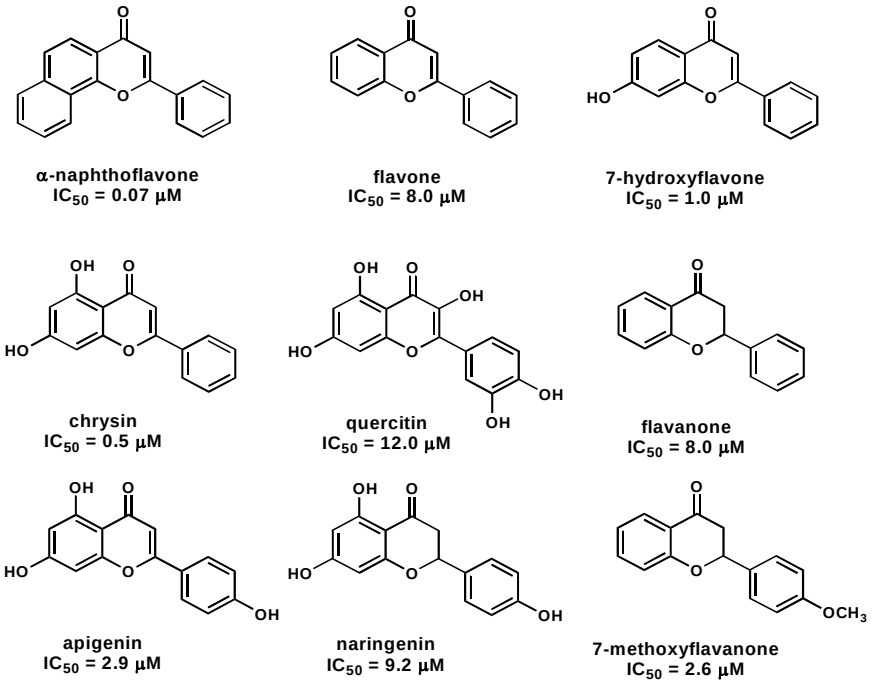


Figure 5. IC_{50} values for selected flavonoids

4.1.2 Human Cell Culture Systems for Measuring Aromatase Activity

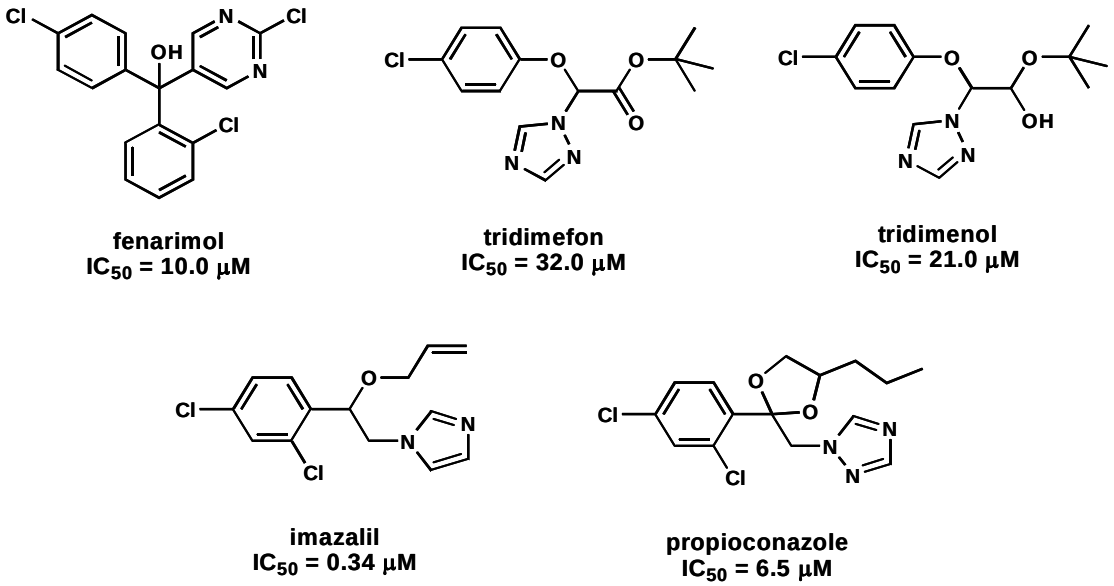


Figure 6. IC_{50} values for selected pesticides

Human JEG-3 and JAR choriocarcinoma cell culture lines, originally isolated from cytotrophoblasts from malignant placental tissues, have been used as *in vitro* systems for measuring the effects of compounds on aromatase activity. These cell lines are also utilized for investigations on the effects of agents in placental toxicology.[99] The cells express a large amount of aromatase activity and have been used extensively in academic labs and pharmaceutical firms for the biological evaluation for potential steroidal and nonsteroidal aromatase inhibitors.[100-103] The JEG-3 and JAR cells are cultured at 37°C and 5% CO_2 atmosphere in standard DMEM media and supplemented with 10% fetal calf serum, and aromatase activity is measured in intact cells using either the radiometric method (most common) or the product isolation method .

Various organochlorine contaminants have been examined in the JEG-3 and/or JAR cell line systems. The contaminants 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and 3,3',4,4',5-pentachlorobiphenyl (PCB126) caused concentration-dependent decreases in the aromatase activity of up to 4.9-fold in the JEG-3 cells.[104] The IC_{50} values for aromatase inhibition were 52 pM and 13 nM for TCDD, and 75 nM and 48 nM for PCB126 in the presence and absence of serum, respectively. These studies were further extended to twenty-one organochlorine PCB derivatives tested in both JEG-3 and JAR cell lines.[105] Aromatase inhibition was observed, but cytotoxicity was also significant. These observations led to the conclusion by the authors that the JEG-3 and JAR cells "appear too sensitive" to the cytotoxic effects of organochlorines for use in evaluating effects on aromatase activity. The fungicide fenarimol was also evaluated in JEG-3 cells,[95] exhibiting an IC_{50} value for aromatase inhibition of 2.0 μM. In general, the results on aromatase inhibition for a wide variety of chemical compounds that have been obtained from an intact cell system such as the JEG-3 or JAR cell culture lines are similar to the results from the human placental microsomal system.

Reports have recently been published on evaluation of environmental contaminants in other human cell culture systems. Yang and Chen have evaluated the organochlorine pesticides toxaphene and chlordane in a transfected SK-BR-3 breast cancer cell system and reported a decrease in aromatase expression, possibly through antagonism of the ERRα-1 transcription factor.[106] The H295R human adrenocortical carcinoma cell line has been used by Sanderson and colleagues to examine the effect the 2-chloro-s-triazine herbicides.[107] In this report, atrazine, simazine, and propazine at concentrations of 0-30 μM resulted in dose-dependent induction of aromatase activity by about 2.5-fold in H295R cells. In another study by these researchers,[108] diindolylmethane (DIM) and analogs, formed in the stomach by an acid-catalyzed condensation product of indole-3-carbinol, induced aromatase activity approximately 2-fold increase at 10 mM in H295R cells. Recently, Sanderson and colleagues reported a significantly less pronounced induction of aromatase by atrazine, simazine, propazine, and metabolites in JEG-3 cells and no induction of aromatase in MCF-7 cells.[109] These studies further illustrate the tissue-specific nature of CYP19 expression and aromatase levels. A third cell line recently reported is a human granulosa tumor cell line KGN,[110] which was used to study the inhibition of aromatase by tributyltin compounds.[111] In this study, tributyltin compounds caused cytotoxicity to KGN cells at concentrations of 1000 ng/ml, whereas concentrations of 20 ng/ml resulted in lowered estrogen biosynthesis and aromatase activity. This decrease in aromatase activity was the result

of lowered aromatase mRNA levels due to downregulation of aromatase gene transcription at promoter II.

4.1.3 Other *In Vitro* Mammalian Cell Systems for Measuring Aromatase Activity

Isolated ovaries, ovarian follicles, ovarian granulosa cells, and ovarian microsomes from various animal species (including humans) have also been utilized to examine the effects of various environmental contaminants on aromatase activity. These *in vitro* ovarian cell systems have been extensively used for decades in reproductive endocrinology studies.[112] Two types of investigations are generally performed that involve the measurement of ovarian aromatase activity. One type of study are those in which the ovaries are isolated from animals treated with a particular agent and then aromatase activity is measured. These assays will be discussed in detail in the next section (Section 3.4). The second type of study involves using ovarian microsomes or ovarian cells in a culture system for an *in vitro* evaluation of the effects of compounds on aromatase inhibition. Odum and Ashby examined nonsteroidal aromatase inhibitors anastrozole, fadrozole, and letrozole in rat ovarian microsomes, found IC_{50} values similar to those reported from human placental microsomes, and observed much lower aromatase activities in the rat ovarian microsomes.[113] Human granulosa cells isolated from follicles were treated with constituents from cigarette smoke, and the smoke extracts produced dose-dependent inhibition of granulosa cell aromatase activity at the 10 μM to 10 mM concentration range.[114] Human granulosa cells were also utilized by Moran et al.[115] to demonstrated that TCDD does not affect aromatase activity but does lower estradiol production by affecting the availability of androgen precursor. Laskey and colleagues[116] studied steroid production by cultured ovarian fragments as a method for assessment of toxicant-induced alterations in ovarian steroidogenesis in Sprague-Dawley rats. Although these researchers did not examine any environmental agents, they did show that the drug aminoglutethimide produced similar inhibitory activity (IC_{50} of 2.43 mM) as observed in cell culture systems. The polycyclic aromatic hydrocarbons (PAHs), 20-methylcholanthrene and β-naphthoflavone, weakly inhibited aromatase activity in ovarian follicles from coho salmon,[117] and several PAHs also demonstrated weak inhibition of aromatase in *in vitro* incubations with ovaries from flounder.[118] In a recent report, phthalates suppressed CYP19 mRNA levels and aromatase activity in rat granulosa cell cultures.[119]

4.2 *IN VIVO* STUDIES OF AROMATASE TO DETECT ENDOCRINE DISRUPTORS

Evaluation of the *in vivo* effects of endocrine disruptors on aromatase are more complicated, and interpretation of results can be problematic for several reasons. The sensitivity of a species or an individual to an endocrine disruptor may be related to circulating concentrations of gonadal steroid hormones. One important contributor to these hormonal levels in the total organism is the enzyme aromatase. In general, ovarian aromatase is the major source of circulating estrogens in adults. On the other hand, extraglandular sources of estrogen can be important in certain species (such as aromatase in adipose tissues in humans). Alterations in estrogen biosynthesis locally in a tissue may produce significant effects on estrogen levels within that tissue while not altering overall circulating hormone levels.[120] Additionally, alterations in estrogen levels *in vivo* are also influenced by the levels and cyclicity of pituitary gonadtropins.

Therefore, it is critical to differentiate effects via aromatase inhibition and effects via disruption of the hypothalamus-pituitary-ovary axis.

Nonetheless, the hazards of potential endocrine disruptors in the environment can not be determined by *in vitro* experiments alone. Results from screening assay can guide further examination of the compounds' potential for inducing endocrine disruption after *in vivo* exposure. Studies to evaluate the effect of endocrine disruptors have been conducted in an extensive number of both mammalian and nonmammalian species.

Administration of potent aromatase inhibitors (such as the drugs anastrozole, letrozole, and exemestane developed for breast cancer therapy) to adult female cycling rats can result in an inhibition of estrogen biosynthesis, decreases in plasma concentrations of estrogens, a disruption of ovarian cyclicity, a reduction in uterine weight and changes in serum LH.[121-124]

Studies have been conducted in mammalian species to evaluate the effect of endocrine disruptors on the reproductive parameters of both males and females. Several pesticides, including fenarimol, imazalil, prochloraz, and triadimefon, have found to inhibit aromatase *in vivo*. In a three-generation study with imazalil in rats, no reproductive effects were found , although, a trend towards a lower number of live births at 80 mg/kg level was observed.[125] In a multigenerational study, rats were fed doses up to 31.2 mg prochloraz per kg per day in the diet.[126] In males receiving the highest dose, a prolonged aggression was noted.In two-generation studies in rats, fenarimol (6.5 mg/kg) caused reduced fertility and a reduction in live-born litter size. Parturition in females was also reduced.[127] Reproduction studies in in different species showed that fenarimol had adverse effects on reproductive parameters and hormone levels in rats and mice, but not in guinea pigs or rabbits. Mechanistic studies showed that fenarimol inhibits male sex differentiation in the CNS by inhibiting CYP19 aromatase activity and therefore the synthesis of estradiol.[128,129] Triadimefon was given to rats in their diet for two generations at levels of 2.5 or 90 mg /kg/day. Reproductive performance was impaired at the high level. The male/female ratio was reduced in the F2 generation and F1 males showed reduced mating and elevated testosterone, whereas FSH levels were unchanged.[130] A two-generation reproduction study in rats using triadimenol was conducted using levels of 1,4, and 20 mg/kg/day and no reproductive endpoints were affected.[131] In other studies, a dose of 120 mg/kg/day gave rise to an increased rate of resorption of foetuses.[131] Vinggaard et al.[95] cite a previous report from the Danish Environmental Protection Agency that shows that, in long term studies with rabbits receiving the pesticide propioconazole, no effects were observed on the classical parameters of reproductive toxicity. In summary, aromatase inhibitors might in some cases be able to affect hormone levels *in vivo* in rodents, but a general reproductive toxicity cannot be predicted solely from inhibition of aromatase *in vitro*.[95]

Averal *et al.* [132] have suggested that the cytoplasm of sperm may be the target of action of cytotoxic drugs. It has also been hypothesized that the cytoplasmic droplet (small amount of cytoplasm adhered to the middle section of mature sperm) has a role in the aromatization of androgen into estrogen, which has a role in the regulation of male reproductive tract function.[133] The droplet is shed before ejaculation, after fulfilling its role, and then sperm obtain optimal

motility. This should occur by the time the sperm reach the cauda epididymis. In a recent study by Akbarsha *et al.*[134] adult male rats receiving ursolic acid, andrographolide, malathion and dichlorvos showed increased numbers of sperm with retained droplets compared to the control values. Sperm were obtained from the distal cauda epididymis and control treated animals had no sperm with retained droplets compared to 65-95% retained droplets in treated males. Motility of the sperm from treated males was also impaired or completely zero. This may provide an interesting approach to studying the relationship of aromatase in males and its relationship to reproductive toxicity of compounds.

Extensive research has shown the implication of steroids in gonadal sex differentiation of non-mammalian vertebrates. Treatments of larvae or embryos with sex steroid hormones have led to various degrees of sex reversal in fishes, amphibians, reptiles, and birds. This effect has been observed in species with temperature-dependent sex determination and those with genotypic sex determination. During the last two decades, the phenomena of imposex has been described in more than 120 species world-wide. Researchers failed to find a difference in the aromatase activity in the gastropod *Bolinus brandaris* in tributyltin polluted sites compared to non-polluted sites on the Mediterranean coast. There were, however, significant reductions in estradiol titers and an increased testosterone/estradiol ratio.[135]

In turtles, aromatase activity strongly correlates with ovarian differentiation.[136] Willingham and Crews[137] have shown that even 0.4 ng of estradiol applied to the eggshells of the red-eared slider turtle will affect sexual development during embryogenesis. Only 0.2 % of the hormone is believed to end up in the actual embryo. These researchers also found that compounds produced different results when tested individually vs in mixtures. Aroclor 1242 significantly altered aromatase activity levels in the brain but not in the adrenal-kidney-gonad complex during a crucial developmental period but did increase the aromatase activity in this complex just prior to hatch. This may emphasize the importance of testing during different periods of development. Sex is determined by the temperature of the incubating egg and the exogenous application of steroid hormones or steroidogenic enzyme inhibitors can reproduce temperature's effects.[138] At 26 °C male hatchlings are produced, but if estrogen is applied to the eggshell, females result. At 29.4 °C, females are produced, but after applying an aromatase inhibitor , males are produced. The aromatase activity during development can also be used as a marker since its activity exhibits temperature dependence and it appears to be an enzyme critical to thermosensitive sex determination and is capable of modification by extrinsic factors. These embryonic systems of many fish, amphibians, and reptiles may be more sensitive to the low doses of ED which may be present in the environment. Hundreds of eggs may be used in these studies so that statistical power of the study is increased above that of mammalian studies.

Studies by Crain *et al.*,[139] have shown that juvenile female alligators from Lake Apopka, a lake historically contaminated with a number of persistent organochlorines, have a significantly lower mean gonadal-adrenal-mesonephros (GAM) aromatase activity when compared to females from Lake Woodruff, a control lake. In juvenile female alligators treated in a laboratory setting, atrazine induced GAM aromatase activity, while 2,4-dichlorophenoxyacetic acid (2,4-D) had no effect. In *in ovo* experiments, aromatase activity in gonadal tissues from

hatchlings incubated at male-producing temperatures (33°C) was elevated by atrazine, while no effect on aromatase activity was observed at female-producing temperatures (30°C). Additionally, no alteration in hepatic aromatase activities was observed in either sex.[140]

The presence and effect of naturally occurring and synthetic aromatase inhibitors are not well studied. Shilling *et al.*,[94] have investigated the ability of 4-hydroxyandrostenedione, letrozole and clotrimazole (an imidazole fungicide) to inhibit aromatase activity *in vivo* by blocking DHEA induced vitellogenesis in the rainbow trout, *Oncorhynchus mykiss.* Although these agents were able to inhibit ovarian aromatase activity *in vitro*, neither letrozole nor clotrimazole blocked vitellogenin production or estradiol synthesis at doses up to 1000 ppm. This lack of observed *in vivo* activity may be due to species differences in pharmacokinetics or to high variability between individual fish, probably due to the lack of inbreeding compared to most mammalian models used for testing. In an experiment involving the feeding of lake trout to rats, the aromatase activity was lowered in all groups compared to the control.[141] Total thyroxine and estrogen were not significantly different in these offspring of dosed dams from that of controls.

4.3 QSAR APPROACHES TO PREDICTING AROMATASE INHIBITION

Aromatase has not been crystallized to date, and thus no x-ray crystal structure is available for a detailed analysis of the enzyme active site. As a result, two general approaches have been used to study the enzyme active site of aromatase and aromatase inhibitors - computational analysis techniques based upon ligand-based molecular modeling and based upon protein homology modeling. The development of both steroidal and nonsteroidal aromatase inhibitors during the 1980's and 1990's provided initial structure-activity relationships and enabled the application of ligand-based molecular modeling. Early studies focused on energy minimization calculations, conformational analysis, molecular volume calculations, and pharmacophore mapping of aromatase inhibitors.[142-144 145,146] Analysis of the steroidal aromatase inhibitors identified restricted sites (e.g., surrounding the steroid A ring) and accessible sites for structural modifications (e.g., lipophilic pocket at 7α-position). Analysis of nonsteroidal agents identified key components as a heterocyclic ring (proposed interaction with heme iron) and lipophilic aromatic ring (mimic of steroidal backbone). Recently, the Comparative Molecular Field Analysis (COMFA) 3D-QSAR method was applied to the analysis of nonsteroidal aromatase inhibitors, correlating inhibitory activity with steric field values.[147,148]

Protein homology modeling analysis of aromatase has involved modeling the aromatase cytochrome P450 based upon sequence comparisons with bacterial cytochrome P450's that have been crystallized, particularly cytochrome $P450_{cam}$, cytochrome $P450_{terp}$, and cytochrome $P450_{BM-3}$, and site-directed mutational studies.[82,149,150] These models have identified the active site of the enzyme located82near the heme-binding region and the I-helix, with the carboxyl-terminal residues of helix F and the – terminal residues of helix G contributing to the structure of the active site. A report on the comparisons of the results of protein homology modeling analysis with COMFA ligand-based molecular modeling revealed several mismatches between the modeling approaches.[148] In summary, further modeling approaches are needed before

docking and *in silico* screening methods can be applied. Particularly, the accuracy of protein homology modeling cannot be easily determined until a crystal structure of aromatase (or other closely similar human cytochrome P450s) is solved. Similarly, ligand-based modeling and COMFA are derived from a finite number of aromatase inhibitors and do not probe the entire active site space.

4.4 ADDITIONAL INFORMATION FROM INTERVIEWEES

Investigators were initially contacted via e-mail in late June 2001 or early July 2001 describing the EPA contract and the title and objectives/goals of this particular work assignment. A document was attached to the e-mail describing in more detail the objectives/goals of this particular work assignment, discussing the two study designs under consideration (human placental microsomal aromatase assay, human choriocarcinoma cell culture assay), and listing questions. Follow-up telephone calls and/or e-mail messages were placed to the investigators in the U.S. Follow-up e-mail messages were sent to investigators in Europe. The original e-mail document containing the questions and the responses are presented in Appendix B.

Discussions were held with Dr. Shiuan Chen (USA), Dr. Raj Tekmal (USA), Dr. Edwin Lephart (USA), and Dr. Sari Mäkelä (Finland). All of the investigators indicated that an *in vitro* assay for aromatase inhibition would be useful as a high-throughput screening method for identifying compounds as potential endocrine disruptors for subsequent evaluation. The advantages of the two assays (the placental microsomal aromatase assay and the human cell aromatase assay) include the availability of specific radiolabeled substrate, use of standard conditions and methodologies, and the ease of performing the assays. Human placenta can be obtained and the microsomal preparations are straightforward. The microsomal assay may show inhibitory activity for compounds that are not capable of crossing the cell membrane and entering the cell. The JEG-3 cells are commercially available, and use of this assay allows for evaluation of issues related to cell membrane transport.

Both Dr. Chen and Dr. Mäkelä discussed alternative cell-based assays using stably transfected cell culture systems exhibiting increased aromatase expression. Such cell lines have been generated by several research groups and exhibit less variation in the aromatase activity. Dr. Chen has used both transfected MCF-7 breast cancer cells, transfected SK-BR-3 breast cancer cells, and transfected CHO cells.[106] He has also generated cell lines expressing mutant aromatase,[83] and he recently has expressed aromatase from other species (not human) in CHO cells[161]. These transfected cell systems are used by the investigators as research tools for investigations on the role of aromatase, and the transfected cells are not available for large scale production and distribution at this time.

Concerns were raised about the sole use of *in vitro* assays. Activity *in vitro* does not necessarily indicate activity *in vivo*. It is difficult to correlate *in vitro* assay activity and activity *in vivo*. Dr. Mäkelä described this concern in her response, and a very recent publication by this research group further emphasizes the matter.[151] Compounds showing activity in the *in vitro* assay must be further tested *in vivo*. Using intact animals, it is relatively difficult and/or time-

consuming to detect aromatase inhibition reliably. Agents producing weak inhibition are not typically identified by measuring changes in circulating estrogen and/or androgen concentrations. Weak inhibitors more likely to act locally in estrogen target tissues are not so easily detected. Also, measurement of small changes in serum estrogen and/or androgen concentrations is difficult. *In vivo* testing may be more easily performed using transgenic animals overexpressing aromatase gene, as they display very prominent reproductive phenotypes that are relatively easily detected (e.g. cryptorchidism and gynecomastia). Both Dr. Tekmal and Dr. Mäkelä have developed and used such animals.[73,152,153] Also, nude mice injected with MCF-7 cells (or other estrogen-responsive cancer cells) overexpressing aromatase gene may be useful here.

Dr. Lephardt and Dr. Mäkelä also discussed the need for *in vivo* endpoints, such as effects on ovarian function, on testicular function, on gonadotropin secretion patterns, on the onset of puberty, on mammary gland development and tumorigenesis, on prostate gland development and tumorigenesis, on reproductive and sexual behavior, and on bone strength and osteoporosis.

Finally, several important questions related to the use of placental aromatase were raised by interviewees. Aromatase activity is regulated in a tissue-specific fashion using various mechanisms that differ in the tissue sites. What are the reasons to use placental tissue/cells in the assay, and what are the reasons that only placental tissue sources are being tested? There may be an endocrine effect on aromatase by endocrine disrupters (in other tissue sites with weak aromatase expression) that would not be picked up in the human placental assay.

4.5 SUMMARY OF DOCUMENTS REVIEWED AND CONTACT INFORMATION FOR INVESTIGATORS

Appendix A lists the documents reviewed (in alphabetical order), and a detailed electronic database of references is provided in Reference Manager format. Also provided in Appendix B is a list of the principal investigators and/or authors contacted, including addresses, phone numbers, and e-mail addresses. Copies of e-mail responses are also included in Appendix B.

5.0 CANDIDATE PROTOCOLS FOR AN *IN VITRO* ASSAY

As described in Sections 3.3 and 3.4, numerous assay systems have been utilized for the examination of the effects of exogenous agents, such as endocrine disruptors, on inhibition of aromatase activity. The human placental microsomal assay is one of the most common assays used for measuring aromatase inhibition. The human placental microsomal aromatase assay is a straightforward, inexpensive, and rapid *in vitro* assay for measuring the effects on chemicals on aromatase activity. The tissue source, normal human term placenta, is readily available as discarded tissues. A possible alternative tissue source for human placenta could be recombinant human aromatase in microsomal preparations from stably-transfected insect cells which is commercially available.[77] The human placental microsomal assay has been utilized to evaluate the ability of various endocrine disruptors to inhibit aromatase. For these reasons, the placental microsomal assay has been selected as one of the candidate protocols for consideration as an *in vitro* assay in the Tier 1 Screening Battery.

Aromatase inhibition of endocrine disruptors have also been evaluated in intact cell systems such as the JEG-3 or JAR choriocarcinoma, the H295R adrenocortical carcinoma or the KGN granulosa cell culture lines; in general, the results for a wide variety of chemical compounds were similar to the results obtained from the human placental microsomal system. Advantages of a whole cell-based assay is that it can provide information on membrane transport abilities of the test agent and the assay can provide information on the effect of the test agent on the expression of the aromatase protein, i.e., if the test compound will induce or suppress the production of new aromatase protein. The choriocarcinoma cell-based aromatase assay has been selected as a second candidate protocol for consideration as an *in vitro* assay in the Tier 1 Screening Battery.

Both the human placental aromatase assay and the JEG-3 or JAR choriocarcinoma cells have been used in the development of potent aromatase inhibitors for breast cancer therapy. Table 1 provides comparative data from these two *in vitro* assays for nonsteroidal aromatase inhibitors,[154,155] several flavonoid derivatives,[151] and the pesticide fenarimol.[95]

Table 1. Comparison of Aromatase Inhibition in *In Vitro* Assays

Compound	*IC_{50} from human placental microsomal assay (μM)*	*IC_{50} from choriocarcinoma cell line assay (μM)*
Therapeutic Agents		
Aminoglutethimide	1.90	15.8
Letrozole	0.011	0.070
Anastrozole	0.023	0.990
Fadrozole	0.005	0.070
Flavanoid Derivatives		
Flavone	8.0	>100
7-Hydroxyflavone	1.0	0.35
Chrysin	0.5	0.5
Apigenin	2.9	0.18
Naringenin	9.2	1.4
Pesticide		
Fenarimol	10.0	2.0

5.1 CANDIDATE ASSAY A - PLACENTAL MICROSOMAL AROMATASE

Figure 7 shows the process used in the placental microsomal aromatase assay.

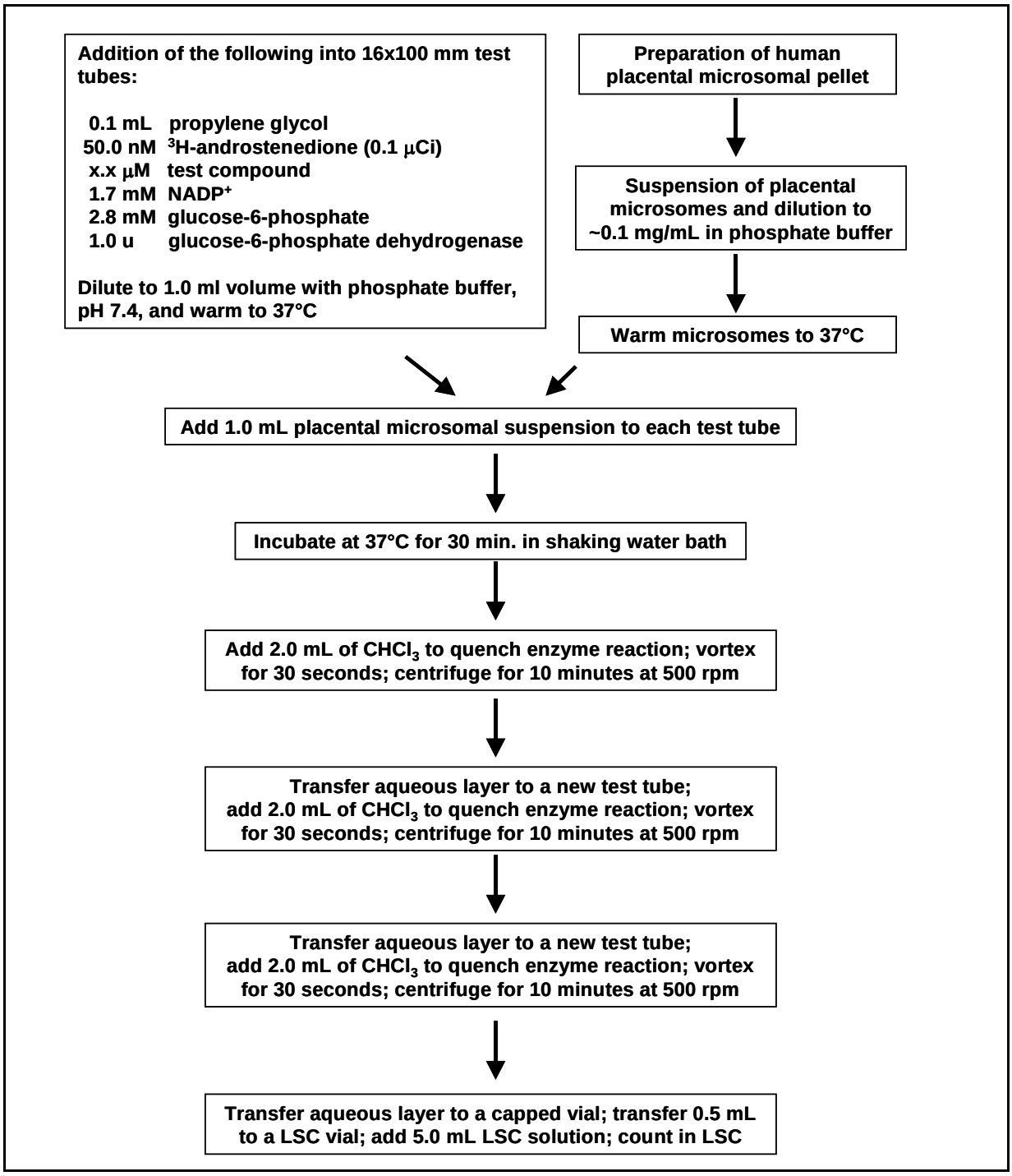


Figure 7. Flow Diagram of the Placental Microsomal Aromatase Assay

5.1.1 Description of the Placental Microsomal Aromatase Assay

Source of Placental Tissue, Methods for Preparation of Microsomes. Normal placenta is obtained after delivery from a local hospital, packed in ice and forwarded immediately to the laboratory. While keeping the placenta at 4°C , the membrane and fibrous material are dissected and removed, and the tissue is then homogenized in a buffer (2:1, w:v) containing 0.25 M sucrose, 0.05 M sodium phosphate, pH 7.0, and 0.04 nicotinamide. The microsomal preparation, is isolated by differential centrifugation procedures. The tissue homogenate is centrifuged at 10,000 x g for 30 minutes; the supernatant is then centrifuged at 105,000 x g for one hour to obtain the crude microsomal pellet. The supernatant is removed, and the microsomal pellet is resuspended in 0.1 M sodium phosphate buffer, pH 7.4, and recentrifuged at 105,000 x g for one hour to wash the microsomes. This washing procedure is repeated one additional time. The twice-washed microsomal pellet is resuspended in 0.1 M sodium phosphate buffer, pH 7.4, containing 0.25 M sucrose, 20% glycerol, and 0.05 mM dithiothreitol, and then frozen at -80°C until needed. Under these storage conditions, the microsomal suspension retains aromatase activity for more than one year. For the enzyme assay, the microsomal suspension is thawed and resuspended in 0.1 M sodium phosphate buffer, pH 7.4, at the appropriate microsomal protein concentration determined using standard methods. Also, a recombinant human aromatase in microsomal preparations from stably-transfected insect cells is commercially available.[77]

Method of Exposure. A stock solution of the chemical and/or product to be evaluated is prepared in 0.1 M sodium phosphate buffer, pH 7.4. If the test chemical is insoluble in aqueous solution, then the chemical is dissolved in ethanol or DMSO and diluted to the appropriate concentration in 0.1 M sodium phosphate buffer, pH 7.4, with the amount of ethanol or DMSO less than 0.5%.

Dose Selection Procedures and Number of Replicates. A screening assay to determine initial aromatase inhibition is performed at concentrations of 0.1, 1.0, and 10 μM in quadruplicate. For compounds exhibiting 10% inhibitory activity or more, full dose-response studies are performed. Full dose-response studies of the chemical and/or product are performed at doses from 1.0 nM (1.0×10^{-9} M) to 1.0 mM (1.0×10^{-3} M). Accurate determination of IC_{50} values for steep dose-response curves may require that the doses tested differ by 0.5 log units (i.e., log values of -9.0, -8.5, -8.0, -7.5, etc.), and the number of replicates to be used is four. Solubility of lipophilic agents may limit the concentration range in the assays

Controls. Both positive and negative controls are required. Negative controls (blank) consist of the use of boiled diluted microsomal suspension. Incubations with the substrate only serve as positive controls and identify the maximum aromatase activity present under the particular enzyme assay conditions. Under these assay conditions using a frozen microsomal preparation as the enzyme source, the range of aromatase activity is typically 40 to 70 pmol (10 to 19 ng) product formed per mg microsomal protein per minute. Equally important in assays is the inclusion of reference compounds, such as a potent steroidal inhibitor, such as 4-hydroxyandrostenedione, exemestane, or a 7α-substituted androstenedione, or a potent

nonsteroidal inhibitor such as anastrozole or letrozole. The concentration of the potent aromatase inhibitor will be 0.1 μM.

Test Conditions. Incubations are performed in 16x100 mm test tubes maintained at 37°C in a shaking water bath. An aliquot (100 μL) of propylene glycol is added to the tubes to serve as a co-solvent. The substrate, [1β-^{3}H]-androstenedione (0.1 μCi, 50 nM), and the inhibitor at test concentrations are added to the tubes. An NADPH-generating system comprised of NADP+ (1.7 mM), glucose-6-phosphate (2.8 mM) and glucose-6-phosphate dehydrogenase (1.0 units) is added to each tube. The tubes are placed at 37°C in the water bath for five minutes. The assay begins with the addition of the diluted placental microsomal suspension (~0.1 mg microsomal protein/mL). The total volume is 2.0 mL, and the tubes are incubated for 30 minutes. The incubations are stopped by the addition of chloroform (2.0 mL) and the tubes vortexed for 30 seconds. The tubes are then centrifuged for 10 minutes at 500 rpm. The aqueous layers are transferred to new test tubes and extracted again with chloroform (2.0 mL). This extraction procedure is performed one additional time. The aqueous layers are transferred to vials, and an aliquot (0.5 mL) is removed and transferred to a 7.0 mL liquid scintillation counting vial. Liquid scintillation solution (emulsion-type, 5.0 mL) is added to each counting vial and shaken to mix the solution. The amount of ^3H_2O in each counting vial is determined using a ^{3}H counting protocol available on a standard liquid scintillation counter.

Endpoint Measured. The amount of ^3H_2O formed in each assay tube is determined from the ^{3}H counts per minute (cpm) detected by the counter. The number of disintegrations per minute (dpm) are calculated by dividing the cpm by the ^{3}H counting efficiency for the liquid scintillation counter (determined using certified sealed ^{3}H standards). The average dpm value of blank (negative control) is subtracted from the dpm value of each tube to give net dpm of ^3H_2O detected.

Data Collected. The total amount of ^3H_2O formed in each assay tube is calculated from the net dpm of ^3H_2O detected by LSC, corrected for the volume of the aliquot sampled. The amount of estrogen product formed in the microsomal assay is determined by dividing the total amount of ^3H_2O formed by the specific activity of the [1β-^{3}H]-androstenedione substrate (expressed in nmol/dpm). The velocity of the enzyme reaction is expressed in nmol mg protein^{-1} min^{-1} and is calculated by dividing the amount of estrogen formed by the product of mg microsomal protein used times 30 minutes.

Incubations with the substrate only are a positive control and identify the maximum aromatase activity or maximum amount of estrogen formed (100% activity) present under the particular enzyme assay conditions. The percentage of enzyme activity for each sample is calculated by dividing the amount of estrogen formed in the sample by the maximum amount of estrogen formed in the positive control. The percentage of aromatase inhibition is determined by subtracting the percentage enzyme activity in each sample from 100%.

Quality Assurance Guidelines. Quality assurance (QA) guidelines are established to ensure that the assay is running at optimal conditions. Factors that influence optimal assay

conditions include sufficient substrate concentrations, sufficient cofactor concentrations, microsomal protein concentrations, test agent solubility, positive controls, and methods to detect inter-assay variability.

Known False Negatives, Known False Positives. Using the proper negative and positive controls, the only false positive for aromatase inhibition is the decrease in enzyme activity as the result of enzyme degradation produced by a test chemical, such as an organic solvent.

Sensitivity of the Assay, Lowest Level of Detection. The microsomal aromatase assay can measure as low as 0.20 pmol of estrogen formed per mg microsomal protein per minute. The minimum limit of aromatase inhibition that can be measured is a difference of 0.20 pmol of estrogen formed per mg microsomal protein per minute.

Statistical Methods. The means, standard errors, and standard deviations are calculated for each concentration of test compound. The unpaired student t-test is used to determine statistically significant differences with a p value of >0.05 between test compound and positive controls. The full dose-response studies are analyzed by nonlinear regression analysis, and dose-response curves are generated. The IC_{50} values are determined from dose-response studies, and the log IC_{50} values, standard err of the log IC_{50} values, and the correlation coefficient also are obtained in this analysis. Standard nonlinear regression analysis of sigmoidal dose-response curves are available on commercial software packages such as SigmaPlot or GraphPad Prism.

Decision Criteria. The activity of a test compound can be classified based upon its IC_{50} value. Common convention in the literature is comparison of the IC_{50} value for a particular agent relative to the IC_{50} value for aminoglutethimide (range from 1.0 to 8.0 μM). For an approximate comparison of agents, many investigators in the field have used the terms no activity (no aromatase inhibition measured), weak activity (IC_{50} value of greater than 20 μM), moderate activity (IC_{50} value between 1 and 20 μM), and strong activity (IC_{50} value less than 1 μM).

5.1.2 Strengths of Placental Microsomal Aromatase Assay

The human placental microsomal aromatase assay is one of the most common *in vitro* assays used for measuring aromatase and aromatase inhibition because of its reliability, reproducibility, and ease of use. The overall protocol of the assay is straightforward for individuals with undergraduate laboratory experiences in biology, biochemistry, and/or molecular biology.

The route of administration/exposure of the test compounds involve dissolving an accurate amount of the compound in ethanol or DMSO and pipetting into appropriate test tubes. The recommended dosing period or duration is 30 minutes.

The measurements of endpoints directly determines the amount of aromatase activity present and the amount of inhibition. The assay involves the use of liquid scintillation counting, which provides excellent efficiency (50%) for the detection of ^{3}H radioisotope used in this assay.

5.1.3 Weaknesses And/or Limitations of Placental Microsomal Aromatase Assay

This *in vitro* enzyme assay is limited to measuring the inhibitory effects of the test agent on aromatase enzymatic activity. It will not provide any information on the effect of the test agent on the expression of the aromatase protein, i.e., if the test compound will induce or suppress the production of new aromatase protein. As described, it is not an enzyme kinetic assay and will not determine the mechanism of aromatase inhibition, i.e., competitive, noncompetitive, or irreversible.

The microsomal fraction of cells is comprised of the smooth endoplasmic reticulum membrane, which also contains other steroid metabolizing enzymes (such as 17β-hydroxysteroid dehydrogenase) and other cytochrome P450 enzymes. The test compounds may influence (inhibit) the activity of these "competing" enzymes, and cause indirect changes in the level of aromatization without any interaction with aromatase enzyme.

A limitation of the assay may arise regarding route of administration/exposure of the test compounds if the test compound is insoluble in aqueous solutions and sparingly soluble in ethanol or DMSO. This solubility problem may limit the doses used in the dose-response studies.

Another limitation is the protein degradation and the loss of aromatase enzymatic activity. This can be minimized by storage of isolated placental microsomes in small aliquots at -80 °C, with the level of enzyme activity stable for approximately 6 to 12 months.

The assay does utilize radioactivity (^{3}H) and the limitations of the use of radioactive materials exist (licensing, record-keeping, personnel training, storage, disposal, etc.).

A final potential limitation is the access and availability of human term placenta as a tissue source. Use of discarded human tissues falls under regulations for research involving human subjects, and thus the research protocols require approval from an institutional human subjects review committee. Additionally, access to such tissues may be limited to certain institutions (e.g., universities but not industry) depending upon hospital policies, and access requires a nearby hospital with a delivery ward. It appeared that this problem with the use of human tissues could be eliminated by using another placental tissue, such as bovine placenta. Bovine placenta can be obtained from slaughterhouses or farms and processed in a similar manner to tissue from humans. Publications using bovine placenta for inhibition measurements or for evaluation of endocrine disruptors are lacking; however, recent studies by RTI showed bovine placenta to be difficult to work with and to have low aromatase activity. It was concluded that bovine placenta is not a practical alternative to human placenta. Only a few published reports on the commercially available recombinant human microsomal aromatase preparations have appeared.[77,160]

Variability in the levels of aromatase cytochrome P450 per mg microsomal protein and in levels of overall cytochrome P450 per mg microsomal protein among placental tissues exist and can also be a concern. Examples include patient heterogeneity and differences between placental

tissues obtained from smokers vs. non-smokers. No aromatase isozymes exist in humans, and only a few mutations of the enzyme have been reported in humans.

5.1.4 Test Method Performance and Test Method Reliability

An active test compound is evaluated in a full dose-response study (1.0 nM to 1.0 mM) with quadruplicate samples at each dose concentration tested. The full dose-response studies will be analyzed by nonlinear regression analysis, and dose-response curves will be generated. The IC_{50} values are determined from dose-response studies, and the log IC_{50} values, standard err of the log IC_{50} values, and the correlation coefficient will also be obtained in this analysis.

This assay endpoint, i.e., measurement of aromatase activity, is accurate and reproducible. The human placental microsomal aromatase assay is one of the most common *in vitro* assays used for measuring aromatase and aromatase inhibition.[25-38] The assay has been utilized extensively and performed reproducibly since the late 1970's. It continues to be used in academic labs and pharmaceutical firms as the initial biological evaluation for potential steroidal and nonsteroidal aromatase inhibitors. The endpoint of the measurement of aromatase activity by the radiometric assay (^3H_2O method) is accurate as the assay measurement and is in agreement with the product isolation method.

The relative inhibitory activities of aromatase inhibitors in the human placental microsomal assay are similar between various laboratories. However, the actual IC_{50} values vary from lab to lab due to variable experiment conditions employed in the particular laboratory. The table below illustrates the variability in the IC_{50} values for aminoglutethimide and for several flavonoids examined in five different laboratories using the human placental microsomal aromatase assays. Experimental conditions varied greatly among the five laboratories, and key differences were observed in substrate concentrations (from 13 nM to 875 nM) and microsomal protein concentrations (from 0.05 mg/mL to 10 mg/mL). Since both the substrate androstenedione and many of the flavonoids are lipophilic, such variability in microsomal lipid membrane preparations in the assay can significant affect the actual concentrations of substrate and inhibitor and result in varying IC_{50} values.

Table 2. Comparisons of IC_{50} Values for Flavonoids

compound	LeBail et al. 2000[88]	Wang et al. 1994[90]	Pelissero et al. 1996[93]	Jeong et al. 1999[85]	Chen et al. 1997[156]
aminoglutethimide*	1.0 μM	5.0 μM	130.0 μM	1.6 μM	5.5 μM
7,8-dihydroxyflavone	8.0 μM			8.8 μM	55.0 μM
chrysin	0.7 μM			4.4 μM	11.0 μM
coumestrol	17.0 μM	25.0 μM			
flavone	48.0 μM		375.0 μM		
flavanone			250.0 μM	34.8 μM	

*Aminoglutethimide is used as a known inhibitor for comparisons, with IC_{50} values typically ranging from 1.0 to 10.0 μM.

5.2 CANDIDATE ASSAY B - AROMATASE ACTIVITY IN CELL LINES

Figure 8 shows an example of the process used in the aromatase assay in the JEG-3 cell line.

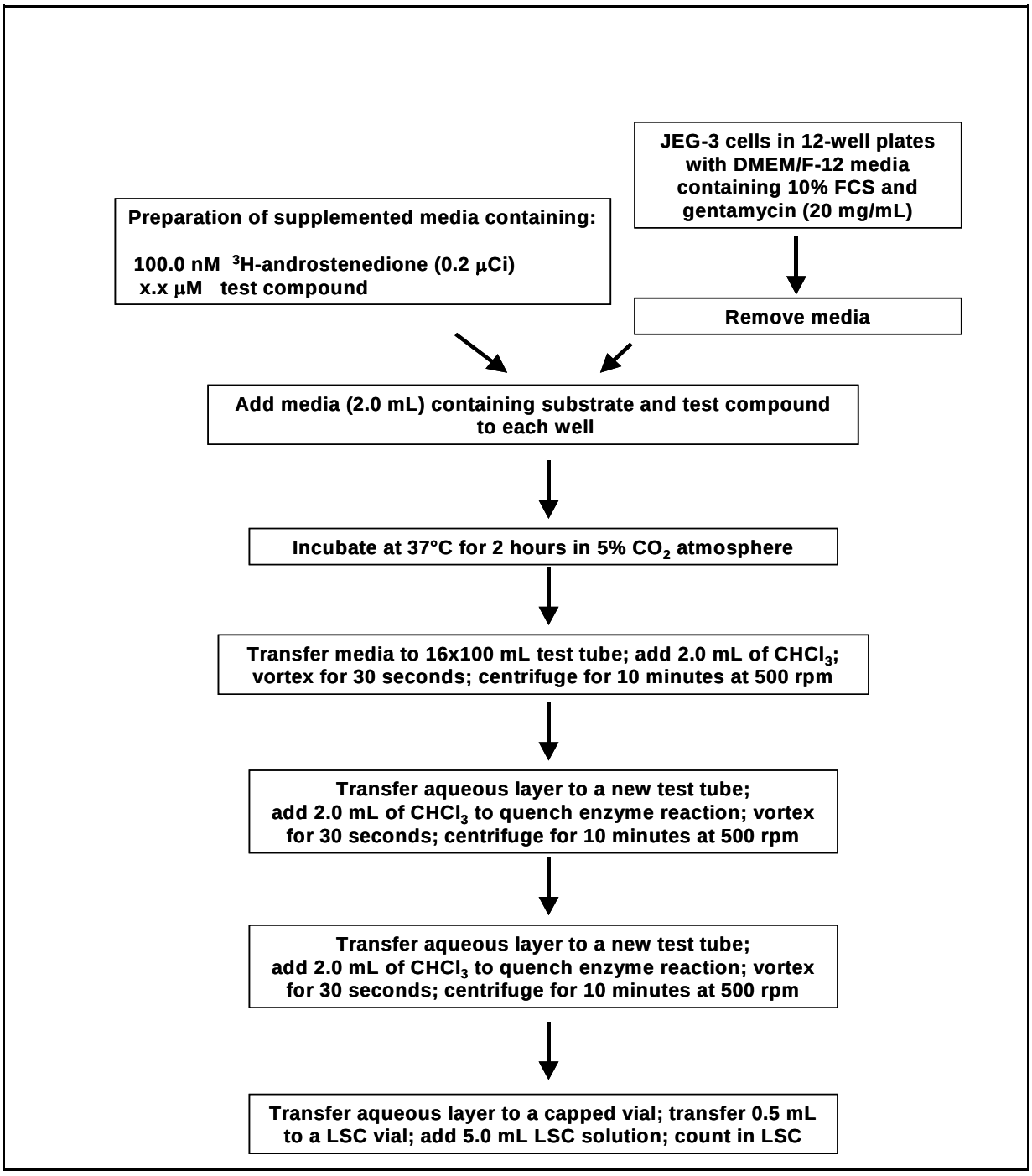


Figure 8. Flow Diagram of the Aromatase Assay in JEG-3 Cells

5.2.1 Description of the Aromatase Assay in JEG-3 Cell Line

Source of Cell Lines. Human JEG-3 cells, ATCC no. HTB-36, are obtained from American Tissue Culture Collection. The cells are maintained in DMEM/F-12 media supplemented with 10% heat-inactivated fetal calf serum and gentamycin (20 mg/mL) and incubated at 37°C in a 5% CO_2 atmosphere. For the determination of aromatase inhibition, JEG-3 cells are seeded into 12-well culture plates at a concentration of 50,000 cells per well in 2.0 mL of supplemented DMEM/F-12 media. The cells are incubated at 37°C in a 5% CO_2 atmosphere for 2 days, reaching approximately 40-50% confluency. Cell viability is determined using a standard formazan dye method, such as MTT or MTS assay.[157-159]

Method of Exposure. A stock solution of the chemical and/or product to be evaluated is prepared in 0.1 M sodium phosphate buffer, pH 7.4 or culture medium. If the test chemical is insoluble in aqueous solution, then the chemical is dissolved in ethanol or DMSO and diluted to the appropriate concentration in 0.1 M sodium phosphate buffer, pH 7.4 or culture medium, such that the concentration of ethanol or DMSO constitutes less than 0.5% of the volume of the incubation mixture.

Dose Selection Procedures and Number of Replicates. A screening assay to determine initial aromatase inhibition is performed at concentrations of 0.1, 1.0, and 10.0 μM in quadruplicate. For compounds exhibiting 10% inhibitory activity or more, full dose-response studies are performed. Full dose-response studies of the chemical and/or product are performed at doses from 1.0 nM (1.0×10^{-9} M) to 1.0 mM (1.0×10^{-3} M). Accurate determination of IC_{50} values for steep dose-response curves may require that the doses tested differ by 0.5 log units (i.e., log values of -9.0, -8.5, -8.0, -7.5, etc.), and the number of replicates to be used is four. Solubility of lipophilic agents may limit the concentration range in the assays to 1.0 or 10.0 μM.

Controls. Both positive and negative controls are required. Negative controls (blank) consist of the media only (no cells). Incubations with the substrate only are a positive control and identify the maximum aromatase activity present under the particular enzyme assay conditions. Under these assay conditions, the range of aromatase activity is typically 20.0 to 25.0 pmol (5.4 to 6.8 ng) product formed per mg cellular protein per hour. Equally important in assays is the inclusion of reference compounds, such as a potent steroidal inhibitor, such as 4-hydroxyandrostenedione, exemestane, or a 7α-substituted androstenedione, or a potent nonsteroidal inhibitor such as anastrozole or letrozole. Potent aromatase inhibitors yield significant enzyme inhibition at concentrations of 1 μM.or lower.

Test Conditions. At the beginning of the experiment, the JEG-3 cells in 12-well plates are taken from the cell culture incubator and the media removed. Fresh supplemented media (2.0 mL) is added to the wells along with the substrate, [1β-^{3}H]-androstenedione (0.2 μCi, 100 nM), and the inhibitor at test concentrations is added to the tubes. The cells are then incubated at 37°C in a 5% CO_2 atmosphere for 2 hours. After 2 hours, the media is transferred to 16x100 mm test tubes for subsequent extraction. Cell viability is determined using a standard formazan dye method, such as MTT or MTS assay. Extraction of the media is accomplished by adding

chloroform (2.0 mL) to each tube, and the tubes vortexed for 30 seconds. The tubes are then centrifuged for 10 minutes at 500 rpm. The aqueous layers are transferred to new test tubes and extracted again with chloroform (2.0 mL). This extraction procedure is performed one additional time. The aqueous layers are transferred to vials, and an aliquot (0.5 mL) is removed and transferred to a 7.0 mL liquid scintillation counting vial. Liquid scintillation solution (emulsion-type, 5.0 mL) is added to each counting vial and shaken to mix the solution. The amount of ^3H_2O in each counting vial is determined using a ^3H counting protocol available on a standard liquid scintillation counter.

Endpoint Measured. The amount of ^3H_2O formed in each assay tube is determined from the ^3H counts per minute (cpm) detected by the counter. The disintegrations per minute (dpm) value is calculated by dividing the cpm by the ^3H counting efficiency for the liquid scintillation counter (determined using certified sealed ^3H standards). The average dpm value of blank (negative control) is subtracted from the dpm value of each tube to give net dpm of ^3H_2O detected.

Data Collected. The total amount of ^3H_2O formed in each assay tube is calculated from the net dpm of ^3H_2O detected by LSC, corrected for the volume of the aliquot sampled. The amount of estrogen product formed in the cell-based assay is determined by dividing the total amount of ^3H_2O formed by the specific activity of the [1β-^3H]-androstenedione substrate (expressed in dpm/nmol). The velocity of the enzyme reaction is expressed in nmol per well.

Incubations with the substrate only are a positive control and identify the maximum aromatase activity or maximum amount of estrogen formed (100% activity) present under the particular assay conditions. The percentage of enzyme activity for each sample is calculated by dividing the amount of estrogen formed in the sample by the maximum amount of estrogen formed in the positive control. The percentage of aromatase inhibition is determined by subtracting the percentage enzyme activity in each sample from 100%.

Quality Assurance Guidelines. Quality assurance (QA) guidelines are established to ensure that the assay is running at optimal conditions. Factors that influence optimal assay conditions include sufficient substrate concentrations, number of viable cells, test agent solubility, positive controls, and methods to detect inter-assay variability.

Known False Negatives, Known False Positives. Using the proper negative and positive controls, the only false positive for aromatase inhibition would be the decrease in enzyme activity as the result of cell cytotoxicity or cell death, such as was observed with certain organochlorines described in Section 3.3.2.

Sensitivity of the Assay, Lowest Level of Detection. The sensitivity of the assay is dependent on the specific activity of the substrate, the limit of detection of the counter (usually as low as twice background - background is typically < 30 dpm), and the non-specific activity found in the negative control samples (usually approaches background levels). The calculation of IC_{50}s is dependent on calculation of the percent of control activity remaining in samples exposed to test substances. Control activities typically range near 15 pmol/mg/min and the

specific activity of the substrate is typically about 1 μCi/nmol. This equates to about 33000 dpm of ^3H_2O formed per well. For the calculation of IC_{50} it is desirable to be able to quantitate values as low as 5% of control (ca. 1700 dpm/well), which is easily accomplished using LSC techniques.

Statistical Methods. The means, standard errors, and standard deviations are calculated for each concentration of test compound. The unpaired student t-test is used to determine statistically significant differences with a p value of >0.05 between test compound and positive controls. The full dose-response studies will be analyzed by nonlinear regression analysis, and dose-response curves will be generated. The IC_{50} values are determined from dose-response studies, and the log IC_{50} values, standard error of the log IC_{50} values, and the correlation coefficient will also be obtained in this analysis. Standard nonlinear regression analysis of sigmoidal dose-response curves are available on commercial software packages such as SigmaPlot or GraphPad.

Decision Criteria. The activity of a test compound will be classified based upon its IC_{50} value. Common convention in the literature is comparison of the IC_{50} value for a particular agent relative to the IC_{50} value for aminoglutethimide (range from 1.0 to 8.0 μM). For an approximate comparison of agents, many investigators in the field have used the terms no activity (no aromatase inhibition measured), weak activity (IC_{50} value of greater than 20 μM), moderate activity (IC_{50} value between 1 and 20 μM), and strong activity (IC_{50} value less than 1 μM).

Strengths of the Aromatase Assay in the JEG-3 Cell Line. Human JEG-3 choriocarcinoma cell culture lines have been used as *in vitro* systems for determination of the effects of compounds on aromatase activity and for investigations on the effects of agents in placental toxicology. The assay involves standard mammalian cell culture techniques and can be performed reliably by individuals with cell biology and culturing experiences.

The route of administration/exposure of the test compounds involves dissolving an known amount of the compound in ethanol or DMSO and pipetting into appropriate wells. The recommended dosing period or duration is 2 hours, but this can be adjusted improve sensitivity or minimize degradation of test agents. The measurement of endpoints directly determines the amount of aromatase activity present and the amount of inhibition.

The assay involves the use of liquid scintillation counting, which provides excellent sensitivity for this assay. One advantage of this cell culture assay is that it can provide information on the effect of the test agent on the expression of the aromatase protein, i.e., if the test compound will induce or suppress the production of new aromatase protein.

Weaknesses and/or Limitations of the Aromatase Assay in the JEG-3 Cell Line. This *in vitro* cell culture assay is limited to measuring the effect of the test agent on aromatase enzymatic activity. It is not an enzyme kinetic assay and will not determine the mechanism of aromatase inhibition, i.e., competitive, noncompetitive, or irreversible.

Aromatase activity in JEG-3 cells varies markedly during the growth phase, particularly at confluence. Also, other steroid metabolizing enzymes are present in JEG-3 cells. The labeled substrate may be metabolized by other enzymes, thus not allowing its aromatization (i.e. the amount of substrate available for aromatase may be modulated by other enzymes). The test compounds may influence (inhibit) the activity of these "competing" enzymes, and cause indirect changes in the level of aromatization without any interaction with aromatase enzyme.

Cytotoxicity from the test agents is also a concern and may limit the use of this method in evaluating effects of certain compounds on aromatase activity. An apparent decrease in aromatase activity may arise from cytotoxic effects of the agents, resulting in lower numbers of JEG-3 cells compared to untreated or control-treated cells.

This assay utilizes various cell culture techniques, and thus is limited to laboratories capable of performing studies with mammalian cell cultures. Essential equipment needed include laminar flow hoods, CO_2 incubators, microscopes, liquid nitrogen storage containers, autoclaves, media storage and/or media preparation facilities, etc.

A limitation of the assay may arise regarding route of administration/exposure of the test compounds if the test compound is insoluble in aqueous solutions and sparingly soluble in ethanol or DMSO. This solubility problem may limit the doses used in the dose-response studies. Additionally, test compounds that are too hydrophilic may not readily pass through biological membranes, whereas highly lipophilic compounds could be preferentially retain in the membrane and insufficient intracellular levels be attained .

Another limitation is that possible metabolism or other transformations of test compounds may occur within intact cells used in this assay, and thus aromatase inhibition may be due to a metabolite and not the parent agent. On the other hand, this may provide additional information of possible activity of metabolites.

As stated earlier under strengths, this cell culture assay is that it can provide information on the effect of the test agent on the expression of the aromatase protein. However, since CYP19 expression is tissue specific, the JEG-3 cells may represent induction or suppression of CYP19 expression only under the control of the placental promoter I.1.

The assay utilizes the JEG-3 human choriocarcinoma cell line available from the American Type Culture Collection (ATCC), and the catalog number for the JEG-3 cells is ATCC HTB 36. The alternative cell line, JAR human choriocarcinoma cell line, is also obtained from ATCC, and the catalog number for the JEG-3 cells is ATCC HTB 144. In order to obtain these cells, an investigator must complete the required ATCC forms and agree to ATCC guidelines regarding use of the cells. These guidelines include (1) the line will be used for research purposes only, (2) cell lines and their products shall not be sold or used for commercial purposes, and (3) cells will not be distributed further to third parties for sale or use for commercial purposes. These restrictions from ATCC may be a significant limitation related to use of the cell lines as a screening assay system in commercial settings. Further clarification from ATCC will

be necessary if this protocol is selected.

The assay does utilize radioactivity (^3H) and the limitations of the use of radioactive materials exist (licensing, record-keeping, personnel training, storage, disposal, etc.).

Test Method Performance and Test Method Reliability. An active test compound is evaluated in a full dose-response study (1.0 nM to 1.0 mM) with quadruplicate samples at each dose concentration tested. The full dose-response studies will be analyzed by nonlinear regression analysis, and dose-response curves will be generated. The IC_{50} values are determined from dose-response studies, and the log IC_{50} values, standard error of the log IC_{50} values, and the correlation coefficient will also be obtained in this analysis.

This assay endpoint, i.e., measurement of aromatase activity, is accurate and reproducible. The JEG-3 cell culture assay is a common *in vitro* assays used for measuring aromatase and aromatase inhibition.[25-38] The assay has been utilized extensively and performed reproducibly since the late 1980's. It continues to be used in academic labs and pharmaceutical firms for aromatase inhibition and for investigations on the effects of agents in placental toxicology. The endpoint of the measurement of aromatase activity by the radiometric assay (^3H_2O method) is accurate as the assay measurement and is in agreement with the product isolation method.

The relative inhibitory activities of aromatase inhibitors in JEG-3 cell culture assay are similar to the activities observed in human placental microsomal assays. However, the actual IC_{50} values can vary from lab to lab due to variable experiment conditions, such as substrate concentrations, cell number, and culture conditions, employed in the particular laboratory.

5.2.2 Description of the Aromatase Assay in H295R Cell Line

Source of Cell Line. Human H295R cells, ATCC no. CRL-2128, are obtained from American Tissue Culture Collection. The cells are maintained in DMEM/F-12 media supplemented with365 mg/mL L-glutamine, 15 mM HEPES, 10 mg/L insulin, 6.7 µg/L sodium selenite, 5.5 mg/L transferrin, 1.25 mg/L BSA, 100 U/L penicillin, 100 µg/L streptomycin and 2% steroid-free replacement serum Ultroser SF and are incubated at 37°C in a 5% CO_2 atmosphere. The cultured cells are trypsinized, suspended in culture medium and then seeded (1 mL/well) in 24-well plates and incubated for 24 h to allow attachment. The nearly confluent cells (ca. 2 x 10^5 cells/well) are then ready for exposure to test chemicals. Cell viability is determined using a standard formazan dye method, such as MTT or MTS assay.[157-159]

Method of Exposure. The same method as describe above (Section 5.2.1) for the JEG-3 cells is used.

Dose Selection Procedures and Number of Replicates. The same procedures and number of replicates as described above (Section 5.2.1) for the JEG-3 cells are used.

Controls. The same type of negative and positive controls as described above (Section

5.2.1) for the JEG-3 cells are used. Under these assay conditions with H295R cells, the aromatase activity in positive control samples is typically about 1.4 pmol product formed per mg cellular protein per hour.

Test Conditions. At the beginning of the experiment, the H295R cells in 24-well plates are taken from the cell culture incubator and the media removed. Fresh supplemented media (1.0 mL) is added to the wells along with the substrate, [1β-^{3}H]-androstenedione (0.2 μCi, 50-100 nM), and the inhibitor at test concentrations is added to the wells. The cells are then incubated at 37°C in a 5% CO_2 atmosphere for 1.5 -2 hours. After the incubation period, the media is transferred to 16x100 mm test tubes for subsequent extraction. Extraction of the media is accomplished by adding chloroform (2.0 mL) to each tube, and the tubes vortexed for 30 seconds. The tubes are then centrifuged for 10 minutes at 500 rpm. The aqueous layers are transferred to new test tubes and extracted again with chloroform (2.0 mL). This extraction procedure is performed one additional time. The aqueous layers are transferred to vials, and an aliquot (0.5 mL) is removed and transferred to a 7.0 mL liquid scintillation counting vial. Liquid scintillation solution (emulsion-type, 5.0 mL) is added to each counting vial and shaken to mix the solution. The amount of ^3H_2O in each counting vial is determined using a ^3H counting protocol available on a standard liquid scintillation counter.

Endpoint Measured. The same endpoints as described above in Section 5.2.1 for the JEG-3 cell assay are measured.

Data Collected. The data collected are the same as those described in Section 5.2.1 for the JEG-3 cell assay..

Quality Assurance Guidelines. Quality assurance (QA) guidelines should be the same as those described in Section 5.2.1 for the JEG-3 cell assay.

Known False Negatives, Known False Positives. Using the proper negative and positive controls, the only false positive for aromatase inhibition would be the decrease in enzyme activity as the result of cell cytotoxicity or cell death.

Sensitivity of the Assay, Lowest Level of Detection. The sensitivity of the assay is dependent on the specific activity of the substrate, the limit of detection of the counter (usually as low as twice background - background is typically < 30 dpm), and the non-specific activity found in the negative control samples (usually approaches background levels). The calculation of IC_{50}s is dependent on calculation of the percent of control activity remaining in samples exposed to test substances. Control activities typically range near 1.4 pmol/mg/h and the specific activity of the substrate is typically about 1 μCi/nmol. This equates to about 3100 dpm of ^3H_2O formed per well per h. For the calculation of IC_{50} it is desirable to be able to quantitate values as low as 5% of control (ca. 155 dpm/well), which may be near the lower limit of detection for LSC techniques, but this would be dependent on the size of the aliquots that are assayed for radiochemical content. The sensitivity can be improved by using substrate of higher specific activity.

Statistical Methods. The statistical methods for this assay would be the same as for the JEG-3 cell assay described in Section 5.2.1.

Decision Criteria. The decision criteria for this assay would be the same as for the JEG-3 cell assay as described in Section 5.2.1

Strengths of the Aromatase Assay in the H295R Cell Line. Human H295R adrenocortical carcinoma cell culture lines have been used as *in vitro* systems for determination of the effects of compounds on aromatase activity. The assay involves standard mammalian cell culture techniques and can be performed reliably by individuals with cell biology and culturing experiences.

The route of administration/exposure of the test compounds involve dissolving a known amount of the compound in ethanol or DMSO and pipetting into appropriate culture dishes/wells. The recommended dosing period or duration is 2 hours, but this can be adjusted improve sensitivity or minimize degradation of test agents. The measurements of endpoints directly determines the amount of aromatase activity present and the amount of inhibition.

The assay involves the use of liquid scintillation counting, which provides excellent sensitivity for this assay. One advantage of this cell culture assay is that it can provide information on the effect of the test agent on the expression of the aromatase protein, i.e., if the test compound will induce or suppress the production of new aromatase protein.

Weaknesses and/or Limitations of the Aromatase Assay in Cell Line. This *in vitro* cell culture assay is limited to measuring the effect of the test agent on aromatase enzymatic activity. It is not an enzyme kinetic assay and will not determine the mechanism of aromatase inhibition, i.e., competitive, noncompetitive, or irreversible.

Other steroid metabolizing enzymes are present in H295R cells. The labeled substrate may be metabolized by other enzymes, thus not allowing its aromatization (i.e. the amount of substrate available for aromatase may be modulated by other enzymes). The test compounds may influence (inhibit) the activity of these "competing" enzymes, and cause indirect changes in the level of aromatization without any interaction with aromatase enzyme.

Cytotoxicity from the test agents is also a concern and may limit the use of this method in evaluating effects of certain compounds on aromatase activity. An apparent decrease in aromatase activity may arise from cytotoxic effects of the agents, resulting in lower numbers of H295R cells compared to untreated or control-treated cells.

This assay utilizes various cell culture techniques, and thus is limited to laboratories capable of performing studies with mammalian cell cultures. Essential equipment needed include laminar flow hoods, CO_2 incubators, microscopes, liquid nitrogen storage containers, autoclaves, media storage and/or media preparation facilities, etc.

A limitation of the assay may arise regarding route of administration/exposure of the test

compounds if the test compound is insoluble in aqueous solutions and sparingly soluble in ethanol or DMSO. This solubility problem may limit the doses used in the dose-response studies. Additionally, test compounds that are too hydrophilic may not readily pass through biological membranes, whereas highly lipophilic compounds could be preferentially retain in the membrane and insufficient intracellular levels be attained .

Another limitation is that possible metabolism or other transformations of test compounds may occur within intact cells used in this assay, and thus aromatase inhibition may be due to a metabolite and not the parent agent. On the other hand, this may provide additional information of possible activity of metabolites.

The assay utilizes the H295R human adrenocortical carcinoma cell line available from the American Type Culture Collection (ATCC). In order to obtain these cells, an investigator must complete the required ATCC forms and agree to ATCC guidelines regarding use of the cells. These guidelines include (1) the line will be used for research purposes only, (2) cell lines and their products shall not be sold or used for commercial purposes, and (3) cells will not be distributed further to third parties for sale or use for commercial purposes. These restrictions from ATCC may be a significant limitation related to use of the cell lines as a screening assay system in commercial settings. Further clarification from ATCC will be necessary if this protocol is selected.

The assay does utilize radioactivity (^3H) and the limitations of the use of radioactive materials exist (licensing, record-keeping, personnel training, storage, disposal, etc.).

Test Method Performance and Test Method Reliability. An active test compound is evaluated in a full dose-response study (1.0 nM to 1.0 mM) with quadruplicate samples at each dose concentration tested. The full dose-response studies will be analyzed by nonlinear regression analysis, and dose-response curves will be generated. The IC_{50} values are determined from dose-response studies, and the log IC_{50} values, standard error of the log IC_{50} values, and the correlation coefficient will also be obtained in this analysis.

This assay endpoint, i.e., measurement of aromatase activity, is accurate and reproducible. The H295R cell culture assay is a common *in vitro* assays used for measuring aromatase and aromatase inhibition.[107-109] The assay has been utilized to test the effects of various flavonoids, herbicides and pesticides on aromatase activity and expression.[162-165] The endpoint of the measurement of aromatase activity by the radiometric assay (^3H_2O method) is accurate as the assay measurement and is in agreement with the product isolation method.

The relative inhibitory activities of aromatase inhibitors in H295R cell culture assay are similar to the activities observed in human placental microsomal assays. However, the actual IC_{50} values can vary from lab to lab due to variable experiment conditions, such as substrate concentrations, cell number, and culture conditions, employed in the particular laboratory.

5.2.3 Description of the Aromatase Assay in the KGN Cell Line

Source of Cell Lines. Human KGN cells (catalog number RCB1154) are obtained from Riken Bioresource Center. The cells are maintained in petri dishes or 12-well plates in DMEM/F-12 media supplemented with 10% fetal calf serum and are incubated at 37°C in a 5% CO_2 atmosphere. For the determination of aromatase inhibition, at confluence, the medium is replaced with DMEM/F-12 media with 10% dextran-coated charcoal treated fetal calf serum prior to treatment with test chemicals or androstenedione. Cell viability is determined using tryphan blue exclusion.

Method of Exposure. The same method as describe above (Section 5.2.1) for the JEG-3 cells is used.

Dose Selection Procedures and Number of Replicates. The same procedures and number of replicates as described above (Section 5.2.1) for the JEG-3 cells are used.

Controls. The same type of negative and positive controls as described above (Section 5.2.1) for the JEG-3 cells are used. Under these assay conditions with KGN cells, the aromatase activity in postiive control samples is typically about 0.07 pmol product formed per mg cellular protein per h.

Test Conditions. Cells with fresh medium containing 10% dextran-coated charcoal treated fetal calf serum may be treated with test chemicals for 24-72 h prior to the addition of substrate, [1β-^{3}H]-androstenedione (0.2 mCi, 100 nM). The cells are then incubated at 37°C in a 5% CO_2 atmosphere for 6-12 hours following substrate addition. At the end of the incubation period, the media is transferred to 16x100 mm test tubes for subsequent extraction. Extraction of the media is accomplished by adding chloroform (2.0 mL) to each tube, and the tubes vortexed for 30 seconds. The tubes are then centrifuged for 10 minutes at 500 rpm. The aqueous layers are transferred to new test tubes and extracted again with chloroform (2.0 mL). This extraction procedure is performed one additional time. The aqueous layers are transferred to vials, and an aliquot (0.5 mL) is removed and transferred to a 7.0 mL liquid scintillation counting vial. Liquid scintillation solution (emulsion-type, 5.0 mL) is added to each counting vial and shaken to mix the solution. The amount of ^3H_2O in each counting vial is determined using a ^{3}H counting protocol available on a standard liquid scintillation counter.

Endpoint Measured. The same endpoints as described above in Section 5.2.1 for the JEG-3 cell assay are measured.

Data Collected. The data collected are the same as those described in Section 5.2.1 for the JEG-3 cell assay.

Quality Assurance Guidelines. Quality assurance (QA) guidelines should be the same as those described in Section 5.2.1 for the JEG-3 cell assay.

Known False Negatives, Known False Positives. Using the proper negative and

positive controls, the only false positive for aromatase inhibition would be the decrease in enzyme activity as the result of cell cytotoxicity or cell death.

Sensitivity of the Assay, Lowest Level of Detection. The sensitivity of the assay is dependent on the specific activity of the substrate, the limit of detection of the counter (usually as low as twice background - background is typically < 30 dpm), and the non-specific activity found in the negative control samples (usually approaches background levels). The calculation of IC_{50}s is dependent on calculation of the percent of control activity remaining in samples exposed to test substances. Control activities typically range near 0 07 pmol/mg/h and the specific activity of the substrate is typically about 1 μCi/nmol. This equates to about 930 dpm of ^3H_2O formed per well during a 6 h incubation. For the calculation of IC_{50} it is desirable to be able to quantitate values as low as 5% of control (ca. 46 dpm/well), which may be near the lower limit of detection for LSC techniques, but this would be dependent on the size of the aliquots that are assayed for radiochemical content. The sensitivity can be improved by using substrate of higher specific activity or by lengthening the incubation time.

Statistical Methods. The statistical methods for this assay would be the same as for the JEG-3 cell assay described in Section 5.2.1.

Decision Criteria. The decision criteria for this assay would be the same as for the JEG-3 cell assay as described in Section 5.2.1.

Strengths of the Aromatase Assay in the KGN Cell Line. The human KGN ovarian granulosa-like tumor cell culture line has been used as an *in vitro* system for determination of the effects of compounds on aromatase activity[110-111]. The assay involves standard mammalian cell culture techniques and can be performed reliably by individuals with cell biology and culturing experiences.

The route of administration/exposure of the test compounds involve dissolving an accurate amount of the compound in ethanol or DMSO and pipetting into appropriate test tubes. The recommended dosing period or duration is 6-12 hours, but this can be adjusted improve sensitivity or minimize degradation of test agents. The measurement of endpoints directly determines the amount of aromatase activity present and the amount of inhibition.

The assay involves the use of liquid scintillation counting, which provides excellent sensitivity for this assay. One advantage of this cell culture assay is that it can provide information on the effect of the test agent on the expression of the aromatase protein, i.e., if the test compound will induce or suppress the production of new aromatase protein.

Weaknesses and/or Limitations of the Aromatase Assay in the KGN Cell Line. This *in vitro* cell culture assay is limited to measuring the effect of the test agent on aromatase enzymatic activity. It is not an enzyme kinetic assay and will not determine the mechanism of aromatase inhibition, i.e., competitive, noncompetitive, or irreversible.

Other steroid metabolizing enzymes are present in KGN cells. The labeled substrate may

be metabolized by other enzymes, thus not allowing its aromatization (i.e. the amount of substrate available for aromatase may be modulated by other enzymes). The test compounds may influence (inhibit) the activity of these "competing" enzymes, and cause indirect changes in the level of aromatization without any interaction with aromatase enzyme.

Cytotoxicity from the test agents is also a concern and may limit the use of this method in evaluating effects of certain compounds on aromatase activity. An apparent decrease in aromatase activity may arise from cytotoxic effects of the agents, resulting in lower numbers of KGN cells compared to untreated or control-treated cells.

This assay utilizes various cell culture techniques, and thus is limited to laboratories capable of performing studies with mammalian cell cultures. Essential equipment needed include laminar flow hoods, CO_2 incubators, microscopes, liquid nitrogen storage containers, autoclaves, media storage and/or media preparation facilities, etc.

A limitation of the assay may arise regarding route of administration/exposure of the test compounds if the test compound is insoluble in aqueous solutions and sparingly soluble in ethanol or DMSO. This solubility problem may limit the doses used in the dose-response studies. Additionally, test compounds that are too hydrophilic may not readily pass through biological membranes, whereas highly lipophilic compounds could be preferentially retain in the membrane and insufficient intracellular levels be attained .

Another limitation is that possible metabolism or other transformations of test compounds may occur within intact cells used in this assay, and thus aromatase inhibition may be due to a metabolite and not the parent agent. On the other hand, this may provide additional information of possible activity of metabolites.

The low basal aromatase activity present in KGN cells presents a possible limitation from the standpoint of assay sensitivity. In order to improve sensitivity for this assay, it would be necessary to either increase incubation times (the 6-12 h recommended for this assay is already among the longest suggested for any assay under consideration) or to use more radioactivity per incubation.

The assay utilizes the KGN human ovarian granulosa-like tumor cell line available from the Riken Bioresource Center (Ibaraki, Japan), catalog number RCB 1154. In order to obtain these cells, an investigator must complete the required Riken Bioresrouce Center (RBC) forms and agree to RBC guidelines regarding use of the cells. These guidelines include (1) the intended use of the line will be disclosed to RBC, (2), the line used for research purposes only, (3) cell lines and their products shall not be sold or used for commercial purposes, and (4) cells will not be distributed further to third parties for sale or use for commercial purposes. In addition, for this cell line the permission of the depositor must be obtained before RBC can distribute the cells. These restrictions from RBC may be a significant limitation related to use of the cell lines as a

screening assay system in commercial settings. Further clarification from RBC will be necessary

if this protocol is selected.

The assay does utilize radioactivity (^3H) and the limitations of the use of radioactive materials exist (licensing, record-keeping, personnel training, storage, disposal, etc.).

Test Method Performance and Test Method Reliability An active test compound is evaluated in a full dose-response study (1.0 nM to 1.0 mM) with quadruplicate samples at each dose concentration tested. The full dose-response studies will be analyzed by nonlinear regression analysis, and dose-response curves will be generated. The IC_{50} values are determined from dose-response studies, and the log IC_{50} values, standard err of the log IC_{50} values, and the correlation coefficient will also be obtained in this analysis.

This assay endpoint, i.e., measurement of aromatase activity, is accurate and reproducible. The KGN cell culture assay is a relatively new *in vitro* assay (2001) used for measuring aromatase and aromatase inhibition.[25-38, 166, 167] The endpoint of the measurement of aromatase activity by the radiometric assay (^3H_2O method) is accurate as the assay measurement and is in agreement with the product isolation method.

There is little data on the relative inhibitory activities of aromatase inhibitors in the KGN cell culture assay to the activities observed in human placental microsomal assays.

Table 3. Comparison of the Two Candidate Assays

Placental Microsomal Aromatase Assay	Aromatase Activity in Cell Lines
Strengths:	*Strengths:*
Common assay in use for over 40 years	Standard mammalian cell culture techniques used
Straightforward enzyme assay	Cell lines available from commercial sources
Directly measures inhibitory activity of test compounds	Excellent sensitivity using ^3H radioisotope
Readily availability of placental tissues	Can measure ability of test compounds to induce or to suppress aromatase mRNA or protein levels
Excellent sensitivity using ^3H radioisotope	
Weaknesses:	*Weaknesses:*
Does not determine mechanism of inhibition	Does not determine mechanism of inhibition
Does not measure ability of test compounds to induce or suppress enzyme levels	Aromatase activity varies markedly during cell growth phase
Microsomal preparations contain other enzymes, including other cytochrome P450s	Cytotoxicity of test compounds to the cells
Requires solubility of test compounds in enzyme solution	Other enzymes present in cells that metabolize substrate
Activity altered by protein degradation	Other enzymes present in cells that can metabolize test compounds
Patient variability of cytochrome P450 content in microsomes	Regulation of aromatase mRNA or protein levels is tissue-specific, thus, any induction or suppression by test compounds may not represent other tissues
Limitations with the use of radioactive materials	Limitations with the use of radioactive materials
	Potential limitation of cell lines for commercial use
	Requires solubility of test compounds in enzyme in solution
	Requires test compounds to permeate cell membrane

6.0 IMPLEMENTATION CONSIDERATIONS

6.1 ESTABLISHMENT OF ASSAY

Candidate Assay A - placental microsomal aromatase: The assay is rather straightforward to establish. Standard equipment in a biochemistry laboratory are needed. This equipment includes a low speed (clinical) bench-top centrifuge, a high speed centrifuge, an ultracentrifuge, a shaking constant-temperature water bath, pH meter, and a liquid scintillation counter. General materials and supplies include a mechanical homogenizer, glassware, test tubes, vials, [1β-^{3}H]-androstenedione, biochemicals (androstenedione, $NADP^{+}$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase), pipettes, buffers, reagents, and organic solvents. Personnel performing the assays would require training in general biology/biochemistry techniques, in enzyme assay methods, and in the use of radioactive materials.

Candidate Assay B - aromatase assay in cell lines: The assay is rather straightforward to establish. Standard equipment in a cell biology laboratory are needed. This equipment includes laminar flow hoods, CO_2 incubators, microscopes, liquid nitrogen storage containers, autoclaves, media storage and/or media preparation facilities, a low speed (clinical) bench-top centrifuge, a high speed centrifuge, a constant-temperature water bath, pH meter, and a liquid scintillation counter. General materials and supplies include a mechanical homogenizer, glassware, cell culture plates, vials, [1β-^{3}H]-androstenedione, androstenedione, DMEM/F-12 media, fetal calf serum, gentamycin (or other standard antibiotics), pipettes, buffers, reagents, and organic solvents. Personnel performing the assays would require training in general cell biology, cell culturing/sterile techniques, and in the use of radioactive materials.

6.2 COST/TIME REQUIRED

Candidate Assay A - placental microsomal aromatase: The time required to establish the assay in a properly equipped biochemistry laboratory would involve one to two months. Each assay, from initiation to data analysis, requires approximately 12-16 hours of labor, and up to 60-100 samples (15-25 doses in quadruplicate) can performed in one assay by one experienced individual. Microsomal preparations from one placenta provide enough material for 2 - 3 months. The recurring costs for operation of the assay (excluding personnel and equipment) are approximately $2,000 to $3,000 per month for radioactive materials, LSC supplies, biochemical reagents, and general supplies.

Candidate Assay B - aromatase assay in cell lines: The time required to establish the assay in a properly equipped biochemistry laboratory would involve three to four months. Cell cultures must be maintained on a daily basis, and cells are plated for an assay 3 to 4 days in advance. Each assay, from initiation to data analysis, requires approximately 12-16 hours of labor, and up to 60-100 samples (15-25 doses in quadruplicate) can performed in one assay by one experienced individual. The recurring costs for operation of the assay (excluding personnel and equipment) are approximately $4,000 to $6,000 per month for radioactive materials, LSC

supplies, cell culture media and sera, culture plasticware, and general supplies..

6.3 ANIMAL WELFARE CONSIDERATIONS

The human placental microsomal aromatase assay uses an enzyme preparation obtained from human term placenta. The cell culture assay uses a stable human choriocarcinoma cell line, JEG-3 cells. The benefit of these assays are that no animals are utilized. For comparison, microsomal preparations from adult rat ovarian tissues typically provide approximately 1.5 mg of microsomal protein per ovary. The level of aromatase activity in rat ovarian microsomes is approximately 6.0 pmol per mg microsomal protein per minute, which is 10% of the activity present in human placental microsomes. One adult female rat would provide ovarian microsomal preparations sufficient for approximately three or four assay tubes, the equivalent of one dose in quadruplicate. Thus, a screening assay for one chemical test compound would use 5 female rats, and a full dose-response curve for one chemical test compound would use a minimum of 10 female rats.

6.4 HUMAN SUBJECTS CONSIDERATIONS

The human placental microsomal aromatase assay uses an enzyme preparation obtained from normal human term placenta, tissue that is discarded after delivery. Use of discarded human tissues falls under regulations for research involving human subjects, and thus the research protocols require approval from an institutional human subjects review committee.

6.5 ASSURANCE OF BIOLOGICAL TEST (*IN VITRO* MODEL) RIGOR AND STABILITY

As stated earlier, these two assays (the human placental microsomal aromatase assay and the cell culture-based assay) are common *in vitro* assays used for measuring aromatase and aromatase inhibition because of their reliability, reproducibility, and ease of use. However, the assays are *in vitro* tests that specifically measure the ability of test agents to inhibit aromatase activity. The assessment of risk of human exposure to potential endocrine disruptors in the environment can not be determined by *in vitro* experiments alone. Such screening results can alert us to the need for further examination of the compounds' potential for inducing endocrine disruption after *in vivo* exposure. This analysis should also include any knowledge of human exposure from use of the compounds to provide a proper risk assessment.

7.0 RECOMMENDATIONS

The "ideal" *in vitro* aromatase assay would be a single assay that measures the effects of test compounds on inhibition of enzyme activity and alteration of aromatase enzyme levels via regulation of CYP19 gene expression. An assay system exhibiting moderate to high enzyme activity would be effective in the evaluation of test compounds for aromatase inhibition and/or for suppression of CYP19 gene expression. However, for examining the ability of test compounds to induce CYP19 gene expression, an assay system exhibiting low basal aromatase levels would be more desirable. Also, the system for examination of CYP19 gene expression should be responsive to the various tissue-specific promoters of aromatase. Furthermore, an "ideal" assay should be straightforward to establish, use readily available tissues or cells, enable the screening of large numbers of test agents in a rapid or high-throughput manner, and be reliable, sensitive, and reproducible. Unfortunately, no single assay meets all these requirements.

7.1 RECOMMENDATIONS FOR AROMATASE INHIBITION: PLACENTAL MICROSOMAL ASSAY

The placental microsomal aromatase assay is recommended as the initial *in vitro* aromatase inhibition screening assay to be one of the Tier 1 Screening Battery Alternate Methods. This assay will enable the assessment of the effects of various environmental toxicants as a screening method for possible inclusion in the Tier 1 Screening Battery. The placental microsomal aromatase assay is recommended based upon ease in establishing the assay, use of standard biochemistry laboratory equipment, ready access to the source tissues, and standard level of training in general biology and/or biochemistry techniques for personnel performing the assays. Also, use of the placental microsomal aromatase assay has an advantage over the use of the cell based assay since the use of each cell line must adhere to guidelines set forth by its suppliers (e.g., research purposes only and no use for commercial purposes).

Aromatase is readily abundant in human term placental tissue and in placenta from other species such as other primates, cattle, horses, and pigs. Bovine placenta can be obtained from slaughterhouses or farms and processed in a similar manner to humans. However, publications using bovine placenta for inhibition measurements or for evaluation of endocrine disruptors are lacking. A commercial source of recombinant human aromatase in microsomal preparations from stably-transfected insect cells is also available.

The overall protocol of the assay is straightforward, and the initial screening assay to determine initial aromatase inhibition will be performed at concentrations of environmental toxicant at 0.1, 1.0, and 10.0 μM in quadruplicate. For compounds exhibiting 10% inhibitory activity or more, full dose-response studies will be performed. Full dose-response studies of the chemical and/or product will be performed at doses from 1.0 nM (1.0×10^{-9} M) to 1.0 mM (1.0×10^{-3} M). The doses tested differ by 0.5 log units (i.e., log values of -9.0, -8.5, -8.0, -7.5, etc.), and the number of replicates to be used is four. The route of administration/exposure of the test compounds involve dissolving an accurate amount of the compound in ethanol or DMSO and pipetting into appropriate test tubes. Incubations will be performed in 16x100 mm test tubes

maintained at 37°C in a shaking water bath. An aliquot (100 μL) of propylene glycol will be added to the tubes to serve as a co-solvent. The substrate, [1β-^{3}H]-androstenedione (0.1 μCi, 50 nM), and the inhibitor at test concentrations will be added to the tubes. An NADPH-generating system comprised of NADP+ (1.7 mM), glucose-6-phosphate (2.8 mM) and glucose-6-phosphate dehydrogenase (1.0 units) will be added to each tube. The tubes will be placed at 37°C in the water bath for five minutes. The assay will begin with the addition of the diluted placental microsomal suspension (~0.1 mg microsomal protein/mL). The total volume will be 2.0 mL, and the tubes will be incubated for 30 minutes. The incubations will be stopped by the addition of chloroform (2.0 mL) and the tubes vortexed for 30 seconds. The tubes will then be centrifuged for 10 minutes at 500 rpm. The aqueous layers will be transferred to new test tubes and extracted again with chloroform (2.0 mL). This extraction procedure will be performed one additional time. The aqueous layers will be transferred to vials, and an aliquot (0.5 mL) will be removed and transferred to a 7.0 mL liquid scintillation counting vial. Liquid scintillation solution (emulsion-type, 5.0 mL) will be added to each counting vial and shaken to mix the solution. The amount of $^{3}H_2O$ in each counting vial will be determined using a ^{3}H counting protocol available on a standard liquid scintillation counter.

Both positive and negative controls are required. Negative controls (blank) consist of the media only (no cells). Incubations with the substrate only is a positive control and identifies the maximum aromatase activity present under the particular enzyme assay conditions. Under these assay conditions, the range of aromatase activity is typically 20.0 to 25.0 pmol (5.4 to 6.8 ng) product formed per mg cellular protein per hour. Equally important in assays is the inclusion of reference compounds, such as a potent steroidal inhibitor, such as 4-hydroxyandrostenedione, exemestane, or a 7α-substituted androstenedione, or a potent nonsteroidal inhibitor such as aminoglutethimide, anastrozole, or letrozole.

7.2 RECOMMENDATIONS FOR AROMATASE GENE EXPRESSION: ADDITIONAL RESEARCH ON CELL LINES

An *in vitro* cell-based assay can provide information on the effect of test compounds on either induction or suppression of CYP19 gene expression, resulting in altered levels of CYP19 mRNA and/or aromatase protein. CYP19 gene expression is tightly regulated in a tissue-specific manner, and test compounds may affect aromatase expression by altering molecular events at one or more of the various tissue-specific promoter regions of aromatase. The JEG-3 and JAR cell lines have sufficient aromatase enzyme levels in order to effectively measure suppression of gene expression as well as measure induction of gene expression. As these cells are derived from trophoblast origin, the JEG-3 and JAR cells may represent induction or suppression of CYP19 expression only under the control of the placental promoter I.1. Since CYP19 expression is tissue specific, studies in JEG-3 cells will not provide information on induction or suppression of CYP19 mRNA and aromatase protein in other critical tissues such as ovary, breast, brain, adipose, and bone. The SK-BR-3 breast cancer cell system also has moderate aromatase enzyme levels and can be used to measure induction and/or suppression of gene expression. However, the cells are breast cancer cells, and aromatase expression is likely regulated at promoter II. Similarly, the KGN cells are granulosa-like cells, and aromatase expression is also likely regulated at promoter II. Cell culture systems exhibiting low basal aromatase levels, such as the

MCF-7 breast cancer cells, the KGN human ovarian granulosa-like tumor cell line and the H295R human adrenocortical carcinoma cell line, would be most useful for examining enzyme induction. However, if these the cells were first exposed to a standard inducing agent to increase aromatase activity, there would be sufficient sensitivity in the assay to examine inhibitory effect. Further research efforts are needed to evaluate various cell culture systems and/or culturing conditions for determination of tissue-specific regulation of CYP19 gene expression and the suitability of cells for studies on induction or suppression of gene expression.

8.0 DEVELOPMENTAL STATUS OF THE ASSAY

8.1 CURRENT STATUS

The various endpoints included in the proposed placental microsomal assay have been evaluated in other studies. However, the protocol itself has not been validated. Pending a final decision on the study design, the protocol would be ready to enter the pre-validation phase.

8.2 RECOMMENDATION FOR PRE-VALIDATION STUDIES

Pre-validation studies following the ICCVAM validation process should be initiated. First, pre-validation studies will be designed to optimize protein, substrate, and cofactor concentrations necessary for the placental microsomal assay. As cited earlier in Section 4.1.5, the concentrations of substrate and microsomal protein used in the assays can influence the IC_{50} values obtained. Therefore, pre-validation studies should be performed to identify the optimal concentrations of substrate, microsomal protein, and co-factors (i.e., the NADPH-generating system). Optimization studies should consist of determination of aromatase activity (tritiated water formation) under conditions of (a) protein concentration ranging from 0.01 to 5 mg microsomal protein per mL, (b) substrate concentrations ranging from 10 nM to 500 nM, and (c) cofactor concentrations of the NADPH-generating system (NADP+ from 0.1 mM to 5.0 mM, glucose-6-phosphate from 0.1 to 5.0 mM, and glucose-6-phosphate dehydrogenase from 0.1 to 5.0 units). Secondly, incubation times should also be optimized to ensure maximum product formation. Thirdly, pre-validation studies should also compare aromatase activities from various placental tissue sources, specifically human, bovine, and porcine, and with the commercially available recombinant aromatase microsomal preparation.

Additional pre-validation studies should include evaluation of 3-5 chemicals as aromatase inhibitors. It is recommended that the study design be performed using known aromatase inhibitors as positive control compounds, such as a potent steroidal inhibitor, such as 4-hydroxyandrostenedione, exemestane, or a 7α-substituted androstenedione, or a potent nonsteroidal inhibitor such as aminoglutethimide, anastrozole, or letrozole.

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9.0 REFERENCES

1. Strauss, J. F. I. and Hsueh, A. J. W. Ovarian Hormone Synthesis. *In* L. J. DeGroot, J. L. Jameson, H. G. Burger, D. L. Loriaux, J. C. Marshall, S. Melmed, W. D. Odell, J. T. Jr. Potts, and A. H. Rubenstein (eds.), Endocrinology, Fourth Edition, pp. 2043-2052. Philadelphia: W.B. Saunders Company, 2001.

2. Weigel, N. L. and Rowan, B. G. Estrogen and Progesterone Action. *In* L. J. DeGroot, J. L. Jameson, H. G. Burger, D. L. Loriaux, J. C. Marshall, S. Melmed, W. D. Odell, J. T. Jr. Potts, and A. H. Rubenstein (eds.), Endocrinology, Fourth Edition, pp. 2053-2060. Philadelphia: W.B. Saunders Company, 2001.

3. Marshall, J. C. Hormonal Regulation of the Menstrual Cycle and Mechanism of Ovulation. *In* L. J. DeGroot, J. L. Jameson, H. G. Burger, D. L. Loriaux, J. C. Marshall, S. Melmed, W. D. Odell, J. T. Jr. Potts, and A. H. Rubenstein (eds.), Endocrinology, Fourth Edition, pp. 2073-2085. Philadelphia: W.B. Saunders Company, 2001.

4. Williams, C. and Stancel, G. M. Estrogens and Progestins. *In* J. G. Hardman, L. E. Limbird, P. B. Molinoff, R. W. Ruddon, and A. G. Gilman (eds.), Goodman and Gilman's The Pharmacological Basis of Therapeutics, Ninth Edition, pp. 1411-1440. New York: McGraw-Hill, 1996.

5. Ruentiz, P. C. Female Sex Hormones and Analogs. *In* M. E. Wolff (ed.), Burger's Medicinal Chemistry, 5th Edition, pp. 553-588. New York: Wiley, 1997.

6. Simpson, E., Rubin, G., Clyne, C., Robertson, K., O'Donnell, L., Jones, M., and Davis, S. The role of local estrogen biosynthesis in males and females. Trends Endocrinol.Metab, *11*: 184-188, 2000.

7. Lombardi, G., Zarrilli, S., Colao, A., Paesano, L., Di Somma, C., Rossi, F., and De Rosa, M. Estrogens and health in males. Mol.Cell Endocrinol., *178*: 51-55, 2001.

8. Brodie, A., Inkster, S., and Yue, W. Aromatase expression in the human male. Mol.Cell Endocrinol., *178*: 23-28, 2001.

9. Lephart, E. D. A review of brain aromatase cytochrome P450. Brain Res.Brain Res.Rev., *22*: 1-26, 1996.

10. Lephart, E. D. Molecular aspects of brain aromatase cytochrome P450. J.Steroid Biochem.Mol.Biol., *61*: 375-380, 1997.

11. Cooke, B., Hegstrom, C. D., Villeneuve, L. S., and Breedlove, S. M. Sexual differentiation of the vertebrate brain: principles and mechanisms. Front Neuroendocrinol., *19*: 323-362, 1998.

12. Ryan, K. J. Biological aromatization of steroids. J.Biol.Chem., *234*: 268-272, 1959.

13. Simpson, E. R., Merrill, J. C., Hollub, A. J., Graham-Lorence, S., and Mendelson, C. R. Regulation of estrogen biosynthesis by human adipose cells. Endocr.Rev., *10*: 136-148, 1989.

14. James, V. H., McNeill, J. M., Lai, L. C., Newton, C. J., Ghilchik, M. W., and Reed, M. J. Aromatase activity in normal breast and breast tumor tissues: in vivo and in vitro studies. Steroids, *50*: 269-279, 1987.

15. Miller, W. R. and O'Neill, J. The importance of local synthesis of estrogen within the breast. Steroids, *50*: 537-548, 1987.

16. Reed, M. J., Owen, A. M., Lai, L. C., Coldham, N. G., Ghilchik, M. W., Shaikh, N. A., and James, V. H. In situ oestrone synthesis in normal breast and breast tumour tissues: effect of treatment with 4-hydroxyandrostenedione. Int.J.Cancer, *44*: 233-237, 1989.

17. Reed, M. J. The role of aromatase in breast tumors. Breast Cancer Res.Treat., *30*: 7-17, 1994.

18. Bulun, S. E., Price, T. M., Aitken, J., Mahendroo, M. S., and Simpson, E. R. A link between breast cancer and local estrogen biosynthesis suggested by quantification of breast adipose tissue aromatase cytochrome P450 transcripts using competitive polymerase chain reaction after reverse transcription. J.Clin.Endocrinol.Metab, *77*: 1622-1628, 1993.

19. Naftolin, F. Brain aromatization of androgens. J.Reprod.Med., *39*: 257-261, 1994.

20. Simpson, E., Jones, M., Davis, S., and Rubin, G. Do intracrine mechanisms regulate aromatase expression? J.Steroid Biochem.Mol.Biol., *69*: 447-452, 1999.

21. Simpson, E. R., Zhao, Y., Agarwal, V. R., Michael, M. D., Bulun, S. E., Hinshelwood, M. M., Graham-Lorence, S., Sun, T., Fisher, C. R., Qin, K., and Mendelson, C. R. Aromatase expression in health and disease. Recent Prog.Horm.Res., *52*: 185-213, 1997.

22. Simpson, E. R., Mahendroo, M. S., Means, G. D., Kilgore, M. W., Hinshelwood, M. M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C. R., Michael, M. D., and . Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr.Rev., *15*: 342-355, 1994.

23. Kellis, J. T., Jr. and Vickery, L. E. Purification and characterization of human placental aromatase cytochrome P-450. J.Biol.Chem., *262*: 4413-4420, 1987.

24. Means, G. D., Kilgore, M. W., Mahendroo, M. S., Mendelson, C. R., and Simpson, E. R. Tissue-specific promoters regulate aromatase cytochrome P450 gene expression in human ovary and fetal tissues. Mol.Endocrinol., *5*: 2005-2013, 1991.

25. Simpson, E. R., Mahendroo, M. S., Means, G. D., Kilgore, M. W., Corbin, C. J., and Mendelson, C. R. Tissue-specific promoters regulate aromatase cytochrome P450 expression. J.Steroid Biochem.Mol.Biol., *44*: 321-330, 1993.

26. McPhaul, M. J., Herbst, M. A., Matsumine, H., Young, M., and Lephart, E. D. Diverse mechanisms of control of aromatase gene expression. J.Steroid Biochem.Mol.Biol., *44*: 341-346, 1993.

27. Simpson, E. R., Clyne, C., Rubin, G., Boon, W. C., Robertson, K., Britt, K., Speed, C., and Jones, M. Aromatase - A Brief Overview. Annu.Rev.Physiol, *64*: 93-127, 2002.

28. Dowsett, M., Macaulay, V., Gledhill, J., Ryde, C., Nicholls, J., Ashworth, A., McKinna, J. A., and Smith, I. E. Control of aromatase in breast cancer cells and its importance for tumor growth. J.Steroid Biochem.Mol.Biol., *44*: 605-609, 1993.

29. Miller, W. R., Mullen, P., Sourdaine, P., Watson, C., Dixon, J. M., and Telford, J. Regulation of aromatase activity within the breast. J.Steroid Biochem.Mol.Biol., *61*: 193-202, 1997.

30. Reed, M. J., Topping, L., Coldham, N. G., Purohit, A., Ghilchik, M. W., and James, V. H. Control of aromatase activity in breast cancer cells: the role of cytokines and growth factors. J.Steroid Biochem.Mol.Biol., *44*: 589-596, 1993.

31. Quinn, A. L., Burak, W. E., Jr., and Brueggemeier, R. W. Effects of matrix components on aromatase activity in breast stromal cells in culture. J.Steroid Biochem.Mol.Biol., *70*: 249-256, 1999.

32. Simpson, E. R. Aromatase 2000: Fifth International Aromatase Conference. 2000. 11-3-2000.

33. Zhao, Y., Agarwal, V. R., Mendelson, C. R., and Simpson, E. R. Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene. Endocrinology, *137*: 5739-5742, 1996.

34. Zhou, C., Zhou, D., Esteban, J., Murai, J., Siiteri, P. K., Wilczynski, S., and Chen, S. Aromatase gene expression and its exon I usage in human breast tumors. Detection of aromatase messenger RNA by reverse transcription- polymerase chain reaction. J.Steroid Biochem.Mol.Biol., *59*: 163-171, 1996.

35. Hughes, R., Timmermans, P., and Schrey, M. P. Regulation of arachidonic acid metabolism, aromatase activity and growth in human breast cancer cells by interleukin-1beta and phorbol ester: dissociation of a mediatory role for prostaglandin E2 in the autocrine control of cell function. Int.J.Cancer, *67*: 684-689, 1996.

36. Brueggemeier, R. W., Quinn, A. L., Parrett, M. L., Joarder, F. S., Harris, R. E., and Robertson, F. M. Correlation of aromatase and cyclooxygenase gene expression in human breast cancer specimens. Cancer Lett., *140*: 27-35, 1999.

37. Harvey, H. A., Lipton, A., and Santen, R. J. Aromatase: New Perspectives for Breast Cancer. Cancer Res., *42*: 3261s-3467s, 1982.

38. Santen, R. J. Aromatase Conference: Future Perspectives. Steroids, *50*: 1-665, 1987.

39. Brodie, A. M., Brodie, H. J., Callard, G., Robinson, C. H., Roselli, C., and Santen, R. J. Proceedings of the Third International Aromatase Conference. J.Steroid Biochem.Mol.Biol., *44*: 321-696, 1993.

40. Simpson, E. R., Bhatnagar, A., Brodie, A. M., Brueggemeier, R. W., Chen, S., DeCoster, R., Dowsett, M., Harada, N., Naftolin, F., Osawa, Y., Plourde, P., Reed, M. J., and Santen, R. J. Proceedings of the Fourth International Aromatase Conference. J.Steroid Biochem.Mol.Biol., *61*: 107-425, 1997.

41. Johnston, J. O. and Metcalf, B. W. Aromatase: A Target Enzyme in Breast Cancer. *In* P. Sunkara (ed.), Novel Approaches to Cancer Chemotherapy, pp. 307-328. New York: Academic Press, 1984.

42. Banting, L., Smith, H. J., James, M., Jones, G., Nazareth, W., Nicholls, P. J., Hewlins, M. J., and Rowlands, M. G. Structure-activity relationships for non-steroidal inhibitors of aromatase. J.Enzyme Inhib., *2*: 215-229, 1988.

43. Covey, D. F. Aromatase Inhibitors: Specific Inhibitors of Oestrogen Biosynthesis. *In* D. Berg and M. Plempel (eds.), Sterol Biosynthesis Inhibitors, pp. 534-571. Chichester, UK: Ellis Horwood, 1988.

44. Banting, L., Nicholls, P. J., Shaw, M. A., and Smith, H. J. Recent developments in aromatase inhibition as a potential treatment for oestrogen-dependent breast cancer. Prog.Med.Chem., *26*: 253-298, 1989.

45. Brueggemeier, R. W. Biochemical and molecular aspects of aromatase. J.Enzyme Inhib., *4*: 101-111, 1990.

46. Cole, P. A. and Robinson, C. H. Mechanism and inhibition of cytochrome P-450 aromatase. J.Med.Chem., *33*: 2933-2942, 1990.

47. Brueggemeier, R. W. Aromatase inhibitors--mechanisms of steroidal inhibitors. Breast Cancer Res.Treat., *30*: 31-42, 1994.

48. Brodie, A. M. and Njar, V. C. Aromatase inhibitors and breast cancer. Semin.Oncol., *23*: 10-20, 1996.

49. Brodie, A., Lu, Q., and Long, B. Aromatase and its inhibitors. J.Steroid Biochem.Mol.Biol., *69*: 205-210, 1999.

50. Santen, R. J. and Harvey, H. A. Use of aromatase inhibitors in breast carcinoma. Endocr.Relat Cancer, *6*: 75-92, 1999.

51. Hickey, G. J., Krasnow, J. S., Beattie, W. G., and Richards, J. S. Aromatase cytochrome P450 in rat ovarian granulosa cells before and after luteinization: adenosine 3',5'-monophosphate-dependent and independent regulation. Cloning and sequencing of rat aromatase cDNA and 5' genomic DNA. Mol.Endocrinol., *4*: 3-12, 1990.

52. Richards, J. S. Hormonal control of gene expression in the ovary. Endocr.Rev., *15*: 725-751, 1994.

53. Richards, J. S., Fitzpatrick, S. L., Clemens, J. W., Morris, J. K., Alliston, T., and Sirois, J. Ovarian cell differentiation: a cascade of multiple hormones, cellular signals, and regulated genes. Recent Prog.Horm.Res., *50*: 223-254, 1995.

54. Valladres, L. E. and Payne, A. H. Induction of testicular aromatization by luteinizing hormone in mature rats. Endocrinology, *105*: 431-436, 1979.

55. Naftolin, F. and MacLusky, N. J. Aromatase in the central nervous system. Cancer Res., *42*: 3274s-3276s, 1982.

56. Lephart, E. D. Molecular aspects of brain aromatase cytochrome P450. J.Steroid Biochem.Mol.Biol., *61*: 375-380, 1997.

57. Gross, T. S. and Williams, W. F. In vitro steroid synthesis by the placenta of cows during late gestation and at parturition. J.Reprod.Fertil. 83, 565-573. 1988.

58. Hinshelwood, M. M., Liu, Z., Conley, A. J., and Simpson, E. R. Demonstration of tissue-specific promoters in nonprimate species that express aromatase P450 in placentae. Biol.Reprod., *53*: 1151-1159, 1995.

59. Hinshelwood, M. M., Dodson, M. M., Sun, T., and Simpson, E. R. Regulation of aromatase expression in the ovary and placenta: a comparison between two species. J.Steroid Biochem.Mol.Biol., *61* : 399-405, 1997.

60. Conley, A., Corbin, J., Smith, T., Hinshelwood, M., Liu, Z., and Simpson, E. Porcine aromatases: studies on tissue-specific, functionally distinct isozymes from a single gene? J.Steroid Biochem.Mol.Biol., *61*: 407-413, 1997.

61. Corbin, C. J., Trant, J. M., and Conley, A. J. Porcine gonadal and placental isozymes of aromatase cytochrome P450: sub-cellular distribution and support by NADPH-cytochrome P450 reductase. Mol.Cell Endocrinol., *172*: 115-124, 2001.

62. Pasmanik, M., Schlinger, B. A., and Callard, G. V. In vivo steroid regulation of aromatase and 5-alpha-reductase activities in bnrain and pituitary of goldfish. Gen.Comp Endocrinol. 71, 175-182. 1988.

63. Callard, G. V. and Tchoudakova, A. Evolutionary and functional significance of two CYP19 genes differentially expressed in brain and ovary of goldfish. J.Steroid Biochem.Mol.Biol., *61*: 387-392, 1997.

64. Trant, J. M., Lehrter, J., Gregory, T., Nunez, S., and Wunder, J. Expression of cytochrome P450 aromatase in the channel catfish, Ictalurus punctatus. J.Steroid Biochem.Mol.Biol., *61*: 393-397, 1997.

65. Kishida, M. and Callard, G. V. Distinct cytochrome P450 aromatase isoforms in zebrafish (Danio rerio) brain and ovary are differentially programmed and estrogen regulated during early development. Endocrinology, *142*: 740-750, 2001.

66. Chiang, E. F., Yan, Y. L., Guiguen, Y., Postlethwait, J., and Chung, B. Two Cyp19 (P450 Aromatase) Genes on Duplicated Zebrafish Chromosomes Are Expressed in Ovary or Brain. Mol.Biol.Evol., *18*: 542-550, 2001.

67. Callard, G. V., Petro, Z., and Ryan, K. J. Identification of aromatase in the reptilian brain. Endocrinology, *100*: 1214-1218, 1977.

68. Jeyasuria, P., Roosenburg, W. M., and Place, A. R. Role of P-450 aromatase in sex determination of the diamondback terrapin, Malaclemys terrapin. J.Exp.Zool., *270*: 95-111, 1994.

69. Fisher, C. R., Graves, K. H., Parlow, A. F., and Simpson, E. R. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. Proc.Natl.Acad.Sci.U.S.A, *95*: 6965-6970, 1998.

70. Robertson, K. M., O'Donnell, L., Jones, M. E., Meachem, S. J., Boon, W. C., Fisher, C. R., Graves, K. H., McLachlan, R. I., and Simpson, E. R. Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. Proc.Natl.Acad.Sci.U.S.A, *96*: 7986-7991, 1999.

71. Jones, M. E., Thorburn, A. W., Britt, K. L., Hewitt, K. N., Wreford, N. G., Proietto, J., Oz, O. K., Leury, B. J., Robertson, K. M., Yao, S., and Simpson, E. R. Aromatase-deficient (ArKO) mice have a phenotype of increased adiposity. Proc.Natl.Acad.Sci.U.S.A, *97*: 12735-12740, 2000.

72. Oz, O. K., Zerwekh, J. E., Fisher, C., Graves, K., Nanu, L., Millsaps, R., and Simpson, E. R. Bone has a sexually dimorphic response to aromatase deficiency. J.Bone Miner.Res., *15*: 507-514, 2000.

73. Tekmal, R. R., Ramachandra, N., Gubba, S., Durgam, V. R., Mantione, J., Toda, K., Shizuta, Y., and Dillehay, D. L. Overexpression of int-5/aromatase in mammary glands of transgenic mice results in the induction of hyperplasia and nuclear abnormalities. Cancer Res., *56*: 3180-3185, 1996.

74. Kirma, N., Mandava, U., Wuichet, K., and Tekmal, R. R. The effects of aromatase overexpression on mammary growth and gene expression in the aromatase x transforming growth factor alpha double transgenic mice. J.Steroid Biochem.Mol.Biol., *78*: 419-426, 2001.

75. Lephart, E. D. and Simpson, E. R. Assay of aromatase activity. Methods Enzymol., *206*: 477-483, 1991.

76. Stresser, D. M., Turner, S. D., McNamara, J., Stocker, P., Miller, V. P., Crespi, C. L., and Patten, C. J. A high-throughput screen to identify inhibitors of aromatase (CYP19). Anal.Biochem., *284*: 427-430, 2000.

77. McNamara, J., Stocker, P., Miller, V. P., Patten, C. J., Stresser, D. M., and Crespi, C. L. CYP19 (aromatase): Characterization of the recombinant enzyme and its role in the biotransformation of xenobiotics. www.gentest.com/products/pdf/post_016.pdf . 9-1-1999.

78. Adlercreutz, H., Bannwart, C., Wahala, K., Makela, T., Brunow, G., Hase, T., Arosemena, P. J., Kellis, J. T., Jr., and Vickery, L. E. Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens. J.Steroid Biochem.Mol.Biol., *44*: 147-153, 1993.

79. Kellis, J. T., Jr. and Vickery, L. E. Inhibition of human estrogen synthetase (aromatase) by flavones. Science, *225*: 1032-1034, 1984.

80. Kellis, J. T., Jr., Nesnow, S., and Vickery, L. E. Inhibition of aromatase cytochrome P-450 (estrogen synthetase) by derivatives of alpha-naphthoflavone. Biochem.Pharmacol., *35*: 2887-2891, 1986.

81. Ibrahim, A. R. and Abul-Hajj, Y. J. Aromatase inhibition by flavonoids. J.Steroid Biochem.Mol.Biol., *37*: 257-260, 1990.

82. Chen, S., Kao, Y. C., and Laughton, C. A. Binding characteristics of aromatase inhibitors and phytoestrogens to human aromatase. J.Steroid Biochem.Mol.Biol., *61*: 107-115, 1997.

83. Kao, Y. C., Zhou, C., Sherman, M., Laughton, C. A., and Chen, S. Molecular basis of the inhibition of human aromatase (estrogen synthetase) by flavone and isoflavone phytoestrogens: A site-directed mutagenesis study. Environ.Health Perspect., *106*: 85-92, 1998.

84. Le Bail, J. C., Laroche, T., Marre-Fournier, F., and Habrioux, G. Aromatase and 17beta-hydroxysteroid dehydrogenase inhibition by flavonoids. Cancer Lett., *133*: 101-106, 1998.

85. Jeong, H. J., Shin, Y. G., Kim, I. H., and Pezzuto, J. M. Inhibition of aromatase activity by flavonoids. Arch.Pharm.Res., *22*: 309-312, 1999.

86. Le Bail, J. C., Pouget, C., Fagnere, C., Basly, J. P., Chulia, A. J., and Habrioux, G. Chalcones are potent inhibitors of aromatase and 17beta-hydroxysteroid dehydrogenase activities. Life Sci., *68*: 751-761, 2001.

87. Le Bail, J. C., Aubourg, L., and Habrioux, G. Effects of pinostrobin on estrogen metabolism and estrogen receptor transactivation. Cancer Lett., *156*: 37-44, 2000.

88. Le Bail, J. C., Champavier, Y., Chulia, A. J., and Habrioux, G. Effects of phytoestrogens on aromatase, 3beta and 17beta-hydroxysteroid dehydrogenase activities and human breast cancer cells. Life Sci., *66*: 1281-1291, 2000.

89. Campbell, D. R. and Kurzer, M. S. Flavonoid inhibition of aromatase enzyme activity in human preadipocytes. J.Steroid Biochem.Mol.Biol., *46*: 381-388, 1993.

90. Wang, C., Makela, T., Hase, T., Adlercreutz, H., and Kurzer, M. S. Lignans and flavonoids inhibit aromatase enzyme in human preadipocytes. J.Steroid Biochem.Mol.Biol., *50*: 205-212, 1994.

91. Chen, S., Sun, X. Z., Kao, Y. C., Kwon, A., and Eng, E. Suppression of breast cancer cell growth with grape juice. Pharmaceutical Biology (Netherlands), *36*: 53-61, 1998.

92. Mak, P., Cruz, F. D., and Chen, S. A yeast screen system for aromatase inhibitors and ligands for androgen receptor: yeast cells transformed with aromatase and androgen receptor. Environ.Health Perspect., *107*: 855-860, 1999.

93. Pelissero, C., Lenczowski, M. J., Chinzi, D., Davail-Cuisset, B., Sumpter, J. P., and Fostier, A. Effects of flavonoids on aromatase activity, an in vitro study. J.Steroid Biochem.Mol.Biol., *57*: 215-223, 1996.

94. Shilling, A. D., Carlson, D. B., and Williams, D. E. Rainbow trout, Oncorhynchus mykiss, as a model for aromatase inhibition. J.Steroid Biochem.Mol.Biol., *70*: 89-95, 1999.

95. Vinggaard, A. M., Hnida, C., Breinholt, V., and Larsen, J. C. Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro. Toxicol.In Vitro, *14*: 227-234, 2000.

96. Mason, J. I., Murry, B. A., Olcott, M., and Sheets, J. J. Imidazole antimycotics: inhibitors of steroid aromatase. Biochem.Pharmacol., *34*: 1087-1092, 1985.

97. Mason, J. I., Carr, B. R., and Murry, B. A. Imidazole antimycotics: selective inhibitors of steroid aromatization and progesterone hydroxylation. Steroids, *50*: 179-189, 1987.

98. Ayub, M. and Levell, M. J. Structure-activity relationships of the inhibition of human placental aromatase by imidazole drugs including ketoconazole. J.Steroid Biochem., *31*: 65-72, 1988.

99. Albrecht, E. D. and Pepe, G. J. Placental steroid hormone biosynthesis in primate pregnancy. Endocr.Rev., *11*: 124-150, 1990.

100. Johnston, J. O. and Metcalf, B. W. Aromatase: A Target Enzyme in Breast Cancer. *In* P. Sunkara (ed.), Novel Approaches to Cancer Chemotherapy, pp. 307-328. New York: Academic Press, 1984.

101. Brueggemeier, R. W. and Katlic, N. E. Aromatase inhibition by an enzyme-activated irreversible inhibitor in human carcinoma cell cultures. Cancer Res., *50*: 3652-3656, 1990.

102. Brueggemeier, R. W., Katlic, N. E., Kenreigh, C. A., and Li, P. K. Aromatase inhibition by 7-substituted steroids in human choriocarcinoma cell culture. J.Steroid Biochem.Mol.Biol., *41*: 85-90, 1992.

103. Brueggemeier, R. W., Gilbert, N. E., Gu, X., O'Reilly, J. M., and Lovely, C. J. Aromatase inhibition in JAr choriocarcinoma cells by 7alpha- arylaliphatic androgens. J.Steroid Biochem.Mol.Biol., *61*: 73-77, 1997.

104. Drenth, H. J., Bouwman, C. A., Seinen, W., and van den, B. M. Effects of some persistent halogenated environmental contaminants on aromatase (CYP19) activity in the human choriocarcinoma cell line JEG-3. Toxicol.Appl.Pharmacol., *148*: 50-55, 1998.

105. Letcher, R. J., van, H., I, Drenth, H. J., Norstrom, R. J., Bergman, A., Safe, S., Pieters, R., and van den, B. M. Cytotoxicity and aromatase (CYP19) activity modulation by organochlorines in human placental JEG-3 and JAR choriocarcinoma cells. Toxicol.Appl.Pharmacol., *160*: 10-20, 1999.

106. Yang, C. and Chen, S. Two organochlorine pesticides, toxaphene and chlordane, are antagonists for estrogen-related receptor alpha-1 orphan receptor. Cancer Res., *59*: 4519-4524, 1999.

107. Sanderson, J. T., Seinen, W., Giesy, J. P., and van den, B. M. 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity? Toxicol.Sci., *54*: 121-127, 2000.

108. Sanderson, J. T., Slobbe, L., Lansbergen, G. W., Safe, S., and van den, B. M. 2,3,7,8-Tetrachlorodibenzo-p-dioxin and Diindolylmethanes Differentially Induce Cytochrome P450 1A1, 1B1, and 19 in H295R Human Adrenocortical Carcinoma Cells. Toxicol.Sci.,

61: 40-48, 2001.

109. Sanderson, J. T., Letcher, R. J., Heneweer, M., Giesy, J. P., and van den, B. M. Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. Environ.Health Perspect., *109*: 1027-1031, 2001.

110. Nishi, Y., Yanase, T., Mu, Y., Oba, K., Ichino, I., Saito, M., Nomura, M., Mukasa, C., Okabe, T., Goto, K., Takayanagi, R., Kashimura, Y., Haji, M., and Nawata, H. Establishment and characterization of a steroidogenic human granulosa-like tumor cell line, KGN, that expresses functional follicle-stimulating hormone receptor. Endocrinology, *142*: 437-445, 2001.

111. Saitoh, M., Yanase, T., Morinaga, H., Tanabe, M., Mu, Y. M., Nishi, Y., Nomura, M., Okabe, T., Goto, K., Takayanagi, R., and Nawata, H. Tributyltin or triphenyltin inhibits aromatase activity in the human granulosa-like tumor cell line KGN. Biochem.Biophys.Res.Commun., *289*: 198-204, 2001.

112. Richards, J. S. Hormonal control of gene expression in the ovary. Endocr.Rev., *15*: 725-751, 1994.

113. Odum, J. and Ashby, J. Detection of aromatase inhibitors in vitro using rat ovary microsomes. Toxicol.In Vitro, in press, 2001.

114. Barbieri, R. L., McShane, P. M., and Ryan, K. J. Constituents of cigarette smoke inhibit human granulosa cell aromatase. Fertil.Steril., *46*: 232-236, 1986.

115. Moran, F. M., Conley, A. J., Corbin, C. J., Enan, E., VandeVoort, C., Overstreet, J. W., and Lasley, B. L. 2,3,7,8-tetrachlorodibenzo-p-dioxin decreases estradiol production without altering the enzyme activity of cytochrome P450 aromatase of human luteinized granulosa cells in vitro. Biol.Reprod., *62*: 1102-1108, 2000.

116. Laskey, J. W., Berman, E., and Ferrell, J. M. The use of cultured ovarian fragments to assess toxicant alterations in steroidogenesis in the Sprague-Dawley rat. Reprod.Toxicol., *9*: 131-141, 1995.

117. Afonso, L. O., Campbell, P. M., Iwama, G. K., Devlin, R. H., and Donaldson, E. M. The effect of the aromatase inhibitor fadrozole and two polynuclear aromatic hydrocarbons on sex steroid secretion by ovarian follicles of coho salmon. Gen.Comp Endocrinol., *106*: 169-174, 1997.

118. Rocha Monteiro, P. R., Reis-Henriques, M. A., and Coimbra, J. Polycyclic aromatic hydrocarbons inhibit in vitro ovarian steroidogenesis in the flounder (Platichthys flesus L.). Aquatic.Toxicol., *48*: 549-559, 2000.

119. Lovekamp, T. N. and Davis, B. J. Mono-(2-ethylhexyl) phthalate suppresses aromatase

transcript levels and estradiol production in cultured rat granulosa cells. Toxicol.Appl.Pharmacol., *172*: 217-224, 2001.

120. Miller, W. R. and O'Neill, J. The importance of local synthesis of estrogen within the breast. Steroids, *50*: 537-548, 1987.

121. Simpson, E. R. Aromatase 2000: Fifth International Aromatase Conference. 2000. 11-3-2000.

122. Santen, R. J. Aromatase Conference: Future Perspectives. Steroids, *50*: 1-665, 1987.

123. Brodie, A. M., Brodie, H. J., Callard, G., Robinson, C. H., Roselli, C., and Santen, R. J. Proceedings of the Third International Aromatase Conference. J.Steroid Biochem.Mol.Biol., *44*: 321-696, 1993.

124. Simpson, E. R., Bhatnagar, A., Brodie, A. M., Brueggemeier, R. W., Chen, S., DeCoster, R., Dowsett, M., Harada, N., Naftolin, F., Osawa, Y., Plourde, P., Reed, M. J., and Santen, R. J. Proceedings of the Fourth International Aromatase Conference. J.Steroid Biochem.Mol.Biol., *61*: 107-425, 1997.

125. WHO. Pesticide residues in food- 1977 evaluations. Part II-Toxicology. WHO/IPCS/10 Sup, 283-303. 1977. World Health Organization, Geneva. International Programme on Chemical Safety.

126. WHO. Pesticide residues in food- 1983 evaluations. Part II-Toxicology. WHO/IPCS/61, 366-442. 1983. World Health Organization, Geneva. International Programme on Chemical Safety.

127. WHO. Pesticide residues in food- 1995 evaluations. Part II-Toxicology. WHO/IPCS/96.48, 75-104. 1995. World Health Organization, Geneva. International Programme on Chemical Safety.

128. Hirsch, K. S., Adams, E. R., Hoffman, D. G., Markham, J. K., and Owen, N. V. Studies to elucidate the mechanism of fenarimol-induced infertility in male rats. Toxicol.Appl.Pharmacol., *86*: 391-399, 1986.

129. Hirsch, K. S., Weaver, D. E., Black, L. J., Falcone, J. F., and MacLusky, N. J. Inhibition of central nervous system aromatase activity: a mechanism for fenarimol-induced infertility in the male rat. Toxicol.Appl.Pharmacol., *91*: 235-245, 1987.

130. WHO. Pesticide residues in food- 1985 evaluations. Part II-Toxicology. WHO/IPCS/72.2, 199-202. 1985. World Health Organization, Geneva. International Programme on Chemical Safety.

131. WHO. Pesticide residues in food- 1989 evaluations. Part II-Toxicology. WHO/IPCS/100.2, 215-235. 1989. World Health Organization, Geneva. International

Programme on Chemical Safety.

132. Averal, H. I., Stanley, A., Murugaian, P., Palanisamy, M., and Akbarsha, M. A. Specific effect of vincristine on epididymis. Indian J.Exp.Biol., *34*: 53-56, 1996.

133. Hess, R. A., Bunick, D., and Bahr, J. M. Sperm, a source of estrogen. Environ.Health Perspect., *103 Suppl 7*: 59-62, 1995.

134. Akbarsha, M. A., Latha, P. N., and Murugaian, P. Retention of cytoplasmic droplet by rat cauda epididymal spermatozoa after treatment with cytotoxic and xenobiotic agents. J.Reprod.Fertil., *120*: 385-390, 2000.

135. Morcillo, Y. and Porte, C. Evidence of endocrine disruption in the imposex-affected gastropod Bolinus brandaris. Environ.Res., *81*: 349-354, 1999.

136. Desvages, G. and Pieau, C. Aromatase activity in gonads of turtle embryos as a function of the incubation temperature of eggs. J.Steroid Biochem.Mol.Biol., *41*: 851-853, 1992.

137. Willingham, E. and Crews, D. The red-eared slider turtle: An animal model for the study of low doses and mixtures. American Zoologist, *40*: 421-428, 2000.

138. Crews, D. Temperature-dependent sex determination: the interplay of steroid hormones and temperature. Zoolog.Sci., *13*: 1-13, 1996.

139. Crain, D. A., Guillette, L. J., Jr., Rooney, A. A., and Pickford, D. B. Alterations in steroidogenesis in alligators (Alligator mississippiensis) exposed naturally and experimentally to environmental contaminants. Environ.Health Perspect., *105*: 528-533, 1997.

140. Crain, D. A., Spiteri, I. D., and Guillette, L. J., Jr. The functional and structural observations of the neonatal reproductive system of alligators exposed in ovo to atrazine, 2,4-D, or estradiol. Toxicol.Ind.Health, *15*: 180-185, 1999.

141. Gerstenberger, S. L., Heimler, I., Smies, R., Hutz, R. J., Dasmahapatra, A. K., Tripoli, V., and Dellinger, J. A. Minimal endocrine alterations in rodents after consumption of lake trout (Salvelinus namaycush). Arch.Environ.Contam Toxicol., *38*: 371-376, 2000.

142. Li, P. K. and Brueggemeier, R. W. 7-substituted steroidal aromatase inhibitors: structure-activity relationships and molecular modeling. J.Enzyme Inhib., *4*: 113-120, 1990.

143. Furet, P., Batzl, C., Bhatnagar, A., Francotte, E., Rihs, G., and Lang, M. Aromatase inhibitors: synthesis, biological activity, and binding mode of azole-type compounds. J.Med.Chem., *36*: 1393-1400, 1993.

144. Ahmed, S., Davis, P. J., and Owen, C. P. Molecular modeling study of the binding of inhibitors of aromatase to the cytochrome P-450 heme. Drug Design and Discovery, *14*:

91-102, 1996.

145. Laughton, C. A., McKenna, R., Neidle, S., Jarman, M., McCague, R., and Rowlands, M. G. Crystallographic and molecular modeling studies on 3-ethyl-3-(4- pyridyl)piperidine-2,6-dione and its butyl analogue, inhibitors of mammalian aromatase. Comparison with natural substrates: prediction of enantioselectivity for N-alkyl derivatives. J.Med.Chem., *33* : 2673-2679, 1990.

146. Nagy, P. I., Tokarski, J., and Hopfinger, A. J. Molecular shape and QSAR analyses of a family of substituted dichlorodiphenyl aromatase inhibitors. J.Chem.Inf.Comput.Sci., *34*: 1190-1197, 1994.

147. Recanatini, M. and Cavalli, A. Comparative molecular field analysis of non-steroidal aromatase inhibitors: an extended model for two different structural classes. Bioorg.Med.Chem., *6*: 377-388, 1998.

148. Cavalli, A., Greco, G., Novellino, E., and Recanatini, M. Linking CoMFA and protein homology models of enzyme-inhibitor interactions: an application to non-steroidal aromatase inhibitors. Bioorg.Med.Chem., *8*: 2771-2780, 2000.

149. Graham-Lorence, S., Khalil, M. W., Lorence, M. C., Mendelson, C. R., and Simpson, E. R. Structure-function relationships of human aromatase cytochrome P-450 using molecular modeling and site-directed mutagenesis. J.Biol.Chem., *266*: 11939-11946, 1991.

150. Laughton, C. A., Zvelebil, M. J., and Neidle, S. A detailed molecular model for human aromatase. J.Steroid Biochem.Mol.Biol., *44*: 399-407, 1993.

151. Saarinen, N., Joshi, S. C., Ahotupa, M., Li, X., Ammala, J., Makela, S., and Santti, R. No evidence for the in vivo activity of aromatase-inhibiting flavonoids. J.Steroid Biochem.Mol.Biol., *78*: 231-239, 2001.

152. Kirma, N., Gill, K., Mandava, U., and Tekmal, R. R. Overexpression of aromatase leads to hyperplasia and changes in the expression of genes involved in apoptosis, cell cycle, growth, and tumor suppressor functions in the mammary glands of transgenic mice. Cancer Res., *61*: 1910-1918, 2001.

153. Li, X., Nokkala, E., Yan, W., Streng, T., Saarinen, N., Warri, A., Huhtaniemi, I., Santti, R., Makela, S., and Poutanen, M. Altered structure and function of reproductive organs in transgenic male mice overexpressing human aromatase. Endocrinology, *142*: 2435-2442, 2001.

154. Yue, W. and Brodie, A. M. Mechanisms of the actions of aromatase inhibitors 4-hydroxyandrostenedione, fadrozole, and aminoglutethimide on aromatase in JEG-3 cell culture. J.Steroid Biochem.Mol.Biol., *63*: 317-328, 1997.

155. Bhatnagar, A. S., Brodie, A. M., Long, B. J., Evans, D. B., and Miller, W. R. Intracellular aromatase and its relevance to the pharmacological efficacy of aromatase inhibitors. J.Steroid Biochem.Mol.Biol., *76*: 199-202, 2001.

156. Chen, S., Kao, Y. C., and Laughton, C. A. Binding characteristics of aromatase inhibitors and phytoestrogens to human aromatase. J.Steroid Biochem.Mol.Biol., *61*: 107-115, 1997.

157. Cory, A. H., Owen, T. C., Barltrop, J. A., and Cory, J. G. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Commun., *3*: 207-212, 1991.

158. Marshall, N. J., Goodwin, C. J., and Holt, S. J. A critical assessment of the use of microculture tetrazolium assays to measure cell growth and function. Growth Regul., *5*: 69-84, 1995.

159. Goodwin, C. J., Holt, S. J., Downes, S., and Marshall, N. J. Microculture tetrazolium assays: a comparison between two new tetrazolium salts, XTT and MTS. J.Immunol.Methods, *179*: 95-103, 1995.

160. Kragie, L., Turner, S. D., Patten, C. J., Crespi, C. L., Stresser, D. M. Assessing preganancy risks of azole antifungals using a high throughput aromatase inhibition assay. Endocr. Res., *28*:129-140, 2002.

161. Zhao, J., Mak, P., Tchoudakova, A., Callard, G., and Chen, S. Different catalytic properties and inhibitor responses of the goldfish brain and ovary aromatase isozymes. Gen. Comp. Endocrinol. *123*: 180-191, 2001.

162. Sanderson, J. T., Hordijk, J., Denison, M. S., Springsteel, M. F., Nants, M. H., van den Berg, M. Induction and inhibition of aromatase (CYP19) activity by natural and synthetic flavonoid compounds in H295R human adrenocortical carcinoma cells. Toxicol Sci, Aug 2004 [epublished ahead of print].

163. Wantanabe, M., and Nakajin, S. Forskolin up-regulates aromatase (CYP19) activity and gene transcripts in the human adrenocortical carcinoma cell line H295R. J. Endocrinol. *180*: 125-133, 2004.

164. Heneweer, M., van den Berg, M., Sanderson, J. T. A comparison of human H295R and rat R2C cell lines as in vitro screening tools for effects on aromatase. Toxicol. Letters, *146*:183-194, 2004.

165. Sanderson, J. T., Boerma, J., Lansbergen, G.W.A., and van den Berg, M. Induction and

inhibition of aromatase activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. Tox. App. Pharmacol. *182*:44-54, 2002.

166. Morinaga, H., Yanase, T., Nomura, M., Okabe, T., Goto, K., Harada, N. and Nawata, H. A benzimidazole fungicide, benomyl, and its metabolite, carbendizim, induce aromatase activity in a human ovarian granulose-like tumor cell line (KGN). Endocrinology, *145*:1860-1869, 2004.

167 Mukasa, C., Nomura, M., Tanaka, T., Tanaka, K., Nishi, Y., Okabe, T., Goto, K., Yanase, T. and Nawata, H Activin signaling through type IB activin receptor stimulates aromatase activity in the ovarian granulosa cell-like human granulosa cell line. Endocrinology, *144*:1603-1611, 2003.

APPENDIX A

MANUSCRIPTS REVIEWED AND CITED (IN ALPHABETICAL ORDER)

Adlercreutz, H., Bannwart, C., Wahala, K., Makela, T., Brunow, G., Hase, T., Arosemena, P. J., Kellis, J. T., Jr., and Vickery, L. E. Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens. J.Steroid Biochem.Mol.Biol., *44*: 147-153, 1993.

Afonso, L. O., Campbell, P. M., Iwama, G. K., Devlin, R. H., and Donaldson, E. M. The effect of the aromatase inhibitor fadrozole and two polynuclear aromatic hydrocarbons on sex steroid secretion by ovarian follicles of coho salmon. Gen.Comp Endocrinol., *106*: 169-174, 1997.

Ahmed, S., Davis, P. J., and Owen, C. P. Molecular modeling study of the binding of inhibitors of aromatase to the cytochrome P-450 heme. Drug Design and Discovery, *14*: 91-102, 1996.

Akbarsha, M. A., Latha, P. N., and Murugaian, P. Retention of cytoplasmic droplet by rat cauda epididymal spermatozoa after treatment with cytotoxic and xenobiotic agents. J.Reprod.Fertil., *120*: 385-390, 2000.

Albrecht, E. D. and Pepe, G. J. Placental steroid hormone biosynthesis in primate pregnancy. Endocr.Rev., *11*: 124-150, 1990.

Averal, H. I., Stanley, A., Murugaian, P., Palanisamy, M., and Akbarsha, M. A. Specific effect of vincristine on epididymis. Indian J.Exp.Biol., *34*: 53-56, 1996.

Ayub, M. and Levell, M. J. Structure-activity relationships of the inhibition of human placental aromatase by imidazole drugs including ketoconazole. J.Steroid Biochem., *31*: 65-72, 1988.

Banting, L., Smith, H. J., James, M., Jones, G., Nazareth, W., Nicholls, P. J., Hewlins, M. J., and Rowlands, M. G. Structure-activity relationships for non-steroidal inhibitors of aromatase. J.Enzyme Inhib., *2*: 215-229, 1988.

Banting, L., Nicholls, P. J., Shaw, M. A., and Smith, H. J. Recent developments in aromatase inhibition as a potential treatment for oestrogen-dependent breast cancer. Prog.Med.Chem., *26*: 253-298, 1989.

Barbieri, R. L., McShane, P. M., and Ryan, K. J. Constituents of cigarette smoke inhibit human granulosa cell aromatase. Fertil.Steril., *46*: 232-236, 1986.

Bhatnagar, A. S., Brodie, A. M., Long, B. J., Evans, D. B., and Miller, W. R. Intracellular aromatase and its relevance to the pharmacological efficacy of aromatase inhibitors. J.Steroid Biochem.Mol.Biol., *76*: 199-202, 2001.

Brodie, A. M., Brodie, H. J., Callard, G., Robinson, C. H., Roselli, C., and Santen, R. J. Proceedings of the Third International Aromatase Conference. J.Steroid Biochem.Mol.Biol., *44*: 321-696, 1993.

Brodie, A. M. and Njar, V. C. Aromatase inhibitors and breast cancer. Semin.Oncol., *23*: 10-20, 1996.

Brodie, A., Lu, Q., and Long, B. Aromatase and its inhibitors. J.Steroid Biochem.Mol.Biol., *69*: 205-210, 1999.

Brodie, A., Inkster, S., and Yue, W. Aromatase expression in the human male. Mol.Cell Endocrinol., *178*: 23-28, 2001.

Brueggemeier, R. W. Biochemical and molecular aspects of aromatase. J.Enzyme Inhib., *4*: 101-111, 1990.

Brueggemeier, R. W. and Katlic, N. E. Aromatase inhibition by an enzyme-activated irreversible inhibitor in human carcinoma cell cultures. Cancer Res., *50*: 3652-3656, 1990.

Brueggemeier, R. W., Katlic, N. E., Kenreigh, C. A., and Li, P. K. Aromatase inhibition by 7-substituted steroids in human choriocarcinoma cell culture. J.Steroid Biochem.Mol.Biol., *41*: 85-90, 1992.

Brueggemeier, R. W. Aromatase inhibitors--mechanisms of steroidal inhibitors. Breast Cancer Res.Treat., *30*: 31-42, 1994.

Brueggemeier, R. W., Gilbert, N. E., Gu, X., O'Reilly, J. M., and Lovely, C. J. Aromatase inhibition in JAr choriocarcinoma cells by 7alpha- arylaliphatic androgens. J.Steroid Biochem.Mol.Biol., *61*: 73-77, 1997.

Brueggemeier, R. W., Quinn, A. L., Parrett, M. L., Joarder, F. S., Harris, R. E., and Robertson, F. M. Correlation of aromatase and cyclooxygenase gene expression in human breast cancer specimens. Cancer Lett., *140*: 27-35, 1999.

Bulun, S. E., Price, T. M., Aitken, J., Mahendroo, M. S., and Simpson, E. R. A link between breast cancer and local estrogen biosynthesis suggested by quantification of breast adipose tissue aromatase cytochrome P450 transcripts using competitive polymerase chain reaction after reverse transcription. J.Clin.Endocrinol.Metab, *77*: 1622-1628, 1993.

Callard, G. V., Petro, Z., and Ryan, K. J. Identification of aromatase in the reptilian brain. Endocrinology, *100*: 1214-1218, 1977.

Callard, G. V. and Tchoudakova, A. Evolutionary and functional significance of two CYP19 genes differentially expressed in brain and ovary of goldfish. J.Steroid Biochem.Mol.Biol., *61*: 387-392, 1997.

Campbell, D. R. and Kurzer, M. S. Flavonoid inhibition of aromatase enzyme activity in human preadipocytes. J.Steroid Biochem.Mol.Biol., *46*: 381-388, 1993.

Cavalli, A., Greco, G., Novellino, E., and Recanatini, M. Linking CoMFA and protein homology models of enzyme-inhibitor interactions: an application to non-steroidal aromatase inhibitors. Bioorg.Med.Chem., *8*: 2771-2780, 2000.

Chen, S., Kao, Y. C., and Laughton, C. A. Binding characteristics of aromatase inhibitors and phytoestrogens to human aromatase. J.Steroid Biochem.Mol.Biol., *61*: 107-115, 1997.

Chen, S., Sun, X. Z., Kao, Y. C., Kwon, A., and Eng, E. Suppression of breast cancer cell growth with grape juice. Pharmaceutical Biology (Netherlands), *36*: 53-61, 1998.

Chiang, E. F., Yan, Y. L., Guiguen, Y., Postlethwait, J., and Chung, B. Two Cyp19 (P450 Aromatase) Genes on Duplicated Zebrafish Chromosomes Are Expressed in Ovary or Brain. Mol.Biol.Evol., *18*: 542-550, 2001.

Cole, P. A. and Robinson, C. H. Mechanism and inhibition of cytochrome P-450 aromatase. J.Med.Chem., *33*: 2933-2942, 1990.

Conley, A., Corbin, J., Smith, T., Hinshelwood, M., Liu, Z., and Simpson, E. Porcine aromatases: studies on tissue-specific, functionally distinct isozymes from a single gene? J.Steroid Biochem.Mol.Biol., *61*: 407-413, 1997.

Cooke, B., Hegstrom, C. D., Villeneuve, L. S., and Breedlove, S. M. Sexual differentiation of the vertebrate brain: principles and mechanisms. Front Neuroendocrinol., *19*: 323-362, 1998.

Corbin, C. J., Trant, J. M., and Conley, A. J. Porcine gonadal and placental isozymes of aromatase cytochrome P450: sub-cellular distribution and support by NADPH-cytochrome P450 reductase. Mol.Cell Endocrinol., *172*: 115-124, 2001.

Cory, A. H., Owen, T. C., Barltrop, J. A., and Cory, J. G. Use of an aqueous soluble tetrazolium/formazan assay for cell growth assays in culture. Cancer Commun., *3*: 207-212, 1991.

Covey, D. F. Aromatase Inhibitors: Specific Inhibitors of Oestrogen Biosynthesis. *In* D. Berg and M. Plempel (eds.), Sterol Biosynthesis Inhibitors, pp. 534-571. Chichester, UK: Ellis Horwood, 1988.

Crain, D. A., Guillette, L. J., Jr., Rooney, A. A., and Pickford, D. B. Alterations in steroidogenesis in alligators (Alligator mississippiensis) exposed naturally and experimentally to environmental contaminants. Environ.Health Perspect., *105*: 528-533, 1997.

Crain, D. A., Spiteri, I. D., and Guillette, L. J., Jr. The functional and structural observations of the neonatal reproductive system of alligators exposed in ovo to atrazine, 2,4-D, or estradiol. Toxicol.Ind.Health, *15*: 180-185, 1999.

Crews, D. Temperature-dependent sex determination: the interplay of steroid hormones and temperature. Zoolog.Sci., *13*: 1-13, 1996.

Desvages, G. and Pieau, C. Aromatase activity in gonads of turtle embryos as a function of the incubation temperature of eggs. J.Steroid Biochem.Mol.Biol., *41*: 851-853, 1992.

Dowsett, M., Macaulay, V., Gledhill, J., Ryde, C., Nicholls, J., Ashworth, A., McKinna, J. A., and Smith, I. E. Control of aromatase in breast cancer cells and its importance for tumor growth. J.Steroid Biochem.Mol.Biol., *44*: 605-609, 1993.

Drenth, H. J., Bouwman, C. A., Seinen, W., and van den, B. M. Effects of some persistent halogenated environmental contaminants on aromatase (CYP19) activity in the human choriocarcinoma cell line JEG-3. Toxicol.Appl.Pharmacol., *148*: 50-55, 1998.

Fisher, C. R., Graves, K. H., Parlow, A. F., and Simpson, E. R. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the cyp19 gene. Proc.Natl.Acad.Sci.U.S.A, *95*: 6965-6970, 1998.

Furet, P., Batzl, C., Bhatnagar, A., Francotte, E., Rihs, G., and Lang, M. Aromatase inhibitors: synthesis, biological activity, and binding mode of azole-type compounds. J.Med.Chem., *36*: 1393-1400, 1993.

Gerstenberger, S. L., Heimler, I., Smies, R., Hutz, R. J., Dasmahapatra, A. K., Tripoli, V., and Dellinger, J. A. Minimal endocrine alterations in rodents after consumption of lake trout (Salvelinus namaycush). Arch.Environ.Contam Toxicol., *38*: 371-376, 2000.

Goodwin, C. J., Holt, S. J., Downes, S., and Marshall, N. J. Microculture tetrazolium assays: a comparison between two new tetrazolium salts, XTT and MTS. J.Immunol.Methods, *179*: 95-103, 1995.

Graham-Lorence, S., Khalil, M. W., Lorence, M. C., Mendelson, C. R., and Simpson, E. R. Structure-function relationships of human aromatase cytochrome P-450 using molecular modeling and site-directed mutagenesis. J.Biol.Chem., *266*: 11939-11946, 1991.

Gross, T. S. and Williams, W. F. In vitro steroid synthesis by the placenta of cows during late gestation and at parturition. J.Reprod.Fertil. 83, 565-573. 1988.

Harvey, H. A., Lipton, A., and Santen, R. J. Aromatase: New Perspectives for Breast Cancer. Cancer Res., *42*: 3261s-3467s, 1982.

Heneweer, M., van den Berg, M., Sanderson, J. T. A comparison of human H295R and rat R2C cell lines as in vitro screening tools for effect on aromatase. Toxicol. Letters, *146*:183-194, 2004.

Hess, R. A., Bunick, D., and Bahr, J. M. Sperm, a source of estrogen. Environ.Health Perspect., *103 Suppl 7*: 59-62, 1995.

Hickey, G. J., Krasnow, J. S., Beattie, W. G., and Richards, J. S. Aromatase cytochrome P450 in rat ovarian granulosa cells before and after luteinization: adenosine 3',5'-monophosphate-dependent and independent regulation. Cloning and sequencing of rat aromatase cDNA and 5' genomic DNA. Mol.Endocrinol., *4*: 3-12, 1990.

Hinshelwood, M. M., Liu, Z., Conley, A. J., and Simpson, E. R. Demonstration of tissue-specific promoters in nonprimate species that express aromatase P450 in placentae. Biol.Reprod., *53*: 1151-1159, 1995.

Hinshelwood, M. M., Dodson, M. M., Sun, T., and Simpson, E. R. Regulation of aromatase expression in the ovary and placenta: a comparison between two species. J.Steroid Biochem.Mol.Biol., *61* : 399-405, 1997.

Hirsch, K. S., Adams, E. R., Hoffman, D. G., Markham, J. K., and Owen, N. V. Studies to elucidate the mechanism of fenarimol-induced infertility in male rats. Toxicol.Appl.Pharmacol., *86*: 391-399, 1986.

Hirsch, K. S., Weaver, D. E., Black, L. J., Falcone, J. F., and MacLusky, N. J. Inhibition of central nervous system aromatase activity: a mechanism for fenarimol-induced infertility in the male rat. Toxicol.Appl.Pharmacol., *91*: 235-245, 1987.

Hughes, R., Timmermans, P., and Schrey, M. P. Regulation of arachidonic acid metabolism, aromatase activity and growth in human breast cancer cells by interleukin-1beta and phorbol ester: dissociation of a mediatory role for prostaglandin E2 in the autocrine control of cell function. Int.J.Cancer, *67*: 684-689, 1996.

Ibrahim, A. R. and Abul-Hajj, Y. J. Aromatase inhibition by flavonoids. J.Steroid Biochem.Mol.Biol., *37*: 257-260, 1990.

James, V. H., McNeill, J. M., Lai, L. C., Newton, C. J., Ghilchik, M. W., and Reed, M. J. Aromatase activity in normal breast and breast tumor tissues: in vivo and in vitro studies. Steroids, *50*: 269-279, 1987.

Jeong, H. J., Shin, Y. G., Kim, I. H., and Pezzuto, J. M. Inhibition of aromatase activity by flavonoids. Arch.Pharm.Res., *22*: 309-312, 1999.

Jeyasuria, P., Roosenburg, W. M., and Place, A. R. Role of P-450 aromatase in sex determination of the diamondback terrapin, Malaclemys terrapin. J.Exp.Zool., *270*: 95-111, 1994.

Johnston, J. O. and Metcalf, B. W. Aromatase: A Target Enzyme in Breast Cancer. *In* P. Sunkara (ed.), Novel Approaches to Cancer Chemotherapy, pp. 307-328. New York: Academic Press, 1984.

Jones, M. E., Thorburn, A. W., Britt, K. L., Hewitt, K. N., Wreford, N. G., Proietto, J., Oz, O. K., Leury, B. J., Robertson, K. M., Yao, S., and Simpson, E. R. Aromatase-deficient (ArKO) mice have a phenotype of increased adiposity. Proc.Natl.Acad.Sci.U.S.A, *97*: 12735-12740, 2000.

Kao, Y. C., Zhou, C., Sherman, M., Laughton, C. A., and Chen, S. Molecular basis of the inhibition of human aromatase (estrogen synthetase) by flavone and isoflavone phytoestrogens: A site-directed mutagenesis study. Environ.Health Perspect., *106*: 85-92, 1998.

Kellis, J. T., Jr. and Vickery, L. E. Inhibition of human estrogen synthetase (aromatase) by flavones. Science, *225*: 1032-1034, 1984.

Kellis, J. T., Jr., Nesnow, S., and Vickery, L. E. Inhibition of aromatase cytochrome P-450 (estrogen synthetase) by derivatives of alpha-naphthoflavone. Biochem.Pharmacol., *35*: 2887-2891, 1986.

Kellis, J. T., Jr. and Vickery, L. E. Purification and characterization of human placental aromatase cytochrome P-450. J.Biol.Chem., *262*: 4413-4420, 1987.

Kirma, N., Gill, K., Mandava, U., and Tekmal, R. R. Overexpression of aromatase leads to hyperplasia and changes in the expression of genes involved in apoptosis, cell cycle, growth, and tumor suppressor functions in the mammary glands of transgenic mice. Cancer Res., *61*: 1910-1918, 2001.

Kishida, M. and Callard, G. V. Distinct cytochrome P450 aromatase isoforms in zebrafish (Danio rerio) brain and ovary are differentially programmed and estrogen regulated during early development. Endocrinology, *142*: 740-750, 2001.

Kragie, L., Turner, S. D., Patten, C. J., Crespi, C. L., Stresser, D. M. Assessing preganancy risks of azole antifungals using a high throughput aromatase inhibition assay. Endocr. Res., *28*:129-

140, 2002.Laskey, J. W., Berman, E., and Ferrell, J. M. The use of cultured ovarian fragments to assess toxicant alterations in steroidogenesis in the Sprague-Dawley rat. Reprod.Toxicol., *9*: 131-141, 1995.

Laughton, C. A., Zvelebil, M. J., and Neidle, S. A detailed molecular model for human aromatase. J.Steroid Biochem.Mol.Biol., *44*: 399-407, 1993.

Laughton, C. A., McKenna, R., Neidle, S., Jarman, M., McCague, R., and Rowlands, M. G. Crystallographic and molecular modeling studies on 3-ethyl-3-(4- pyridyl)piperidine-2,6-dione and its butyl analogue, inhibitors of mammalian aromatase. Comparison with natural substrates: prediction of enantioselectivity for N-alkyl derivatives. J.Med.Chem., *33* : 2673-2679, 1990.

Letcher, R. J., van, H., I, Drenth, H. J., Norstrom, R. J., Bergman, A., Safe, S., Pieters, R., and van den, B. M. Cytotoxicity and aromatase (CYP19) activity modulation by organochlorines in human placental JEG-3 and JAR choriocarcinoma cells. Toxicol.Appl.Pharmacol., *160*: 10-20, 1999.

Le Bail, J. C., Laroche, T., Marre-Fournier, F., and Habrioux, G. Aromatase and 17beta-hydroxysteroid dehydrogenase inhibition by flavonoids. Cancer Lett., *133*: 101-106, 1998.

Le Bail, J. C., Aubourg, L., and Habrioux, G. Effects of pinostrobin on estrogen metabolism and estrogen receptor transactivation. Cancer Lett., *156*: 37-44, 2000.

Le Bail, J. C., Champavier, Y., Chulia, A. J., and Habrioux, G. Effects of phytoestrogens on aromatase, 3beta and 17beta-hydroxysteroid dehydrogenase activities and human breast cancer cells. Life Sci., *66*: 1281-1291, 2000.

Le Bail, J. C., Pouget, C., Fagnere, C., Basly, J. P., Chulia, A. J., and Habrioux, G. Chalcones are potent inhibitors of aromatase and 17beta-hydroxysteroid dehydrogenase activities. Life Sci., *68*: 751-761, 2001.

Lephart, E. D. Molecular aspects of brain aromatase cytochrome P450. J.Steroid Biochem.Mol.Biol., *61*: 375-380, 1997.

Lephart, E. D. A review of brain aromatase cytochrome P450. Brain Res.Brain Res.Rev., *22*: 1-26, 1996.

Lephart, E. D. Molecular aspects of brain aromatase cytochrome P450. J.Steroid Biochem.Mol.Biol., *61*: 375-380, 1997.

Lephart, E. D. and Simpson, E. R. Assay of aromatase activity. Methods Enzymol., *206*: 477-483, 1991.

Li, P. K. and Brueggemeier, R. W. 7-substituted steroidal aromatase inhibitors: structure-activity relationships and molecular modeling. J.Enzyme Inhib., *4*: 113-120, 1990.

Li, X., Nokkala, E., Yan, W., Streng, T., Saarinen, N., Warri, A., Huhtaniemi, I., Santti, R., Makela, S., and Poutanen, M. Altered structure and function of reproductive organs in transgenic male mice overexpressing human aromatase. Endocrinology, *142*: 2435-2442, 2001.

Lombardi, G., Zarrilli, S., Colao, A., Paesano, L., Di Somma, C., Rossi, F., and De Rosa, M. Estrogens and health in males. Mol.Cell Endocrinol., *178*: 51-55, 2001.

Lovekamp, T. N. and Davis, B. J. Mono-(2-ethylhexyl) phthalate suppresses aromatase transcript levels and estradiol production in cultured rat granulosa cells. Toxicol.Appl.Pharmacol., *172*: 217-224, 2001.

Mak, P., Cruz, F. D., and Chen, S. A yeast screen system for aromatase inhibitors and ligands for androgen receptor: yeast cells transformed with aromatase and androgen receptor. Environ.Health Perspect., *107*: 855-860, 1999.

Marshall, J. C. Hormonal Regulation of the Menstrual Cycle and Mechanism of Ovulation. *In* L. J. DeGroot, J. L. Jameson, H. G. Burger, D. L. Loriaux, J. C. Marshall, S. Melmed, W. D. Odell, J. T. Jr. Potts, and A. H. Rubenstein (eds.), Endocrinology, Fourth Edition, pp. 2073-2085. Philadelphia: W.B. Saunders Company, 2001.

Marshall, N. J., Goodwin, C. J., and Holt, S. J. A critical assessment of the use of microculture tetrazolium assays to measure cell growth and function. Growth Regul., *5*: 69-84, 1995.

Mason, J. I., Murry, B. A., Olcott, M., and Sheets, J. J. Imidazole antimycotics: inhibitors of steroid aromatase. Biochem.Pharmacol., *34*: 1087-1092, 1985.

Mason, J. I., Carr, B. R., and Murry, B. A. Imidazole antimycotics: selective inhibitors of steroid aromatization and progesterone hydroxylation. Steroids, *50*: 179-189, 1987.

McNamara, J., Stocker, P., Miller, V. P., Patten, C. J., Stresser, D. M., and Crespi, C. L. CYP19 (aromatase): Characterization of the recombinant enzyme and its role in the biotransformation of xenobiotics. www.gentest.com/products/pdf/post_016.pdf , 1999.

McPhaul, M. J., Herbst, M. A., Matsumine, H., Young, M., and Lephart, E. D. Diverse mechanisms of control of aromatase gene expression. J.Steroid Biochem.Mol.Biol., *44*: 341-346, 1993.

Means, G. D., Kilgore, M. W., Mahendroo, M. S., Mendelson, C. R., and Simpson, E. R. Tissue-specific promoters regulate aromatase cytochrome P450 gene expression in human ovary and fetal tissues. Mol.Endocrinol., *5*: 2005-2013, 1991.

Miller, W. R., Mullen, P., Sourdaine, P., Watson, C., Dixon, J. M., and Telford, J. Regulation of aromatase activity within the breast. J.Steroid Biochem.Mol.Biol., *61*: 193-202, 1997.

Miller, W. R. and O'Neill, J. The importance of local synthesis of estrogen within the breast. Steroids, *50*: 537-548, 1987.

Moran, F. M., Conley, A. J., Corbin, C. J., Enan, E., VandeVoort, C., Overstreet, J. W., and Lasley, B. L. 2,3,7,8-tetrachlorodibenzo-p-dioxin decreases estradiol production without altering the enzyme activity of cytochrome P450 aromatase of human luteinized granulosa cells in vitro. Biol.Reprod., *62*: 1102-1108, 2000.

Morcillo, Y. and Porte, C. Evidence of endocrine disruption in the imposex-affected gastropod Bolinus brandaris. Environ.Res., *81*: 349-354, 1999

Morinaga, H., Yanase, T., Nomura, M., Okabe, T., Goto, K., Harada, N. and Nawata, H. A benzimidazole fungicide, benomyl, and its metabolite, carbendizim, induce aromatase activity in a human ovarian granulose-like tumor cell line (KGN). Endocrinology, *145*:1860-1869, 2004.

Mukasa, C., Nomura, M., Tanaka, T., Tanaka, K., Nishi, Y., Okabe, T., Goto, K., Yanase, T. and Nawata, H Activin signaling through type IB activin receptor stimulates aromatase activity in the ovarian granulosa cell-like human granulosa cell line. Endocrinology, *144*:1603-1611, 2003..

Naftolin, F. and MacLusky, N. J. Aromatase in the central nervous system. Cancer Res., *42*: 3274s-3276s, 1982.

Naftolin, F. Brain aromatization of androgens. J.Reprod.Med., *39*: 257-261, 1994.

Nagy, P. I., Tokarski, J., and Hopfinger, A. J. Molecular shape and QSAR analyses of a family of substituted dichlorodiphenyl aromatase inhibitors. J.Chem.Inf.Comput.Sci., *34*: 1190-1197, 1994.

Nishi, Y., Yanase, T., Mu, Y., Oba, K., Ichino, I., Saito, M., Nomura, M., Mukasa, C., Okabe, T., Goto, K., Takayanagi, R., Kashimura, Y., Haji, M., and Nawata, H. Establishment and characterization of a steroidogenic human granulosa-like tumor cell line, KGN, that expresses functional follicle-stimulating hormone receptor. Endocrinology, *142*: 437-445, 2001.

Odum, J. and Ashby, J. Detection of aromatase inhibitors in vitro using rat ovary microsomes. Toxicol.In Vitro, in press, 2001.

Oz, O. K., Zerwekh, J. E., Fisher, C., Graves, K., Nanu, L., Millsaps, R., and Simpson, E. R. Bone has a sexually dimorphic response to aromatase deficiency. J.Bone Miner.Res., *15*: 507-514, 2000.

Pasmanik, M., Schlinger, B. A., and Callard, G. V. In vivo steroid regulation of aromatase and 5-alpha-reductase activities in bnrain and pituitary of goldfish. Gen.Comp Endocrinol. 71, 175-182. 1988.

Pelissero, C., Lenczowski, M. J., Chinzi, D., Davail-Cuisset, B., Sumpter, J. P., and Fostier, A. Effects of flavonoids on aromatase activity, an in vitro study. J.Steroid Biochem.Mol.Biol., *57*: 215-223, 1996.

Quinn, A. L., Burak, W. E., Jr., and Brueggemeier, R. W. Effects of matrix components on aromatase activity in breast stromal cells in culture. J.Steroid Biochem.Mol.Biol., *70*: 249-256, 1999.

Recanatini, M. and Cavalli, A. Comparative molecular field analysis of non-steroidal aromatase inhibitors: an extended model for two different structural classes. Bioorg.Med.Chem., *6*: 377-388, 1998.

Reed, M. J., Owen, A. M., Lai, L. C., Coldham, N. G., Ghilchik, M. W., Shaikh, N. A., and James, V. H. In situ oestrone synthesis in normal breast and breast tumour tissues: effect of treatment with 4-hydroxyandrostenedione. Int.J.Cancer, *44*: 233-237, 1989.

Reed, M. J., Topping, L., Coldham, N. G., Purohit, A., Ghilchik, M. W., and James, V. H. Control of aromatase activity in breast cancer cells: the role of cytokines and growth factors. J.Steroid Biochem.Mol.Biol., *44*: 589-596, 1993.

Reed, M. J. The role of aromatase in breast tumors. Breast Cancer Res.Treat., *30*: 7-17, 1994.

Robertson, K. M., O'Donnell, L., Jones, M. E., Meachem, S. J., Boon, W. C., Fisher, C. R., Graves, K. H., McLachlan, R. I., and Simpson, E. R. Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. Proc.Natl.Acad.Sci.U.S.A, *96*: 7986-7991, 1999.

Rocha Monteiro, P. R., Reis-Henriques, M. A., and Coimbra, J. Polycyclic aromatic hydrocarbons inhibit in vitro ovarian steroidogenesis in the flounder (Platichthys flesus L.). Aquatic.Toxicol., *48*: 549-559, 2000.

Richards, J. S. Hormonal control of gene expression in the ovary. Endocr.Rev., *15*: 725-751, 1994.

Richards, J. S., Fitzpatrick, S. L., Clemens, J. W., Morris, J. K., Alliston, T., and Sirois, J. Ovarian cell differentiation: a cascade of multiple hormones, cellular signals, and regulated genes. Recent Prog.Horm.Res., *50*: 223-254, 1995.

Ruentiz, P. C. Female Sex Hormones and Analogs. *In* M. E. Wolff (ed.), Burger's Medicinal Chemistry, 5th Edition ed, pp. 553-588. New York: Wiley, 1997.

Ryan, K. J. Biological aromatization of steroids. J.Biol.Chem., *234*: 268-272, 1959.

Saarinen, N., Joshi, S. C., Ahotupa, M., Li, X., Ammala, J., Makela, S., and Santti, R. No evidence for the in vivo activity of aromatase-inhibiting flavonoids. J.Steroid Biochem.Mol.Biol., *78*: 231-239, 2001.

Saitoh, M., Yanase, T., Morinaga, H., Tanabe, M., Mu, Y. M., Nishi, Y., Nomura, M., Okabe, T., Goto, K., Takayanagi, R., and Nawata, H. Tributyltin or triphenyltin inhibits aromatase activity in the human granulosa-like tumor cell line KGN. Biochem.Biophys.Res.Commun., *289*: 198-204, 2001.

Sanderson, J. T., Boerma, J., Lansbergen, G.W.A., and van den Berg, M. Induction and inhibition of aromatase activity by various classes of pesticides in H295R human adrenocortical carcinoma cells. Tox. App. Pharmacol. *182*:44-54, 2002.

Sanderson, J. T., Hordijk, J., Denison, M. S., Springsteel, M. F., Nants, M. H., van den Berg, M. Induction and inhibition of aromatase (CYP19) activity by natural and synthetic flavonoid compounds in H295R human adrenocortical carcinoma cells. Toxicol Sci, Aug 2004 [epublished ahead of print].

Sanderson, J. T., Seinen, W., Giesy, J. P., and van den, B. M. 2-Chloro-s-triazine herbicides induce aromatase (CYP19) activity in H295R human adrenocortical carcinoma cells: a novel mechanism for estrogenicity? Toxicol.Sci., *54*: 121-127, 2000.

Sanderson, J. T., Slobbe, L., Lansbergen, G. W., Safe, S., and van den, B. M. 2,3,7,8-Tetrachlorodibenzo-p-dioxin and Diindolylmethanes Differentially Induce Cytochrome P450 1A1, 1B1, and 19 in H295R Human Adrenocortical Carcinoma Cells. Toxicol.Sci., *61*: 40-48, 2001.

Sanderson, J. T., Letcher, R. J., Heneweer, M., Giesy, J. P., and van den, B. M. Effects of chloro-s-triazine herbicides and metabolites on aromatase activity in various human cell lines and on vitellogenin production in male carp hepatocytes. Environ.Health Perspect., *109*: 1027-1031, 2001.

Santen, R. J. Aromatase Conference: Future Perspectives. Steroids, *50*: 1-665, 1987.

Santen, R. J. and Harvey, H. A. Use of aromatase inhibitors in breast carcinoma. Endocr.Relat Cancer, *6*: 75-92, 1999.

Shilling, A. D., Carlson, D. B., and Williams, D. E. Rainbow trout, Oncorhynchus mykiss, as a model for aromatase inhibition. J.Steroid Biochem.Mol.Biol., *70*: 89-95, 1999.

Simpson, E. R., Merrill, J. C., Hollub, A. J., Graham-Lorence, S., and Mendelson, C. R. Regulation of estrogen biosynthesis by human adipose cells. Endocr.Rev., *10*: 136-148, 1989.

Simpson, E. R., Mahendroo, M. S., Means, G. D., Kilgore, M. W., Corbin, C. J., and Mendelson, C. R. Tissue-specific promoters regulate aromatase cytochrome P450 expression. J.Steroid Biochem.Mol.Biol., *44*: 321-330, 1993.

Simpson, E. R., Mahendroo, M. S., Means, G. D., Kilgore, M. W., Hinshelwood, M. M., Graham-Lorence, S., Amarneh, B., Ito, Y., Fisher, C. R., Michael, M. D., and . Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocr.Rev., *15*: 342-355, 1994.

Simpson, E. R., Bhatnagar, A., Brodie, A. M., Brueggemeier, R. W., Chen, S., DeCoster, R., Dowsett, M., Harada, N., Naftolin, F., Osawa, Y., Plourde, P., Reed, M. J., and Santen, R. J. Proceedings of the Fourth International Aromatase Conference. J.Steroid Biochem.Mol.Biol., *61*: 107-425, 1997.

Simpson, E. R., Zhao, Y., Agarwal, V. R., Michael, M. D., Bulun, S. E., Hinshelwood, M. M., Graham-Lorence, S., Sun, T., Fisher, C. R., Qin, K., and Mendelson, C. R. Aromatase expression in health and disease. Recent Prog.Horm.Res., *52*: 185-213, 1997.

Simpson, E., Jones, M., Davis, S., and Rubin, G. Do intracrine mechanisms regulate aromatase expression? J.Steroid Biochem.Mol.Biol., *69*: 447-452, 1999

Simpson, E. R. Aromatase 2000: Fifth International Aromatase Conference. 2000. 11-3-2000.

Simpson, E., Rubin, G., Clyne, C., Robertson, K., O'Donnell, L., Jones, M., and Davis, S. The role of local estrogen biosynthesis in males and females. Trends Endocrinol.Metab, *11*: 184-188, 2000.

Simpson, E. R., Clyne, C., Rubin, G., Boon, W. C., Robertson, K., Britt, K., Speed, C., and Jones, M. Aromatase - A Brief Overview. Annu.Rev.Physiol, *64*: 93-127, 2002.

Strauss, J. F. I. and Hsueh, A. J. W. Ovarian Hormone Synthesis. *In* L. J. DeGroot, J. L. Jameson, H. G. Burger, D. L. Loriaux, J. C. Marshall, S. Melmed, W. D. Odell, J. T. Jr. Potts, and A. H. Rubenstein (eds.), Endocrinology, Fourth Edition, pp. 2043-2052. Philadelphia: W.B. Saunders Company, 2001.

Stresser, D. M., Turner, S. D., McNamara, J., Stocker, P., Miller, V. P., Crespi, C. L., and Patten, C. J. A high-throughput screen to identify inhibitors of aromatase (CYP19). Anal.Biochem., *284*: 427-430, 2000.

Tekmal, R. R., Ramachandra, N., Gubba, S., Durgam, V. R., Mantione, J., Toda, K., Shizuta, Y., and Dillehay, D. L. Overexpression of int-5/aromatase in mammary glands of transgenic mice results in the induction of hyperplasia and nuclear abnormalities. Cancer Res., *56*: 3180-3185, 1996.

Trant, J. M., Lehrter, J., Gregory, T., Nunez, S., and Wunder, J. Expression of cytochrome P450 aromatase in the channel catfish, Ictalurus punctatus. J.Steroid Biochem.Mol.Biol., *61*: 393-397, 1997.

Valladres, L. E. and Payne, A. H. Induction of testicular aromatization by luteinizing hormone in mature rats. Endocrinology, *105*: 431-436, 1979.

Vinggaard, A. M., Hnida, C., Breinholt, V., and Larsen, J. C. Screening of selected pesticides for inhibition of CYP19 aromatase activity in vitro. Toxicol.In Vitro, *14*: 227-234, 2000.

Wang, C., Makela, T., Hase, T., Adlercreutz, H., and Kurzer, M. S. Lignans and flavonoids inhibit aromatase enzyme in human preadipocytes. J.Steroid Biochem.Mol.Biol., *50*: 205-212, 1994.

Wantanabe, M., and Nakajin, S. Forskolin up-regulates aromatase (CYP19) activity and gene transcripts in the human adrenocortical carcinoma cell line H295R. J. Endocrinol. *180*: 125-133, 2004

WHO. Pesticide residues in food- 1977 evaluations. Part II-Toxicology. WHO/IPCS/10 Sup, 283-303. 1977. World Health Organization, Geneva. International Programme on Chemical Safety.

WHO. Pesticide residues in food- 1983 evaluations. Part II-Toxicology. WHO/IPCS/61, 366-442. 1983. World Health Organization, Geneva. International Programme on Chemical Safety.

WHO. Pesticide residues in food- 1995 evaluations. Part II-Toxicology. WHO/IPCS/96.48, 75-104. 1995. World Health Organization, Geneva. International Programme on Chemical Safety.

WHO. Pesticide residues in food- 1985 evaluations. Part II-Toxicology. WHO/IPCS/72.2, 199-202. 1985. World Health Organization, Geneva. International Programme on Chemical Safety.

WHO. Pesticide residues in food- 1989 evaluations. Part II-Toxicology. WHO/IPCS/100.2, 215-235. 1989. World Health Organization, Geneva. International Programme on Chemical Safety.

Weigel, N. L. and Rowan, B. G. Estrogen and Progesterone Action. *In* L. J. DeGroot, J. L. Jameson, H. G. Burger, D. L. Loriaux, J. C. Marshall, S. Melmed, W. D. Odell, J. T. Jr. Potts, and A. H. Rubenstein (eds.), Endocrinology, Fourth Edition, pp. 2053-2060. Philadelphia: W.B. Saunders Company, 2001.

Williams, C. and Stancel, G. M. Estrogens and Progestins. *In* J. G. Hardman, L. E. Limbird, P. B. Molinoff, R. W. Ruddon, and A. G. Gilman (eds.), Goodman and Gilman's The Pharmacological Basis of Therapeutics, Ninth Edition ed, pp. 1411-1440. New York: McGraw-Hill, 1996.

Willingham, E. and Crews, D. The red-eared slider turtle: An animal model for the study of low doses and mixtures. American Zoologist, *40*: 421-428, 2000.

Yang, C. and Chen, S. Two organochlorine pesticides, toxaphene and chlordane, are antagonists for estrogen-related receptor alpha-1 orphan receptor. Cancer Res., *59*: 4519-4524, 1999.

Yue, W. and Brodie, A. M. Mechanisms of the actions of aromatase inhibitors 4-hydroxyandrostenedione, fadrozole, and aminoglutethimide on aromatase in JEG-3 cell culture. J.Steroid Biochem.Mol.Biol., *63*: 317-328, 1997.

Zhao, J., Mak, P., Tchoudakova, A., Callard, G., and Chen, S. Different catalytic properties and inhibitor responses of the goldfish brain and ovary aromatase isozymes. Gen. Comp. Endocrinol. *123*: 180-191, 2001.

Zhao, Y., Agarwal, V. R., Mendelson, C. R., and Simpson, E. R. Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene. Endocrinology, *137*: 5739-5742, 1996.

Zhou, C., Zhou, D., Esteban, J., Murai, J., Siiteri, P. K., Wilczynski, S., and Chen, S. Aromatase gene expression and its exon I usage in human breast tumors. Detection of aromatase messenger RNA by reverse transcription- polymerase chain reaction. J.Steroid Biochem.Mol.Biol., *59*: 163-171, 1996.

APPENDIX B

INVESTIGATORS CONTACTED AND QUESTIONNAIRE

Investigators contacted

Investigator	*Expertise*
Shiuan Chen, Ph.D. Division of Immunology Beckman Research Institute, City of Hope 1450 E. Duarte Road Duarte, CA 91006, U.S.A. E-mail: schen@smtplink.coh.org	Biochemistry and molecular biology of aromatase; structure-function studies; aromatase inhibition by flavonoids
Rajeshwar R. Tekmal, Ph.D. Department of Ob/Gyn Emory University School of Medicine 4219 WMB, 1639 Pierce Drive Atlanta, GA 30322, U.S.A. E-mail: rtekmal@emory.edu	Molecular biology of aromatase; development of transgenic mice expressing aromatase
Edwin D. Lephart, Ph.D. 633 WIDB, Neuroscience Center Brigham Young University Provo, UT 84602, U.S.A. E-mail: edwin_lephart@byu.edu	Brain aromatase; flavonoids and aromatase
Gerard Habrioux, Ph.D. Laboratory of Biochemistry Faculty of Pharmacy 2 rue du Dr. Marcland 87025 Limoges Cedex, FRANCE E-mail: habrioux@unilim.fr	Biochemistry and pharmacology of aromatase; aromatase inhibition by flavonoids
Sari Mäkelä, Ph.D., and **Risto Santti**, Ph.D. University of Turku Institute of Biomedicine and Medicity Research Laboratory Turku, FINLAND E-mail: sarmak@utu.fi	Biochemistry and pharmacology of aromatase; aromatase inhibition by flavonoids; endocrine disruptors
A.M. Vinnggaard, Ph.D. Institute of Food Safety and Toxicology Division of Biochemistry and Molecular Biology Danish Veterinary and Food Administration Morkhoj Bygade 19 DK-2860 Soborg, DENMARK E-mail: amv@vfd.dk	Endocrine toxicology; endocrine disruptors
Martin van den Berg, Ph.D. Research Institute of Toxicology Utrecht University P.O. Box 80.176, Yaleaan 2 Utrecht 3508 TD, The NETHERLANDS E-mail: r.letcher@ritox.vet.uu.nl	Endocrine toxicology; endocrine disruptors

Questionnaire sent via e-mail

Questionnaire: Aromatase as a possible target for endocrine disrupters and use of an *in vitro* aromatase assay for screening chemicals

From: Bob Brueggemeier, The Ohio State University (E-mail: Brueggemeier.1@osu.edu)

The United States Environmental Protection Agency (EPA) is implementing an Endocrine Disruptor Screening Program (EDSP). In this program, comprehensive toxicological and ecotoxicological screens and tests are being developed for identifying and characterizing the endocrine effects of various environmental contaminants, industrial chemicals, and pesticides. One potential endocrine target for environmental chemicals is the enzyme aromatase, the cytochrome P450 enzyme complex responsible for estrogen biosynthesis. The EPA is considering employing an *in vitro* aromatase assay as an alternative for possible inclusion in the Tier 1 Screening Battery. Battelle Research Institute, Columbus, Ohio, has the EPA contract for reviewing, establishing, and performing validation studies on the toxicological and ecotoxicological screens and tests. I am serving as a consultant to Battelle because of my research expertise in aromatase and aromatase inhibitors.

Two *in vitro* aromatase assays being evaluated for their use in measuring endocrine disrupters that affect estrogen biosynthesis are:

Human Placental Microsomal Aromatase assay: Determines the effects of environmental chemicals/toxicants on aromatase activity in human placental microsomal incubations by measuring inhibition of the release of ^3H_2O from [1β-^{3}H]-androstenedione. Initial screening bioassays will determine %inhibition, and IC_{50} values and/or Ki values will be determined for active test compounds.

Human JEG-3 (or JAR) choriocarcinoma cell culture assay: Determines the effects of environmental chemicals/toxicants on aromatase activity in choriocarcinoma cell cultures by measuring inhibition of the release of ^3H_2O from [1β-^{3}H]-androstenedione. Initial screening bioassays will determine %inhibition, and IC_{50} values will be determined for active test compounds.

Part of the process of reviewing relevant literature includes contacting experts in aromatase pharmacology and toxicology. We would appreciate your comments to the following interview questions:

- What are the strengths/weaknesses of the study designs under consideration?
- Based on your expertise and experience, what endpoints will be most appropriate?

- What changes would you recommend to the study designs under consideration and why?

- If appropriate, what statistical methods would you employ or suggest that we employ?
- Based on your experience, what chemicals, routes, duration, and doses should we use to validate the assay?

- Can you suggest published references (yours or others) to aid us in our study and endpoint selection? If so, which?

- Do you have any unpublished data relevant to these assays that you would be willing to share? If so, are there any restrictions?

- Would you be willing/like to be involved in the study progress, results, and interpretation?

- Is there anyone else you think we should contact? If so, whom? Can we mention your name when we contact him/her?

Responses received

- Dr. Shiuan Chen (USA) met with Dr. Brueggemeier at the 2001 Hormonal Carcinogenesis Gordon Conference held July 8-13, 2001 and provided his verbal responses to the questions and additional input.

- Dr. Raj Tekmal (USA) met with Dr. Brueggemeier at the 2001 Hormonal Carcinogenesis Gordon Conference held July 8-13, 2001 and provided his verbal responses to the questions and additional input.

- Dr. Edwin Lephart (USA) sent an response to the questions on August 10, 2001. He also provided additional information as a result of his recent visit to the Reproductive Endocrinology Section, EPA, Research Triangle Park, NC.

- Dr. Sari Mäkelä (Finland) met with Dr. Brueggemeier at the 2001 Hormonal Carcinogenesis Gordon Conference held July 8-13, 2001. She also talked with Dr. Risto Santti upon her return to Finland and sent an e-mail response on August 22, 2001.

- Dr. Gerard Habrioux (France) did not respond.

- Dr. A.M. Vinnggaard (Denmark) did not respond.

- Dr. Martin van den Berg (Netherlands) did not respond.

E-mail Responses from Dr. Edwin Lephardt are indicated in bold print

What are the strengths/weaknesses of the study designs under consideration?

Strengths- ease of availability of substrate and assay conditions.
Weakness- androstenedione is a weak androgen in very low circulating levels with relatively low affinity to the aromatase enzyme vs. that of testosterone, etc.

Based on your expertise and experience, what endpoints will be most appropriate?

Parameters- Reproductive and behavioral.; Reproductive- female, puberty onset, cyclicity, gonadotrophin levels and steroid hormone levels; Male, gonadotrophin levels and steroid hormone levels (especially circulating estradiol levels); Female and Male- reproductive behaviors

What changes would you recommend to the study designs under consideration and why?

What are the reasons to use placental tissue/cells in each assay? Aromatase activity is regulated by various mechanisms, especially in brain, bone, etc., that is different than other tissue sites. Also, what are the reasons that only placental tissue sources are being tested? There may be an endocrine effect on aromatase by endocrine disrupters (in other tissue sites with weak aromatase expression) that would not be picked up in the human placental assay.

If appropriate, what statistical methods would you employ or suggest that we employ?

This is a difficult question to address without knowing more about the design of the experiment (i.e. number of different tissues assayed, affinity and saturating concentration of substrate, etc.).

Based on your experience, what chemicals, routes, duration, and doses should we use to validate the assay?

For endocrine disrupters, circulating levels should be determined or estimated, then doses should be established. Routes should simulate oral (in a food matrix or nutritional capsule) or skin cream/patch, etc.

Can you suggest published references (yours or others) to aid us in our study and endpoint selection? If so, which?

Please search: KDR Setchell, H Adlercruetz, S. Barnes

Do you have any unpublished data relevant to these assays that you would be willing to share? If so, are there any restrictions?

We have several endocrine parameters that we have measured, but I do not feel

comfortable sharing this information, since I don't know how this information will be used.

Would you be willing/like to be involved in the study progress, results, and interpretation?

Sure

Is there anyone else you think we should contact? If so, whom? Can we mention your name when we contact him/her?

[No answer provided.]

E-mail Responses from Dr. Sari Mäkelä are indicated in bold print

Strengths and weaknesses of the in vitro study designs

Strengths:

Suitable for high-throughput screening
JEG-3 cells are available commercially
Human placenta can be obtained easily

Weaknesses:

Activity *in vitro* does not necessarily indicate activity *in vivo*. It is difficult to determine how high activity in *in vitro* assay is required to indicate activity *in vivo* (i.e. the level of inhibitory activity *in vitro* required, for the compound to be classified as a putative aromatase inhibitor, and selected for further testing).

In placental microsome assay compounds that are not capable to enter the cell may show activity ("false positive result").

Aromatase activity in JEG-3 cells varies markedly, depending on the growth phase (confluency). This causes variation between individual experiments.

Compounds that modulate aromatization by influencing the expression of aromatase gene/protein will most likely not be recognized in these assays.

In JEG-3 cells other steroid metabolizing enzymes are present, and the labeled substrate may be metabolized by other enzymes, thus not allowing its aromatization (i.e. the amount of substrate available for aromatase may be modulated by other enzymes). The test compounds may influence (inhibit) the activity of these "competing" enzymes, and cause indirect changes in the level of aromatization without any interaction with aromatase enzyme.

Most appropriate endpoints (In general? Not necessarily in this order)

testicular function
ovarian function
onset of puberty
mammary gland development, growth and tumorigenesis
prostate gland development, growth and tumorigenesis
gonadotropin secretion patterns
sexual behavior
bone strength / osteoporosis (?)

Suggested changes to the study designs under consideration:

Use of cells stably transfected with human aromatase, instead of JEG-3 cells. Such cell lines have been generated by several research groups, and (at least in our hands) they are easier to work with, as there is less variation in the aromatase activity.

Validation of the assay:

Use of one or two well characterized aromatase inhibitors (e.g. letrozole), proven to be active *in vivo*, as a positive control, is necessary.

It would be nice to include a model EDC, proven to be aromatase inhibitor *in vitro* and *in vivo*, in the assays as another positive control. However, such compound may not be available, as none of the natural or manmade compounds showing aromatase inhibitory activity *in vitro* (such as chrysin and apigenin), have been shown to act as aromatase inhibitors *in vivo*.

Compounds showing activity in the *in vitro* assay must be further tested *in vivo*. Using intact animals, it is relatively difficult and/or time-consuming to detect aromatase inhibition reliably. In order to observe marked changes in circulating estrogen and/or androgen concentrations, potent inhibitors are needed. Weak inhibitors more likely to act locally in estrogen target tissues are not so easily detected. Furthermore, measurement of small changes in serum estrogen and/or androgen concentrations is not easy (in particular, measurement of estrogen concentrations in male serum is very challenging). *In vivo* testing may be more easily performed using transgenic animals overexpressing aromatase gene (two models published so far), as they display very prominent reproductive phenotypes that are relatively easily detected (e.g. cryptorchidism and gynecomastia). Also, nude mice injected with MCF-7 cells (or other estrogen-responsive cancer cells) overexpressing aromatase gene may be useful here.

Recent references

Li X, Nokkala E, Yan W, Streng T, Saarinen N, Warri A, Huhtaniemi I, Santti R, Mäkelä S, Poutanen M: Altered structure and function of reproductive organs in transgenic male mice overexpressing human aromatase. Endocrinology 142: 2435-2442, 2001.

Saarinen N, Joshi SC, Ahotupa M, Li X, Ämmälä J, Mäkelä S, Santti R: No evidence for the in vivo activity of aromatase-inhibiting flavonoids. J Steroid Biochem Mol Biol, in press.

Unpublished data:

We have recently tested the effects of a synthetic aromatase inhibitor, finrozole, in our aromatase-overexpressing mouse model. It appears that the phenotype in males can be reversed by long-term aromatase inhibitor treatment. Similar experiments with some natural polyphenolics that are active *in vitro*, are ongoing.