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Recent Progress in the Discovery of Next Generation Inhibitors of Aromatase from the Structure–Function Perspective

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Abstract

Human aromatase catalyzes the synthesis of estrogen from androgen with high substrate specificity. For the past 40 years, aromatase has been a target of intense inhibitor discovery research for the prevention and treatment of estrogen-dependent breast cancer. The so-called third generation aromatase inhibitors (AIs) letrozole, anastrozole, and the steroidal exemestane were approved in the U.S. in the late 1990s for estrogen-dependent postmenopausal breast cancer. Efforts to develop better AIs with higher selectivity and lower side effects were handicapped by the lack of an experimental structure of this unique P450. The year 2009 marked the publication of the crystal structure of aromatase purified from human placenta, revealing an androgen-specific active site. The structure has reinvigorated research activities on this fascinating enzyme and served as the catalyst for next generation AI discovery research. Here, we present an account of recent developments in the AI field from the perspective of the enzyme's structure–function relationships.

Graphical Abstract

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Notes

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1. INTRODUCTION

Cytochrome P450 aromatase (AROM) catalyzes with high substrate specificity the conversion of androstenedione (ASD), testosterone (TST), and 16α -hydroxytestosterone (all with the same androgen backbone) to estrone (E1), 17β -estradiol (E2), and $17\beta,16\alpha$ -estril (all with the same estrogen backbone), respectively. It is the only known enzyme in vertebrates capable of catalyzing the aromatization of a six-membered ring. The reaction requires coupling with its redox partner cytochrome P450 reductase (CPR), which uses NADPH as the source of electrons. AROM has been the subject of intense biochemical, biophysical, and clinical investigations for the past 50 years.^{1–3} Inhibition of estrogen biosynthesis by AROM inhibitors (AI) constitutes one of the foremost therapies for postmenopausal estrogen-dependent breast cancer today.^{4,5}

Nevertheless, many aspects of the aromatization reaction remained poorly understood. Until 2009,⁶ the absence of a crystal structure for human AROM had led to a number of homology models for the enzyme based on other experimental P450 structures and site-directed mutagenesis data.^{7–11} Several androgen-binding scenarios at the active site, possible involvements of side chains in the catalytic process, and models for enzyme's mechanism of action were proposed based on homology structures and functional analyses. However, none of these models could adequately explain the unique characteristics of AROM that set this P450 apart from all others. Details of the substrate and inhibitor binding interactions at the active site were crucial for the development of next generation AIs.

Despite concerted efforts in many laboratories, no experimental molecular structure of AROM emerged for a very long time. The major impediments to AROM crystallization were its strong hydrophobic character and susceptibility to rapid denaturation in the absence of the protective lipid bilayer. Using term human placenta as a rich source of AROM and a purification technique that employs a highly specific monoclonal antibody-based affinity chromatography,¹² we were able to purify large quantities of the enzyme in a pristine, active

form that permitted the growth of diffraction-quality single crystals. This was the first and only microsomal P450 purified from a native source to date and the first full length P450 to be crystallized.^{6,13} Employing the same crystallization protocols, a recombinant form of human AROM has more recently been crystallized.¹⁴

Needless to say, the real atomic model has reinvigorated the field of AROM research, from perspectives of re-examining the reaction mechanism¹⁵ and novel AI discovery.¹⁶ One of many fundamental questions that remained unresolved for decades was the following: What are the two proton donors to the 3C=O and 17C=O groups involved in binding of the natural substrate ASD and its conversion to E1? Evidently, a direct structural observation of the binding interactions of ASD in the active site was necessary for the unequivocal answer in this case. Indeed, it would have been impossible to envision that one of the most unlikely candidates the Asp309 carboxylate, protonated at the physiological pH, is the proton donor to 3C=O and the equally imperceptible Met374 backbone amide to 17C=O⁶ (Figure 1). The predicted protonation of Asp309 side chain and its involvement in the aromatization reaction have since been validated by other works.^{17,18} These data, along with the nature of hydrophobic interactions that define the highly androgen-specific, tight active site cleft (Figure 1), are crucial to the discovery of future AIs.

Here we describe the recent advancements made toward design and discovery of novel AIs from the perspective of current understanding of the enzyme's structure–function relationships, giving a brief context to the known AIs developed prior to the AROM structure. We summarize most, if not all, inhibitors that were developed since 2009 utilizing rational means, such as structure-based design, molecular docking, and virtual screening. We present a brief account of our own structure-guided efforts not only for design but also for validation of design. We compare and contrast the reported potencies of the inhibitors and the measurement protocols in an attempt to stress that the numbers are not always comparable. We conclude by describing the limitations in the current knowledge base and future directions.

2. BRIEF HISTORY OF CURRENT AROM INHIBITORS

2.i. First and Second Generation Inhibitors

The chronology of discovery of early generation AIs, inhibitory properties, clinical trial history, side effects, etc. is summarized in Table 1. Shown in Figure 2, aminoglutethimide (AGT) was the first known potent AI.¹⁹ It was reported to reversibly suppress AROM activity in placental microsomes.²⁰ In fact, AGT also was a potent inhibitor of other P450s, such as P450scc²¹ and enzymes involved in the biosynthesis of cortisol, aldosterone, and thyroid hormones.²² Although AGT treatment lacked AROM specificity,²³ doses of 250–1000 mg of AGT effectively inhibited peripheral AROM activity and suppressed plasma E1 levels in postmenopausal women.^{24,25} Formestane (4-hydroxy-4-androstene-3,17-dione, Figure 2), first isolated as a byproduct of 2-hydroxy-steroid 4-en-3-ones synthesis,²⁶ was one of the most promising second generation AIs that underwent multiple clinical trials.^{27–31} It was also the first effective steroidal inhibitor of AROM³² with limited side effects^{27,29,30} but was never approved for clinical use in the U.S. Formestane was reported as a competitive, irreversible, mechanism-based AROM inhibitor due to its time-dependent

suppression of E1 biosynthesis in placental microsomes preincubated with ASD, NADPH, and inhibitor.^{20,33} Fadrozole (FDZ, Figure 2), the prominent second generation AI, was more potent and specific than the other nonsteroidal AI AGT.^{34,35} It was reported to be a competitive and reversible AI,³⁵ which had weak inhibitory effects on P450scc.^{36,37} It also underwent multiple clinical trials in the U.S. and other countries.^{31,35,37–42} Although approved in Japan for treatment in estrogen-dependent postmenopausal breast cancer, FDZ never became a drug in the U.S.

2.ii. Third Generation AIs

Anastrozole (ANZ; Arimidex), letrozole (LTZ; Femara), and exemestane (EXM; Aromasin) are the three AIs that received the FDA approval for use in estrogen-dependent breast cancer in the late 1990s (Table 1, Figure 2). Unlike their predecessors, these so-called third generation inhibitors all have high selectivity for AROM and low cross-reactivity to other targets.^{4,43–45} EXM was reported to elicit time-dependent inactivation of AROM and classified as a suicide inhibitor.^{20,33} However, the crystal structure of the AROM–EXM complex at 3.2 Å resolution did not reveal a covalent bond between AROM and EXM.⁴⁶ EXM binds in competition with the substrate but does not covalently link to protein in the absence of the reductase and electron source.⁴⁶ There is no structural evidence suggesting that any of the AIs are either reversible or irreversible. We have reported the inhibition kinetics of LTZ and utilized statistical analyses to show that its mode of action could be either noncompetitive or mixed.⁴⁷ Furthermore, clinical data do not show superior efficacy of reversible AIs over irreversible AIs or of steroidal AIs over nonsteroidal AIs and vice versa. In the head-to-head trials evaluating the clinical efficacy of EXM and ANZ,⁴⁸ and LTZ and ANZ⁴⁹ in postmenopausal breast cancer, all were found to be equally effective. All three third generation AIs are more potent than the previous inhibitors and performed much better in clinical trials.^{4,5,43–45,50–68} Also noteworthy is the fact that all three AIs have shown better efficacies in major clinical trials in head-to-head comparison with the estrogen-receptor antagonist tamoxifen in the treatment of postmenopausal estrogen-dependent breast cancer.^{4,43–45} They are proven to be more effective than tamoxifen in estrogen receptor (ER) positive tumors that upregulate progesterone receptor (PR) and epidermal growth factor receptors (EGFRs or HER-2/neu) as escape survival mechanisms from prolonged tamoxifen therapy.^{69–72} Regardless of growth factor receptor status, the third-generation AIs could circumvent endocrine resistance that might occur due to estrogen genotoxicity,⁷³ activation of additional estrogen-dependent signaling pathways by tamoxifen metabolites,^{69–72} activation of growth factor receptor signaling pathways by partial agonistic activity of tamoxifen,^{69–72} and greater disease free survival compared to tamoxifen.⁷⁴ The negative effects of third-generation AIs include total body loss of estrogen, joint pain, osteoporosis, and low bone mineral density.^{4,43–45,50–56,58,60–64} Vorozole (Rivizor), another third-generation AI, underwent clinical trials for use in treating estrogen-dependent postmenopausal breast cancer in Europe and Canada.⁷⁵ However, it never gained FDA approval in the U.S.⁷⁶

3. CRYSTAL STRUCTURE OF HUMAN AROM: HOW THE ACTIVE SITE ARCHITECTURE PRESENTED NEW IDEAS

3.i. Active Site

The crystal structure of the highly active human placental AROM in complex with the substrate ASD was originally determined at 2.90 Å resolution.⁶ More recently, the resolution has been extended to 2.75 Å.⁴⁶ The tertiary structure of AROM follows the characteristic cytochrome P450 fold. The active site of AROM is located at the heme-distal cavity, buried deep within the roughly spherical molecule near its geometrical center. ASD binds with its β -face oriented toward the heme group and C19 of the methyl group positioned 4.0 Å from the Fe atom.

The steroid binding cleft is defined by residues Ile305, Ala306, Asp309, and Thr310 from I-helix, Phe221 and Trp224 from F-helix, Ile133 and Phe134 from the B–C loop, Val370, Leu372, and Val373 from the K- β loop, Met374 from β 3, and Leu477 and Ser478 from the β 8– β 9 loop. These hydrophobic side chains and the heme porphyrin rings surround the steroid backbone to form a cavity complementary in shape to ASD (Figures 1 and 3). The side chains of residues Arg115, Ile133, Phe134, Phe221, Trp224, Ala306, Thr310, Val370, Val373, Met374, and Leu477 make direct van der Waals contacts with the bound ASD. Ile133, Phe134, Phe221, Trp224, and Leu477 approach the substrate from the α -face and follow contour and puckering of the steroid backbone, while Arg115, Ala306, and Met374 make contacts at its edge, and Thr310, Val370, and Val373 on the β -face of ASD. The combined surface creates a pocket of roughly 400 Å³ that tightly encloses the bound ASD. This is considerably smaller than the volumes of the active site space in cytochrome P450s 3A4,⁷⁷ 2D6,^{77,78} 11A1,⁷⁹ 11B2,⁷⁹ 17A1,⁸⁰ 21A1,⁸¹ 24A1,⁸² and 51A1.⁸³

The 17-keto oxygen of ASD makes a hydrogen bond with the backbone amide of Met374. It is also at a distance of 3.4 Å from the NH1 of Arg115 side chain. The 3-keto oxygen at the other end is hydrogen bonded to a protonated Asp309 side chain at the crystallization pH of 7.4. The proposed roles of Asp309 side chain protonation in substrate binding, enolization of the 3-keto group, and proton relay network⁶ have been supported by recent findings.^{17,18} The Asp309 side chain is linked by solvent molecules to the salt bridging Arg192-Glu478 side chain pair at the end of the solvent access channel at the lipid interface.⁶ The “gatekeeper” residue Arg192, predicted to play an important catalytic role in proton relay,⁶ has since been shown to be critical for maintaining the enzyme activity.¹⁴ Interestingly, recent clinical case reports establish that mutations of Arg192 result in AROM deficiency and virilization in human.^{84,85}

Although the binding pocket is sealed tight, it has a doorway where water molecules are located (Figure 3), leading to an access channel that opens to the exterior of the protein surface. The Arg192-Glu478 side chain pair, polar residues Asp309, Thr310, and Ser478, and several hydrophobic side chains Phe221, Val313, and His480 line the channel (Figure 3). This channel hosts the proton relay network and is also the major transport route to and from the active site for water, oxygen, and steroid molecules. It was shown that flexibility and fluctuations in the channel-bordering regions permit the channel to “breathe” and swell,

perhaps allowing the passage of steroids.⁸⁶ This entire region adjacent to atoms C4 and C6 of the substrate leading to the access channel presents an intriguing mix of functional groups for AROM-specific inhibitor design ideas.

3.ii. New Perspective on Reaction Mechanism: A Tool for Inhibitor Design

The unique active site architecture, the novelty of steroid-binding interactions, and the proposed 3-keto enolization as well proton relay mechanisms⁶ are all unprecedented in the previous homology models.^{7–11} The structure, thus, has not only validated some of the previously held ideas but also provided a platform for further mechanistic studies on the aromatization reaction.^{18,87–90} In the experimentally observed substrate-bound state, the hydrogen atoms of C19 methyl group are at the viable H-abstraction distances⁹¹ from the modeled oxygen of the reactive oxyferryl moiety (Fe(III) modeled as Fe(IV) =O, compound **I**), shown in Figure 4a. The residues that are directly involved in catalysis are Ala306, Asp309, and Thr310. In addition, Arg192, Glu483, Ser478, and His480 have critical roles in proton relay and/or aromatization. The location of Thr310 with respect to the heme iron is similar to other P450s and performs a similar role in the first two steps of hydroxylation yielding C19-aldehyde derivative of ASD and retaining the *pro-S* hydrogen.⁸⁷ The structure revealed a specific interaction involving Thr310 suggesting its crucial role in all three steps of catalysis. The C=O oxygen of 306Ala and O γ H of Thr310 are at 3.7 and 3.8 Å, respectively, from H2 β (Figure 4b) suggesting 306AlaC=O alone or in conjunction with a deprotonated Thr310O γ^- acts as a nucleophile for the abstraction of H2 β . This action at C2 in conjunction with a protonated Asp309 side chain acting as an electrophile and interacting strongly with the C3-keto oxygen could promote H2 β abstraction and enolization of the 3-keto. The presence of a water molecule could facilitate the deprotonation of the Thr310 side chain by weakening the Ala306CO $\cdots$ HO γ Thr310 hydrogen bond. This would free carbonyl for the nucleophilic attack for H2 β abstraction by -H₂C2-C3-keto to -HC2=C3-enol tautomerization (Figure 4c). H1 β is too far from this carbonyl (6.2 Å) to be abstracted by the same mechanism. It points at the heme Fe (4.2 Å) and is more likely abstracted during the peroxoferric nucleophilic attack on 19-aldehyde (Figure 4c) as previously postulated.⁸⁷ The deprotonated Asp309 side chain is quickly reprotonated by the proton relay network. The direct involvement of Asp309 in protonation and aromatization appears to be unique to AROM.

Many groups have attempted to determine the critical intermediate by theoretical calculations, electron paramagnetic resonance,⁹² resonance Raman spectroscopy,^{93,94} kinetic solvent isotope effect,⁹⁵ and molecular oxygen labeling experiments.^{36,96} These results have shown the possibility that either the oxy-ferryl or peroxo-ferric can be involved in the aromatization reaction. These findings have also been previously summarized.⁹⁷ None of the transition states have been directly observed by X-ray diffraction. Atomistic details of the functional groups in and around the active site, as well as understanding of the organometallics of aromatization chemistry, are critical to the design of superior inhibitors that are mechanism-based and/or transition state analogs and, therefore, exclusive to AROM.

4. RECENT ADVANCES IN THE DISCOVERY OF STEROIDAL AROM INHIBITORS

The third generation AIs are remarkably effective against breast cancer. AIs have also been used for the treatments of endometriosis^{98,99} and ovarian^{98,100} and lung¹⁰¹ cancers. These AIs have high affinities for AROM but also show some cross-reactivity to other P450s. CYP1A2, CYP2C9, and CYP3A4 are inhibited by ANZ, and CYP2A6 and CYP2C19 are inhibited by LTZ.^{102–105} CYP2A6 and CYP3A4 metabolize LTZ.^{106–108} Furthermore, EXM is androgenic¹⁰⁹ and also has weak ER α agonistic activity.¹¹⁰ Despite high efficacy, some patients may fail to respond to AIs, which is known as AI resistance.¹⁰⁶ Rational approaches such as structure-guided design of next generation AIs could minimize the nonspecific and adverse effects.⁵

4.i. Structure-Based Design

Crystallographic and computational results have shown that AROM structure has a rigid core; ligands are thus accommodated by modest adjustments of the catalytic cavity.⁴⁶ By exploiting the active site architecture described in section 3.i above, derivatives of androsta-1,4-diene-3,17-dione have been synthesized. These compounds have undergone in vitro characterization in a MCF-7 breast cancer cell-based antiproliferation system and cell-free purified enzyme inhibition assays.⁴⁶ Three C6 β -alkoxy/alkynyloxy derivatives, in particular, have exhibited the most potent AROM inhibition to date.⁴⁶ Table 2 summarizes the inhibitory and antiproliferative potencies of the three most potent compounds **4**, **5**, and **9** (Figure 5, upper panel), in comparison to EXM. Compound **5**, the best in the series, has IC₅₀ and EC₅₀ roughly 4- and 187- fold, respectively, better than EXM (Table 2). The X-ray structures of the complexes of **4** and **5** with AROM reveal that the 2-alkynyloxy side chains fit tightly within the hydrophobic environment of the channel.^{6,46} Both the structural and functional data are consistent in that **5** and **9** possess the right dimensions to traverse the access channel. The C24 methyl end of the **5** alkyne chain packs against the Val313 side chain, Asp309 main chain carbonyl, arene ring of Phe221 and His480 imidazole. The side group of **5** nearly extends to the polar residues Arg192 and Glu483 at the channel entrance at the membrane–protein interface. A C25 methyl and longer derivatives of **5** have progressively reduced inhibitory and antiproliferative potencies.⁴⁶

4.ii. Other Rational Approaches

Several other groups have utilized the AROM structure to design new inhibitors. A summary of these newly designed inhibitors and their properties (all inhibition assays of the novel steroidal AIs are performed in vitro) is provided in Table 3, and the chemical structures are shown in Figure 6 (compound numbers are the same as in Table 3). However, most of the binding modes and/ or mechanisms are hypothetical. The inhibitors synthesized as derivatives of ASD¹¹¹ and TST¹¹¹ (compounds **10–16**) are reported to have submicromolar potencies in placental microsomes. The modifications are at the positions C3, C4, C5, C6, and C17, as well as C7 α of allylandrostanes (**17–20**).¹¹² Other designs include 16-imidazolyl substituted steroidal derivatives (**23, 24**),¹¹³ pyrazole and isoxazole substitutions

at C2 and C3 positions (**25**, **26**),¹¹⁴ and synthesis of 16 β -cyano-17 β -hydroxy-4-phenylthia-4-androsten-3-one as a novel inhibitor (**27**).¹¹⁵

5. RECENT ADVANCES IN THE DISCOVERY OF NONSTEROIDAL AROM INHIBITORS

5.i. Binding Site Ambiguity in the Absence of Structural Data

No structural data are available yet for unequivocal elucidation of the binding modes of nonsteroidal AIs, such as LTZ and ANZ. In the absence of experimentally determined binding modes of the nonsteroidal inhibitors, virtual docking results to the AROM active site widely reported in the literature are tentative at best. Our modeling data indicate that LTZ and ANZ do not easily fit into the AROM active site pocket¹¹⁶ that resembles an androgen backbone (Figure 1). Furthermore, recent inhibition kinetics data on LTZ and other azole compounds are indicative of mixed modes of inhibition, structural changes, and allostery.⁴⁷ Possibility of allosteric sites for nonsteroidal compounds has also been proposed from computational studies.¹¹⁷

5.ii. Recent Reports

Table 3 summarizes the reported nonsteroidal inhibitors. All inhibition/kinetic assays of the novel nonsteroidal AIs were performed in vitro. The chemical structures are shown in Figure 6a. A brief description of each subclass of AI is provided below.

a. Azole Derivatives—Several imidazole, indole, and imidazolylbenzyl derivatives of LTZ were reported to have potencies similar to LTZ.¹¹⁸ Vorozole is another older azole compound that causes a reversible inhibition of AROM with IC₅₀ value of 1.4 nM.¹¹⁸ The major AROM inhibitory activity of vorozole is attributable to the dextro isomer. Replacement of the 1*H*-benzo[*d*][1,2,3]triazole functional group in the vorozole molecule by benzofuran presented a potent racemic triazole derivative with IC₅₀ value of 10 nM.¹¹⁸

b. Quinoline Derivatives—AI treatment of postmenopausal breast cancer could increase the risk of cardiovascular diseases, which could be brought about by high aldosterone levels as a consequence of the estrogen deficiency.¹¹⁹ Dual inhibitors of AROM (CYP19A1) and aldosterone synthase (CYP11B2) could be useful as adjuvant therapy to reduce the risk of cardiovascular disease for patients undergoing AI treatment. The design utilized the common feature of heme iron coordination by pyridinylmethyl moiety and the hydrophobic core of 1,2,5,6-tetrahydropyrrolo[3,2,1-*ij*]quinolin-4-one, important for potent CYP11B2 inhibition.¹¹⁹ The compound 8-[(3-methylphenyl)(pyridin-4-yl)methyl]-1,2,5,6-tetrahydro-4*H*-pyrrolo[3,2,1-*ij*]quinolin-4-one (**28**) exhibited strong dual inhibition.¹¹⁹ The design of other potent dual inhibitors included combining the pyrroloquinoline core of antimitotic phenylpyrroloquinolinone derivatives with elements of potent nonsteroidal AIs' imidazole and triazole rings (**29–32**).¹²⁰ A high-throughput approach identified several compounds with imidazolyl quinolone skeleton (**33**) as potent AIs.¹²¹

c. Flavone Analogs—Imidazole derivatives of flavonoids were shown to have AROM inhibitory properties.¹¹⁸ More recently, various modifications of this basic design have been

made to synthesize potent AIs, including isoflavanone derivatives with functional groups such as methoxy, phenoxy, chloride, pyridyl, etc. (34–36).¹²² In addition, flavans from the roots of *Desmos cochinchinensis* exhibited potent AROM inhibitory activity at nanomolar levels, the most potent of them being 5-methoxy-8-formyl-4,7-dihydroxyflavan (37).¹²³

d. Coumarins—Position 4-substituted coumarin derivatives were found to potently inhibit AROM.¹¹⁸ One of the most potent compounds in this series was 4-benzyl-3-(4'-chlorophenyl)-7-methoxycoumarin, a competitive inhibitor of AROM with a K_i of 84 nM.¹¹⁸ Some of the imidazolyl derivatives of 4,7-substituted coumarins (38, 39) were shown to be potent inhibitors of AROM with IC₅₀ values in the nanomolar range, higher selectivity over CYP17A1, and favorable interactions at the active site.¹²⁴

e. STS/AROM Dual Inhibitors—Dual AROM/STS could prove more effective than the AIs, since both enzymes were reported to be simultaneously present in E2-dependent breast tumors.¹²⁵ Sulfamate moieties containing EMATE and 667-COUMATE are known potent inhibitors of STS.¹¹⁸ The first of these compounds generated by introducing the AI pharmacophore into a known biphenyl STS inhibitor template (i.e., sulfamate derivatives of LTZ and ANZ templates) was shown to have nanomolar level half-maximum effect.^{126–129} Sulfamate derivatives of other AI templates, such as vorozole, their halide substitutions, and enantiomorphic compounds were synthesized (40–45).¹³⁰ However, the reported inhibition and antiproliferation potencies of these compounds measured in JEG3 cells with low AROM expression levels are not comparable to the others in the absence of standardized controls.

f. Tamoxifen Metabolites—Metabolites of tamoxifen, a well-known selective estrogen receptor modulator (SERM), were shown to inhibit AROM.^{131,132} Such compounds (46, 47) could serve dual purposes as a SERM agent and an AI. Among the metabolites tested, norendoxifen was found to be the most potent and more AROM selective over other liver CYPs and claimed to have favorable interactions at the active site.¹³¹ Various isomers of norendoxifen were also prepared and tested (48–51).¹³²

g. Others (Sulfonamide, Indole–Imidazole, Aryl Halide, Casimiroin, Xanthone Derivatives)—A series of 1,4-disubstituted-1,2,3-triazoles containing sulfonamide moiety (52) were synthesized and shown to have anti-AROM inhibitory properties.¹³³ A meta analog of triazole-benzene-sulfonamide bearing 6,7-dimethoxy substituents on the isoquinoline ring was most potent and fit in the AROM active site.¹³³ Aryl halide derivatives of a centrally flexible, five-component 1,2,3-triazole containing moiety (55) were prepared and evaluated as potential AIs.¹³⁴ Among novel indole–imidazole derivatives, 2-(imidazol-1-ylmethyl)-1-[4-(trifluoromethyl)phenyl]indole (56) was shown to be a highly potent AI.¹³⁵ Casimiroin derivatives (57–59) were shown to inhibit both AROM and quinone reductase 2. *N*-Methyl casimiroin analogue (57) exhibited potency in the micromolar range.¹³⁶ Some imidazolyl derivatives of xanthone scaffold (60, 61) were shown to be highly potent AIs.¹³⁷ Additionally, high-throughput in silico docking approach was used to identify existing compounds as potent AIs.^{138,139} These include compounds with sulfonamide groups (53, 54), and morpholino ethanone derivatives (62) and imidazole groups (63).¹³⁸

Several other compounds identified by in silico docking were reported to have antiproliferative properties in cancer cells.^{140,141} Among them, compounds 3-oxo-16,17-secoandros-ta-1,4-diene-16,17a-dinitrile (**64**)¹⁴⁰ and 4-[2-(3-chlorophenyl)-1-(1*H*-1,2,4-triazol-1-yl)ethenyl]benzonitrile (**65**)¹⁴¹ (Figure 6b) containing AI scaffolds exhibited antiproliferative potency in the micromolar range against MCF7 cells. However, these compounds were not shown to inhibit AROM and hence cannot be classified as new AIs.

6. NEW INSIGHTS FROM MOLECULAR DOCKING OF KNOWN AIS

Several groups utilized the 3D structure of AROM to predict the binding modes and/or deduce quantitative structure–activity relationships of known inhibitors/ligands by computational approaches. However, no inhibitory and/or antiproliferative properties for these compounds were experimentally measured. Therefore, these compounds were excluded from Table 3 and Figure 6. The compounds attempted include flavonoids,¹⁴² isoflavanone derivatives,¹⁴³ imidazolylmethyl-substituted flavone,¹⁴⁴ flavanone and isoflavones,¹⁴³ benzofuran scaffold,¹⁴⁵ steroidal backbones,¹⁴⁶ ASD derivatives,¹⁴⁷ non-steroidal AIs based on an AROM structure-guided pharmacophore model,¹⁴⁸ and tetracyclic triterpenoids,¹⁴⁹ to name a few. There were also efforts to develop better strategy or algorithm for in silico screening and discovering novel AIs.^{16,144,150}

7. EFFECTS OF MEMBRANE INTEGRATION AND DYNAMICS ON RATIONAL DESIGN

7.i. Membrane Integration

The 3D topology allows the enzyme to integrate into the endoplasmic reticulum and/or golgi membrane such that the substrates could utilize the lipid bilayer to gain access to the active site.⁶ Lipophilicity of the AI scaffold is critical to integration and translocation through the lipid bilayer, as the inhibitors have to translocate through the endoplasmic reticulum membrane to gain access to the substrate-binding site from the access channel that opens toward the lumen of the endoplasmic reticulum. Membrane integration topology, the architecture of the access channel, and its flexibility are, therefore, useful information for the design of new AIs. The amino-terminal helix A_{NT} of AROM spans the membrane and positions a putative glycosylation site at Asn12 in the luminal space,^{6,47,86} as shown in Figure 7. The hydrophobic helix A' (residues 57–68) and parts of helix A (residues 69–80) are embedded into the membrane, thereby positioning several arginine (Arg64, Arg79, and Arg86) and tryptophan (Trp67 and Trp88, as well as Trp239 from the F–G loop) residues at the lipid–protein interface, similar to other lipid integrating proteins.¹⁵¹ In this model, the entrance to the active site access channel and the amphipathic F–G loop including the G' helix sits on the membrane surface (Figure 7), while the hydrophobic loop of residues 462–471 between $\beta 7$ and $\beta 8$ touches the interior of the membrane.⁴⁷ The side group of compound **5** nearly extends to the “gatekeeper” residues Arg192 and Glu483 at the channel entrance at the membrane–protein interface. The juxtaposition of these catalytically important residues^{6,14} at the end of a hydrophobic channel and lipid interface suggests that membrane integration has an important role not only in steroid passage and proton relay critical to aromatization⁶ but in substrate and inhibitor selectivity as well. The terminal C24 methyl of

the 2-pentynyloxy derivative **5** has a favorable surrounding of large hydrophobic groups Phe221 and His480.⁴⁶ However, a hydroxyl group at this position in **9** promotes polar interactions with the polar moieties at the end of the channel. Membrane integration of AROM determines the shape and size of the access channel and should be considered in inhibitor design.

7.ii. Dynamics

Normal mode analysis (NMA) of the X-ray structure revealed the intrinsic fluctuations of AROM, the internal modes in membrane-free and membrane-integrated monomers.⁸⁶ The results confirmed the rigid-core structure of AROM centered at the active site is maintained in spite of the changes in steroid binding interactions. The NMA results showed that the N-terminal helix is the most mobile and flexible structural element identified, in agreement with the X-ray observation. The F–G loop is the next most flexible region of the AROM structure that is not significantly influenced by membrane integration. The F–G loop flexibility is one of the common features of P450s.¹⁵² Evidence of the flexible loop undergoing an open/close motion, perhaps to allow steroids to enter into or leave from the active site through the access channel,⁶ was observed.⁸⁶ Furthermore, the NMA of a monomer revealed that the access channel could serve as a hinge for intramolecular bending and an interface for twisting motions. These motions, together with the intrinsic flexibility of the access channel, are likely to contribute to channel “breathing”, opening and closing of the channel mouth and the cavity, perceived necessary for entry and exit of steroids to and from the active site.^{6,86} The hinge bending and twisting motions at the access channel are also present in the lipid-embedded AROM but at a higher frequency. Results from other dynamical simulations also have demonstrated similar flexibility of the AROM structure, especially in the access channel region, even on membrane insertion.^{153,154} Despite the observed rigidity of the active site core, the likelihood of some ligand-induced flexibility cannot be ruled out. In addition, inhibition kinetics data⁴⁷ as well as computational analysis¹¹⁷ suggest possible secondary binding sites for nonsteroidal ligands. Membrane integration, motion, and flexibility of AROM thus pose additional challenges to designing of AIs by rational means.

8. MISSING INFORMATION AND NEW CONCEPTS IN FUTURE DESIGN

8.i. Structural Data on Nonsteroidal Inhibitor–AROM Complexes

As discussed earlier, no experimental structure data on a nonsteroidal inhibitor complex of AROM are available yet. This is a major handicap to the development of novel nonsteroidal AIs with superior clinical properties. Computational methodologies such as docking of LTZ and ANZ into the AROM active site cavity, molecular dynamics simulations, and energy minimization are inadequate for an unequivocal elucidation of the true binding mode. Our data suggest that a major conformational rearrangement of the androgen-binding cleft is necessary to accommodate these triazole ligands at the heme distal site.¹¹⁶ Recent experimental data suggest¹⁷ that the binding mode of ANZ is different from EXM in that the triazole does not use Asp309, the critically important active site residue, which is protonated at physiological pH and makes a hydrogen bond to the 3-keto oxygen of ASD and EXM in their binding modes.^{6,46} Although the conventional wisdom suggests that the triazole ring

nitrogen would coordinate with heme iron as the sixth ligand from the distal site, additional experimental structural data are necessary to settle this question once and for all.

8.ii. AROM Phosphorylation

Phosphorylation of the proximal cavity residue Tyr361 shown in Figure 8 has recently been reported to increase AROM activity in breast cancer cells.¹⁵⁵ Short exposure of estrogen-dependent MCF-7 and ZR75 breast cancer epithelial cells to E2 induces an increase of AROM enzymatic activity. E2-induced enhancement of AROM activity does not correlate with increase in AROM mRNA and protein content. Site-directed mutagenesis experiments reveal that phosphorylation of Tyr361 is crucial in the up-regulation of enzymatic activity. E2 treatment enhances Tyr361 phosphorylation and activity of AROM by activating c-Src kinase or blocking the tyrosine phosphatase PTP1B. In the absence of E2, PTP1B reduces AROM activity by dephosphorylation of Tyr361.¹⁵⁶ Rapid E2 synthesis by a phosphorylated AROM and nongenomic autocrine E2 loop signaling via the plasma membrane-associated ER has long been proposed to be a mechanism by which estrogen performs neuroprotective, neurogenerative, and neurotransmitter roles in the brain and CNS.¹⁵⁷ This positive feedback loop involving phosphorylation of AROM, elevated enzyme activity, and rapid E2 synthesis was first reported in the brains of songbirds and other mammals.^{158–160}

The heme proximal cavity plays key roles in enzyme function during the transfer of electrons to heme.¹⁶¹ We have shown that Tyr361 lies in the path of transfer of electrons from CPR to heme (Figure 8) and Y361F mutation drastically reduces enzyme activity.¹⁴ X-ray data show unexplained residual electron density inside the proximal cavity,¹³ which could serve as a binding scaffold for small molecules. If AROM phosphorylation plays any role in AI resistance, the future AI design criteria would have to take the phosphorylation site(s) into consideration. The mechanism of modulation of the enzyme activity by phosphorylation–dephosphorylation could influence the inhibitory properties of AIs depending on their binding sites. Furthermore, targeting the proximal cavity at the AROM–CPR coupling interface is a concept that could lead to the discovery of a novel class of inhibitors of estrogen biosynthesis. Additionally, the putative secondary sites^{47,117} could also be targeted for inhibition of enzyme activity.

9. CONCLUDING REMARKS

Recent progress on structure–function relationships of AROM has revitalized the next generation AI discovery research. Since 2009 when the AROM structure came to light, roughly several hundred new compounds designed from structure-based modifications to existing inhibitors and belonging to 30 classes have been reported in 46 major publications. Overall, during this period roughly 1000 pieces of documents on design and discovery of novel AIs have appeared in the public domain. The evidence is clear that through rational design, three of the C6-substituted 2-alkynyloxy compounds are more potent than EXM *in vitro*.⁴⁶ However, there are no *in vivo* data yet to suggest superiority to EXM. Structural data show that these novel AROM inhibitors bind in competition with the substrate at the small, tight, androgen-specific active site of AROM and also fill the access channel as they were designed to.^{6,13,46} They could, therefore, be more specific to AROM over other receptors.

While the structure has resolved many outstanding issues, it has also raised new questions. The mechanism of conversion of androgens to estrogens by AROM is very complex and much remains to be achieved in this regard. Additionally, more comprehensive understanding of the aromatization process requires analysis of the structure of electron-transfer complex between AROM and CPR. This has proven to be difficult for any P450. Although steroidal inhibitor complexes of AROM have been crystallized, no experimental structural data on LTZ and ANZ complexes are available yet. This creates a void in our understanding of the molecular basis of inhibition and a hindrance to the discovery of rationally designed nonsteroidal compounds with superior efficacy. Paradoxically, crystallization of these complexes with AROM has been unsuccessful under the conditions that yielded the steroidal complex crystals. Conformational reorganization of the active site cavity induced by the nonsteroidal agents and/or mixed modes of binding resulting structural changes are distinct possibilities. The missing structural information is crucial for designing superior nonsteroidal AIs. Modulation of the enzyme activity by phosphorylation is another emerging area that could reshape the future of AI discovery research.

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ABBREVIATIONS USED

³H₂O	tritiated water
AI	AROM inhibitor
ANZ	anastrozole
AROM	cytochrome P450 aromatase
ASD	androstenedione
CPR	cytochrome P450 reductase
E1	estrone
E2	17 β -estradiol
EGFR	epithelial growth factor receptor
ER	estrogen receptor
EXM	exemestane
FCA	fluorescent CYP19 inhibition assay
HER2/neu	human EGFR2
LTZ	letrozole
MCF-7a	MCF-7 cell line stably expressing AROM

MD	molecular docking
mER	plasma membrane associated estrogen receptor
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NCI	National Cancer Institute
NNC	National Nanjing Center for Drug Screening
NMA	normal-mode analysis
PR	progesterone receptor
PMTW	pacental microsomes, tritiated water assay
TST	testosterone

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Figure 1.

The AROM active site is shaped like an androgen backbone. A van der Waals interaction surface cast by the protein and heme atoms at the active site is shown. The semitransparent surface closely resembles the shape, size, and puckering of the steroid backbone. The surfaces are color-coded green for hydrophobic and magenta for polar. The protein atoms defining the van der Waals surface are also shown: C gray, N blue, and O red. The only opening to the pocket is the one to the active site access channel indicated by an arrow. Water oxygen atoms in the channel and bound to the protonated Asp309 carboxylate are shown as red spheres. Adapted from ref 6.

Figure 2.
Previous and current generation AIs. The chemical structures of known AIs are shown.

Figure 3.

Androgen-binding environment: schematic diagram of the X-ray structure depicting the residues lining the ASD binding pocket, and ASD making hydrophobic and hydrogen-bonding contacts (hydrophobic, green; acidic, red; basic, blue; polar, purple; S-containing, yellow). Exposure at the C4 and C6 positions of the steroid to the access channel opening is indicated. Also shown schematically is a water molecule trapped between Asp309 and Arg192 side chains, postulated to have a role in the proton relay network and enolization of 3-keto. Arg192 and its salt bridge partner Glu483 are the “gatekeepers” of the access channel. Adapted from ref 46.

Figure 4.

Reaction center and perspective on catalysis. (a) Modeling of Fe (III) as an oxyferryl Fe(IV) =O (compound I) moiety. The C19-methyl hydrogen atoms are shown at the calculated ideal positions. Important side chains, heme, and water molecules are depicted in element colors: C, gray; N, blue; O, red; S, yellow; Fe, firebrick; H, orange. The C atoms of ASD are colored in cornflower blue. (b) Close-up view of the 306AlaCO...HOThr310 pair that may have a role in aromatization of the A-ring of ASD. Calculated hydrogen atom positions of C2 of the bound ASD are shown. The distances shown are in Å. (c) Possible mechanism for H2β abstraction and 2,3-enolization. The direction of proton flow from the proton relay network through Asp309 carboxylate is indicated. Adapted from ref 6.

Figure 5.

Structural basis for the design of potent inhibitors. The chemical structures of the three most potent steroidal inhibitors **4**, **5**, and **9** of the 6 β -2-alkynyloxy series are shown in the upper panel. Lower panel: Schematic diagram depicting the X-ray structure of the tight hydrophobic binding pocket for the designed steroidal inhibitor **5**, the proton donors at the 3- and 17-keto positions, and the 6 β -2-alkynyloxy side group that nearly fills the access channel. Compound **4** also binds the same way. The residues lining the binding pocket making hydrophobic and hydrogen-bonding contacts are shown. Adapted from ref 46.

Figure 6.

(a) Recently developed steroidal and nonsteroidal AIs. The compounds are numbered and grouped in the same way as referenced in the text and in Table 3. (b) Compounds that exhibit antiproliferative effects on breast cancer cells. Compounds **65** and **66** exhibit antiproliferative effects on breast cancer cells. No direct aromatase inhibition was measured; therefore, they are not classified as AIs.

Figure 7.

Influence of lipid bilayer. In this membrane integration model, the opening to the active-site access channel rests on the lipid bilayer surface, allowing the steroids to enter the AROM active site directly from within the bilayer. The backbone ribbon is drawn in rainbow color: the N terminus is blue, and the C terminus is red. The model suggests lipid integration/association of the N-terminal helices A_{NT} , A' , and A , the F–G loop that includes helix G' , and loop 7–8 between $\beta 7$ and $\beta 8$ near the C terminus. Asn12 is shown as facing the lumen of the endoplasmic reticulum. Atom color scheme is the following: oxygen in red; nitrogen blue; phosphorus orange; ASD in cornflower blue; heme and protein side chains in gray; lipid in light blue.

Figure 8. Strategic location of Y361 in the proximal cavity. The redox partner cytochrome P450 reductase is presumed to bind at this interface, and electrons are transferred from FMN to heme as illustrated by the dashed line. Y361 side chain sits in the path of electron transfer. Atom color code is the same as in other figures.

Table 1

Chronology of Discovery of Earlier Generation AIs

occurrence	first generation AI	second generation AIs		third generation AIs		
	aminogluthethimide	formestane	fadrozole	exemestane	letrozole	anastrozole
Discovery/earliest reported use	1961 (treatment of epilepsy) ¹⁶² 1966 (antithyroidal and antiadrenocortical activity) ^{21,22}	1973 (synthesis) ²⁶ 1977 (preclinical testing) ^{32,163,164}	1987 (preclinical testing) ¹⁶⁵	1988 (preclinical testing) ^{166,167}	1990 (preclinical testing) ¹⁶⁸	1994 (summary of preclinical testing and clinical studies) ^{60,61}
First reported use of AIs	1974 (in the U.S.) ¹⁹	1984 (clinical trials in the UK first) ^{27-31,169}	1989 (clinical trials in the U.S. and later in Europe, South Africa, Japan) ^{35,37-42}	1995 (clinical trials in Italy first and later in Europe and U.S.) ⁵⁰⁻⁵⁹	1993 (clinical trials in the U.S. and UK) ^{67,68,74,170,171}	1994 (clinical trials in the U.S.) ⁶⁰⁻⁶⁴
IC ₅₀ against pure enzyme	Not determined (5–7 μ M against human placental microsomes ¹⁷²)	49 nM ⁴⁶	Not determined (5 nM against human placental microsomes ¹⁷³)	50 nM ⁴⁶	10 nM ⁴⁶	Not determined (23 nM against human placental microsomes ¹⁷³)
IC ₅₀ /EC ₅₀ in breast cancer cell line	10 μ M against MCF-7 cells transfected with AROM, ¹⁷⁴ 20 μ M in particulate fractions of breast cancer; ⁴³ 10 μ M in culture of mammary adipose tissue fibroblasts ⁴³	30 nM (particulate fractions of human breast cancer tissues) ¹⁷³	50 pM in MCF-7 cells ¹⁷³	5.6 nM in MCF-7 cells over-expressing AROM ⁴⁶ (antiproliferation assay)	4 pM in MCF-7 cells overexpressing AROM ⁴⁶ (antiproliferation assay)	3.6 nM in MCF-7 cells ¹⁷³
Dose in treatment	250 mg/4 $\times$ daily orally ^{24,45,175}	250 mg daily orally or 500 mg via intramuscular injection every 2 weeks ^{29,45,175}	1.8–2 mg daily orally ^{41,45,175}	25 mg daily orally ^{45,175}	2.5 mg daily orally ^{45,175}	1 mg daily orally ^{45,175}
Side effects/issues ^{45,175}	Low selectivity for AROM Inhibition of cortisol, aldosterone, thyroid hormone synthesis. Induction of hepatic enzymes. Minor side effects: nystagmus, ataxia, lethargy, skin rash ^{2,4,176-178}	Poor oral bioavailability ²⁹ Local side effects due to intramuscular injection ⁴⁰ Severe neutropenia (transient and reversible upon discontinuation of treatment) ²⁷	Suppression of aldosterone release ¹⁷⁹ Mild hot flashes, nausea and vomiting, fatigue, and mild loss of appetite ⁸⁰	Hot flashes, fatigue, arthralgia (joint pain), headache, insomnia (difficulty sleeping), increased sweating, hypertension (high blood pressure), dizziness, cardiac ischemic events, reduced bone mineral density/osteoporosis ^{50-59,181}	Low-grade hot flashes, arthritis, arthralgia, and myalgia, and new diagnoses of osteoporosis ^{67,68,74,170,171}	Hepatic toxicity, blurred vision, angina pain, irregular heartbeat, edema in legs, headaches, dizziness, nervousness, shortness of breath ⁶⁰⁻⁶⁴
Year of FDA approval as an AI	Not FDA-approved in the U.S.	Not FDA approved in the U.S.	Not FDA approved in the U.S.	1999 ⁵⁷⁻⁵⁹	1998 ^{65,66}	1995 ⁶⁴

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occurrence	first generation AI		second generation AIs		third generation AIs	
	aminoglutethimide	formestane	fadrozole	exemestane	letrozole	anastrozole
Current FDA-approval status	FDA-approved for Cushing syndrome	Initially approved as an AI in Europe Withdrawn due to poor oral bioavailability and discovery of third-generation orally active AIs	Approved as an AI in Japan ¹⁸²	Approved as an AI in the U.S.	Approved as an AI in the U.S.	Approved as an AI in the U.S.

Table 2
Summary of IC₅₀ and EC₅₀ of the Most Potent C6 β -Alkoxy/2-Alkynyloxy Series of Steroidal Aromatase Inhibitors and Controls

compd	IC ₅₀ (nM)	95% confidence interval	potency relative to EXM	EC ₅₀ (nM)	95% confidence interval	potency relative to EXM
exemestane (EXM)	50.1	40.9–61.4		5.6	2.7–6.5	
4 (HDDG029)	112.3	78.2–161.3	0.5	1.7	1.2–2.2	3.3
5 (HDDG046)	11.8	9.3–14.9	4.2	0.03	0.02–0.06	187.0
9 (HDDG065)	20.0	18.1–22.0	2.5	0.3	0.2–0.4	18.7

Summary of New AROM Inhibitors Since 2009

Table 3

compd	type	name	assayed against, assay type	IC ₅₀	exptl binding mode verification
1–9	C6-substituted 2-alkynyloxy compounds ⁴⁶	C6-substituted androsta-1,4-diene-3,17-dione	Purified AROM and MCF-7 ^a , ^a ³ H ₂ O, antiproliferation	20–18100 nM	Yes: X-ray
10	A- and D-ring ASD derivative ¹¹¹	3 β -Hydroxyandrost-4-en-17-one	PMTW	0.18 μ M	No
11	A- and D-ring ASD derivative ¹¹¹	Androst-4-en-17-one	PMTW	0.14 μ M	No
12	A- and D-ring ASD derivative ¹¹¹	4 α ,5 α -Epoxyandrostan-17-one	PMTW	0.97 μ M	No
13	A- and D-ring ASD derivative ¹¹¹	5 α -Androst-3-en-17-one	PMTW	0.23 μ M	No
14	A- and D-ring ASD derivative ¹¹¹	3 α ,4 α -Epoxy-5 α -androstan-17-one	PMTW	0.15 μ M	No
15	A- and D-ring ASD derivative ¹¹¹	5 α -Androst-2-en-17-one	PMTW	1.7 μ M	No
16	A- and D-ring ASD derivative ¹¹¹	2 α ,3 α -Epoxy-5 α -androstan-17-one	PMTW	1.2 μ M	No
17	7 α -allylandrostanes ¹¹²	7 α -Allylandrost-4-ene-3,17-dione	Placental microsomes, ³ H ₂ O (PMTW) ^{b,c}	0.59 μ M	No
18	7 α -allylandrostane ¹¹²	7 α -Allylandrost-4-en-17-one	PMTW	0.75 μ M	No
19	7 α -allylandrostane ¹¹²	7 α -Allyl-3-oxoandrosta-1,4-dien-17 β -ol	PMTW	0.45 μ M	No
20	7 α -allylandrostane ¹¹²	7 α -Allylandrosta-1,4-diene-3,17-dione	PMTW	0.47 μ M	No
21	NCI ^e compound (B-nor-androstenedione scaffold) ¹³⁹	3 α ,5 β ,10-Trimethyl-1,2,4,5,5 α ,6,7,10,10 α ,10 β -decalhydrocyclopental[<i>a</i>]fluorene-3,8-dione	PMTW	0.27 μ M	No
23	Imidazolyl substituted steroidal derivative ¹¹³	16 β -(Imidazol-1-yl)-4-androstene-3,17-dione	PMTW	0.18 μ M	No
24	Imidazolyl substituted steroidal derivative ¹¹³	16 β -(Imidazol-1-yl)androsta-1,4-diene-3,17-dione	PMTW	0.168 μ M	No
25	Androstane ¹¹⁴	17 β -Hydroxy-4-oxo-5 α -androstanol[2,3- <i>d</i>]pyrazole	PMTW	512.0 nM	No: MD ^d
26	Androstane ¹¹⁴	2-Cyano-3,17 β -dihydroxy-5 α -androst-2-en-4-one	PMTW	1019.8 nM	No: MD
27	Carbonitrile ¹¹⁵	16 β -Cyano-17 β -hydroxy-4-phenylthia-4-androsten-3-one	PMTW	169.3 nM	No: MD
Nonsteroidal AROM Inhibitors					
22	NCI ^e compound NSC613604 ¹³⁹	5-(4-Chloroanilino)-2-methyl-9-nitro-5 <i>H</i> -chromeno[4,3- <i>b</i>]pyridine-3-carbonitrile	PMTW	0.67 μ M	No
28	Quinoline derivative-dual CYP11B2/CYP19 inhibitor ¹¹⁹	8-[(3-Methylphenyl)(pyridin-4-yl)methyl]-1,2,5,6-tetrahydro-4 <i>H</i> -pyrrolo[3,2,1- <i>ij</i>]quinolin-4-one	PMTW	32 nM	No: MD
29	Azolylmethylpyrroloquinoline ¹²⁰	4-((1 <i>H</i> -Imidazol-1-yl)methyl)-7-ethyl-2-phenyl-7 <i>H</i> -pyrrolo[2,3- <i>h</i>]quinoline	Supersomes ^g /H295R cells, FCA ^h /antiproliferation	5.3 nM	No: MD
30	Azolylmethylpyrroloquinoline ¹²⁰	4-((1 <i>H</i> ,2,4-Triazol-1-yl)methyl)-2-(4-methoxyphenyl)-7 <i>H</i> -pyrrolo [2,3- <i>h</i>]quinoline	Supersomes/H295R cells, FCA/antiproliferation	>10000 nM	No: MD

compd	type	name	IC ₅₀	assayed against, assay type	exptl binding mode verification
31	AzolyImethylpyrroloquinoline ¹²⁰	9-(((1 <i>H</i> -Imidazol-1-yl)methyl)-3-ethyl-7-phenyl-3 <i>H</i> -pyrrolo- [3,2- <i>f</i>]quinoline	3.1 nM	Supersomes/H295R cells, FCA/antiproliferation	No: MD
32	AzolyImethylpyrroloquinoline ¹²⁰	9-(((1 <i>H</i> -1,2,4-Triazol-1-yl)methyl)-3-ethyl-7-phenyl-3 <i>H</i> -pyrrolo [3,2- <i>f</i>]quinoline	13.3 nM	Supersomes/H295R cells, FCA/antiproliferation	No: MD
33	NNC ^l compound (imidazolyl quinoline) ¹²¹	2-(4-Fluorophenyl)-4-(imidazol-1-yl)quinoline	0.8 μ M	Supersomes/T47cells, E2 ELISA/antiproliferation	No: MD
34	Isoflavanone derivative ¹²²	3-(4-Phenoxyphenyl)chroman-4-one	2.4 μ M	Supersomes/FCA	No: MD
35	Isoflavanone derivative ¹²²	6-Methoxy-3-phenylchroman-4-one	0.3 μ M	Supersomes/FCA	No: MD
36	Isoflavanone derivative ¹²²	3-(Pyridin-3-yl)chroman-4-one	5.8 μ M	Supersomes/FCA	No: MD
37	Flavans ¹²³	5-Methoxy-8-fornyl-4,7-dihydroxyflavan	40 nM	Supersomes/FCA	No: MD
38	4,7-disubstituted coumarin ¹²⁴	7-(3,4-Difluorophenoxy)-4-(1 <i>H</i> -imidazol-1-ylmethyl)-2 <i>H</i> -chromen-2-one	47 nM	PMTW	No: MD
39	4,7-disubstituted coumarin ¹²⁴	4-(1 <i>H</i> -Imidazol-1-ylmethyl)-7-phenoxy-2 <i>H</i> -chromen-2-one	150 nM	PMTW	No: MD
40	Dual AROM/STS inhibitor ¹²⁶	4-[[[(4-Cyanophenyl)(1 <i>H</i> -imidazol-1-yl)amino]methyl]-3-fluorophenyl sulfamate	0.2 nM	JEG3 cells, ³ H ₂ O ^c	No: MD
41	Dual AROM/STS inhibitor ¹³⁰	2-Bromo-4-(2-(4-cyanophenyl)-2-(1 <i>H</i> -1,2,4-triazol-1-yl)ethyl) phenyl sulfamate	0.9 nM	JEG3 cells, ³ H ₂ O ^c	No
42	Dual AROM/STS inhibitor ¹²⁹	5'-((1 <i>H</i> -1,2,4-Triazol-1-yl)methyl)-2'-cyanobiphenyl-4-yl sulfamate	2 nM	JEG3 cells, ³ H ₂ O ^c	No
43	Dual AROM/STS inhibitor ¹²⁹	5'-((1 <i>H</i> -1,2,4-Triazol-1-yl)methyl)-3-chloro-2'-cyanobiphenyl- 4-yl sulfamate	0.5 nM	JEG3 cells, ³ H ₂ O ^c	No
44	Dual AROM/STS inhibitor ¹²⁸	2-Chloro-4-(((6-cyanobiphenyl-3-yl)(4 <i>H</i> -1,2,4-triazol-4-yl) amino)methyl)phenyl sulfamate	15 pM	JEG3 cells, ³ H ₂ O ^c	No: MD
45	Dual AROM/STS inhibitor ¹²⁸	2-Bromo-4-(((6-cyanobiphenyl-3-yl)(4 <i>H</i> -1,2,4-triazol-4-yl) amino)methyl)phenyl sulfamate	18 pM	JEG3 cells, ³ H ₂ O ^c	No: MD
46	Tamoxifen metabolite ¹³¹	Z-4-Hydroxytamoxifen	530 μ M	Supersomes/T to E2 ⁱ	No: MD
47	Tamoxifen metabolite ¹³¹	Z-Norendoxifen	30 nM	Supersomes/T to E2	No: MD
48	Tamoxifen metabolite ¹³¹	Z-Endoxifen	6 μ M	Supersomes/T to E2	No: MD
49	Tamoxifen metabolite ¹³²	(<i>Z</i>)-Norendoxifen	1029 nM	Supersomes/FCA	No: MD
50	Tamoxifen metabolite ¹³²	(<i>E</i>)-Norendoxifen	77 nM	Supersomes/FCA	No: MD
51	Tamoxifen metabolite ¹³²	(<i>E,Z</i>)-Norendoxifen	102 nM	Supersomes/FCA	No: MD
52	Sulfonamide derivative ¹³³	7-((1-(3-((6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1 <i>H</i> -yl) sulfonyl)phenyl)- 1 <i>H</i> -1,2,3-triazol-4-yl)methoxy)-2 <i>H</i> -chromen-2-one	0.2 μ M	Supersomes/T to E2	No: MD
53	ASINEX compounds (sulfonamide derivative) ¹³⁸	1-(3- <i>tert</i> -Butyl-4-methoxybenzenesulfonyl)-3-(imidazol-1yl)methyl)piperdine	9.4 nM	Supersomes/FCA	No: MD
54	ASINEX compounds (sulfonamide derivative) ¹³⁸	<i>N</i> -(3-(Imidazol-1-yl)propyl)-2,3-dihydro-1,4-benzodioxine-6-sulfonamide	119 nM	Supersomes/FCA	No: MD
55	Aryl halide ¹³⁴	2-[7-Bromo-1-(3-ethoxypropyl)imidazo[4,5- <i>c</i>] pyridin-5-ium-5-yl]-1-phenylethanone	20 nM	Supersomes/FCA	No: MD
56	Indole-imidazole derivative ¹³⁵	2-(Imidazol-1-ylmethyl)-1-[4-(trifluoromethyl)phenyl]indole	4.9 nM	Placental microsomes, ELISA	No

compd	type	name	IC ₅₀	assayed against, assay type	exptl binding mode verification
57	Casimiroin analogue ¹³⁶	N-Methylated casimiroin analogues: 5,6,8-trimethoxy-1,4-dimethylquinolin-2(1 <i>H</i>)-one	0.1 μ M	Supersomes/FCA	No: MD
58	Casimiroin analogue ¹³⁶	Non-N-methylated casimiroin analogues: 6-methyl[1,3]dioxolo [4,5- <i>h</i>]quinolin-8(9 <i>H</i>)-one	>98.5 μ M	Supersomes/FCA	No: MD
59	Casimiroin analogue ¹³⁶	Casimiroin	3.9 μ M	Supersomes/FCA	No: MD
60	Xanthone scaffold ¹³⁷	4-Imidazol-1-ylmethylthioxanthen-9-one	4 nM	PMTW	No
61	Xanthone scaffold ¹³⁷	1-(4-Nitro-2-phenylsulfonylbenzyl)-1 <i>H</i> -imidazole	5.6 nM	PMTW	No
62	ASINEX compounds (morpholinoethanone derivative) ¹³⁸	1-[2-(Imidazol-1-ylmethyl)morpholin-4-yl]-2-(3,4,5-trimethoxyphenyl)ethanone	59.2 nM	Supersomes/FCA	No: MD
63	ASINEX compounds (imidazolylacetamide) ¹³⁸	<i>N</i> -[2-(4-Fluorophenoxy)phenyl]-2-(imidazol-1-yl)acetamide	248 nM	Supersomes/FCA	No: MD

^aMCF-7a: a MCF-7 cell line stably expressing AROM as described.⁵⁹

^bPMTW: placental microsomes, tritiated water assay.

^c³H₂O: tritiated water.

^dMD: molecular docking.

^eNCI: National Cancer Institute.

^fNNC: National Nanjing Center for Drug Screening.

^gSupersomes: commerial mixture of baculovirus AROM microsomes and reductase.

^hFCA: fluorescent CYP19 inhibition assay, which measures the conversion of a fluorescence substrate to its metabolite, a hydroxylation reaction.

ⁱ_T to E2 assay, which measures T to E2 conversion by HPLC.