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Structure–function studies of aromatase and its inhibitors: a progress report☆

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Abstract

The utilization of computer modeling, site-directed mutagenesis, inhibition kinetic analysis and reaction metabolite analysis allows us to better understand the structure–function relationship between aromatase and its inhibitors. Our results have helped in determining how steroidal and nonsteriodal aromatase inhibitors bind to the active site of the enzyme. This information has also aided in the understanding of the reaction mechanism of aromatase. Furthermore, our structure–function studies of aromatase have generated important information for predicting how environmental chemicals interact with the enzyme. During the last 2 years, a new aromatase computer model based on the X-ray structure of rabbit cytochrome P450 2C5 has been generated and used to evaluate the results obtained from new aromatase mutants produced in this laboratory. In addition, we have succeeded in the expression and purification of functionally active aromatase using an *Escherichia coli* expression method. The catalytic properties of this recombinant aromatase are similar to those properties exhibited by the human placental aromatase preparation and the mammalian cell-expressed enzyme. The *E. coli* expressed aromatase will be very useful for further structure–function studies of aromatase. Our laboratory has also evaluated the growth-inhibiting activity of aromatase inhibitors in estrogen receptor-positive breast cancer using three-dimensional cell cultures of aromatase-over expressing MCF-7 and T-47D cell lines (i.e. MCF-7aro and T-47Daro). Our results demonstrate that these three-dimensional cultures are valuable approaches to assess the growth-inhibiting activity of aromatase inhibitors. Finally, we have identified several phytochemicals to be potent inhibitors of aromatase. To demonstrate the impact of the phytochemicals on estrogen formation in vivo, we showed that the intake of anti-aromatase chemicals from red wine was capable of suppressing MCF-7aro-mediated tumor formation in nude mice and aromatase-induced hyperplasia in a transgenic mouse model in which aromatase is over-expressed in the mammary tissue.

Keywords: Aromatase; Structure–function studies of aromatase; Expression of aromatase in *E. coli*

1. Introduction

Aromatase inhibitors have been found to be valuable to treat these estrogen-dependent and aromatase-mediated diseases including breast cancer [1]. The third generation nonsteroidal aromatase inhibitors, letrozole and anastrozole, were recently approved by the US Food and Drug Administration for use as first-line agents against estrogen-responsive cancer. As first-line therapy in postmenopausal women with advanced or metastatic breast cancer, letrozole is more active than tamoxifen (an antiestrogen), achieving higher response rates, longer time to progression, and an early survival advantage, while treatment is well tolerated [2,3]. In the same setting, anastrozole and tamoxifen have had at least equivalent activity and tolerability [4–6].

Previous aromatase inhibitor development has been based primarily on inhibitor structure–activity relationship studies. In order to provide useful information about the active site of aromatase for designing aromatase inhibitors with a high specificity and potency, we and other groups have carried out structure–function and mechanism studies of aromatase and its inhibitors utilizing computer modeling, site-directed mutagenesis, inhibition kinetic analysis and reaction metabolite analysis. The site-directed mutagenesis has been carried out mainly using the mammalian cell-expression method [7–19].

2. Aromatase-expression methods

For structure–function studies of aromatase, this and other laboratories have evaluated a series of aromatase-expression

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Table 1
Kinetic properties of aromatase in mammalian cells and *E. coli*

	K_m (nM)	V_{max} (pmol (mg h^{-1})
MCF-7[a]	52	0.08
T-47D[a]	41	0.01
MCF-7aro[a]	55 ± 10	73 ± 6
T-47Daro[a]	25 ± 3	48 ± 4
CHOaro[b]	72 ± 13	339 ± 34
Recombinant aromatase from *E. coli*[c]	62 ± 24	5800

[a] [25].
[b] [18].
[c] [26].

methods. Expression of functionally active human aromatase in insect cells [20–22] and in yeast [23] has been reported. To date, these expression methods have not yet been demonstrated to be practical approaches for the large-scale expression of aromatase and its mutants for detailed analysis. In a recent publication [24], Conley et al. have reported the expression and purification of porcine aromatase isozymes in insect cells. However, it is difficult to evaluate the yield and quality of the recombinant aromatase isozymes from insect cells because essential data was not included in the paper. The mammalian cell-expression method has been chosen for our structure–function studies of aromatase. We have chosen Chinese hamster ovary (CHO) cells as the host cells to express the wild-type or mutated human aromatase because we have found that CHO cell-expressed aromatase has kinetic properties comparable to those of the enzyme purified from human placenta (Table 1). The enzyme assay can be conveniently performed using an "in-cell" method utilizing the endogenous NADPH-cytochrome P450 reductase as the source of the reductase. In addition, the level of aromatase activity was well maintained in the stable aromatase-expressing CHO cells, allowing us to perform multiple analyses of expressed aromatase and its mutants. Although important information regarding the structure of the aromatase active site and reaction mechanism of aromatase has been generated using this approach, the mammalian cell-expression system cannot produce adequate amounts of the enzyme for a detailed structural analysis.

Table 3
Aromatase P450 contents in the membrane-bound fractions

	P450 content (pmol mg^{-1})	Aromatase activity (pmol (min mg^{-1}))	Turnover number (min^{-1})
Full-length	35.4	30.8	0.9
Del-20	15.9	17.0	1.1
Del-38	234	618	2.6
Host	<0.5	0	–

2.1. Expression of human aromatase in Escherichia coli

Expression of protein in *E. coli* is a cost-effective method. This laboratory, as well as others, have made a number of attempts to express human aromatase in *E. coli*. In our initial experiments, we found that significant amounts of aromatase protein could be generated using the *E. coli* expression method; however, the recombinant protein was found to be catalytically inactive and present mainly in the inclusion bodies [13]. Previous protein sequence analysis from our laboratory revealed that the N-terminal sequence of aromatase P450 carries a membrane anchoring hydrophobic region [27]. It was thought that deleting this region might increase the solubility of the enzyme while retaining biological function. Therefore, an amino N-terminal 38 amino acid sequence deleted form (Del-38) was expressed in *E coli*. While this enzyme form still remains in the inclusion body, it was found to be catalytically active when reconstituted with NADPH-cytochrome P450 reductase [28]. In an extensive analysis, Toma et al. [28] found that the membrane-bound fraction of *E. coli* expressing Del-38 had a significantly higher aromatase activity than those expressing the full-length or Del-20 forms (Table 2). Using a sandwich ELISA method with monoclonal antibody MAD3-212 and rabbit antiserum to human aromatase, the aromatase P450 contents of membrane-bound fractions of *E. coli* expressing different aromatase forms were measured (Table 3). The turnover number for Del-38 (i.e. 2.6 min^{-1}) was found to be very similar to that estimated with human placental microsomes (i.e. 2.5 min^{-1}). These results suggest that Del-38 is a useful form of aromatase for further structure–function studies.

Last year, we decided to revisit the possibility of expressing and purifying functionally active human aromatase in *E. coli*. We have now succeeded in not only the ex-

Table 2
Aromatase activity obtained from 3 l culture of *E. coli*

	E. coli homogenate			Membrane-bound fraction (125,000 × *g* pellet)		
	Protein (mg)	Specific activity (pmol (min mg^{-1}))	Total activity (nmol min^{-1})	Protein (mg)	Specific activity (pmol (min mg^{-1}))	Total activity (nmol min^{-1})
Full-length	626 ± 40[a]	12.1 ± 1.5	7.6 ± 1.2	175 ± 20	36.0 ± 6.4	6.2 ± 0.9
Del-20	1058 ± 23	5.9 ± 0.4	6.2 ± 0.3	305 ± 22	16.9 ± 0.8	5.2 ± 0.1
Del-38	1578 ± 72	174.2 ± 32.7	275.0 ± 52.0	642 ± 22	397.4 ± 82.5	254.7 ± 49.2
Host	841	<0.05	<0.04	198	<0.2	<0.04

[a] Mean ± S.D.; $n = 15$ for the full-length protein, 4 for Del-20, and 12 for Del-38.

pression, but also the purification of functionally active aromatase from *E. coli*. In a recent report [26], we described the construction and expression of a recombinant derivative of human aromatase in *E. coli* using the pET vector system, and the purification of the enzyme by means of nickel–agarose affinity chromatography. The addition of two separate hexameric histidine tags at both the amino and the carboxyl-termini (i.e. NC-6xHis-tagged Del-38) increased the binding affinity of the recombinant enzyme to the nickel–agarose. The expressed aromatase (i.e. NC-6xHis-tagged Del-38 aromatase) was eluted from the nickel–agarose with 80 mM EDTA. The total aromatase activity of the 80 mM EDTA-eluted fractions was significantly higher than the detergent-solublized protein extract, indicating a renaturation process during the nickel–agarose affinity chromatography. Purified aromatase exhibited a single band when analyzed by SDS polyacrylamide gel electrophoresis (PAGE), and an activity up to 5.8 nmol (mg min^{-1}) was obtained using the tritiated water release assay. The K_m value for androstenedione was determined at 62 ± 24 nM by enzyme kinetic analysis. The recombinant aromatase preparation was also characterized using reduced CO-difference spectral analysis, reaction product extraction assay, and inhibition studies using two aromatase inhibitors (letrozole and anastrozole). It was determined that 340 pmol of functionally active recombinant aromatase (estimated by reduced CO-difference spectral analysis) could convert 830 pmol of androstenedione to estrone per minute. Therefore, the turnover number of the recombinant aromatase purified from *E. coli* is calculated to be 2.4 min^{-1}. As indicated above, the turnover number for human placental aromatase was estimated to be 2.5 min^{-1}. Our reduced CO-difference spectral analyses of the recombinant aromatase preparations also indicate the presence of a small amount of inactive form of the enzyme. We are evaluating experimental conditions to generate fully active aromatase preparations.

We feel that our *E. coli* expression study is important for the following reasons: for the first time, a functionally active aromatase can be generated and purified from *E. coli*. Deletion of the first 38 amino acids and introduction of two 6-His tags at the N- and C-termini of the aromatase facilitate the solublization and purification of the recombinant aromatase. This *E. coli* expression method provides an easy way to produce significant quantities of aromatase for research because it is easier and cheaper to carry out a large-scale *E. coli* culture than either insect or mammalian cells. In addition, our study reveals that a renaturation process takes place during the purification of the recombinant enzyme. The development of this purification procedure is essential for the isolation of functionally active aromatase from *E. coli*. Finally, our biochemical characterization indicates that our recombinant aromatase carries out an efficient aromatization of androgen and has catalytic properties identical to aromatase found in human tissue. This recombinant form of aromatase is a very useful tool for evaluating the molecular action of aromatase inhibitors such as letrozole and anastrozole.

3. Computer modeling of aromatase

While we were busy with the isolation of a significant quantity of *E. coli* recombinant human aromatase preparation for X-ray structural analysis, we decided to simultaneously perform structure–function studies of aromatase using the well-established CHO cell-aromatase-expression method. Numerous molecular models of aromatase have already been published, but all are based on soluble bacterial cytochrome P450s, whose percent sequence identity with aromatase ranges from 13 to 18%. Laughton et al. [29] based their model on P450 CAM, as did Koymans et al. [30]. Graham-Lorence et al. [15] based their model primarily on P450 BM-3, but also used P450 CAM and P450 TERP as templates. A recent model by Auvray et al. [31] was likewise based on P450 BM-3. Our model [14] was generated 10 years ago based on P450 CAM and has helped us greatly in our aromatase structure–function studies. When the X-ray structure of the first mammalian cytochrome P450, i.e. rabbit P450 2C5, was published recently [32], we decided to generate a new model of aromatase based on this mammalian template. The rabbit P450 2C5 catalyzes the hydroxylation 21-methyl group on the 17 side chain of progesterone. Since aromatase is also membrane-bound and catalyzes the hydroxylation of a steroid (androstenedione or testosterone), it was anticipated that a model based on P450 2C5 would likely be far more reliable.

The new model based on rabbit P450 2C5 differs significantly from all previous models based on bacterial P450 templates. Residues previously assigned to the B–C loop, the F and G helices, and the C-terminal β hairpin, all of which contribute to the active site, have been reassigned due to differences in the alignment between the target sequence (aromatase) and the new modeling template. As a result, peripheral residues previously identified as being important based on mutagenesis data now fall within the active site. Of the 20 residues that line the active site pocket, 12 are hydrophobic and 8 are hydrophilic. Asp-309, ser-478, and his-480, which flank the A-ring of androstenedione, form a catalytic triad consistent with our previously proposed mechanism [18]. The C-19 methyl group, which undergoes oxidation during the hydroxylation reaction, is centered over the heme-bound iron atom, placing the C-2 carbon adjacent to asp-309, which supposedly extracts a proton from it during the subsequent aromatization reaction. The D-ring is anchored by hydrogen bonds between the O-17 keto group and lys-119 and arg-435. Docking studies suggest that these positively charged residues also participate in binding various electronegative groups of inhibitors. Glu-302, previously thought to bind substrate but now judged as being too distant, forms a buried salt bridge with his-128 of the B–C loop, thus explaining its importance.

This new model is currently being validated by site-directed mutagenesis.

4. Aromatase suppression with phytochemicals in fruits and vegetables

Phytoestrogens, such as flavones and isoflavones, are plant chemicals that bind to ER and induce many components of estrogen action. They may function as antiestrogens or weak estrogens by competing with estrogens to bind to estrogen receptor (ER). However, it is also possible that some of these compounds may act in an indirect fashion by inhibiting aromatase activity, resulting in a decrease in the level of estrogen in women. Flavones have been demonstrated to be competitive inhibitors of aromatase with respect to the androgen substrate, with K_i values at low micromolar concentrations [33–37]. The binding characteristics and the structural requirement necessary for the inhibition of human aromatase by flavone and isoflavone phytoestrogens were obtained using computer modeling and confirmed by site-directed mutagenesis [38]. It was found that these compounds bind to the active site of aromatase in an orientation in which their rings-A and -C mimic rings-D and -C of the androgen substrate, respectively. The study also provides a molecular basis describing why isoflavones are significantly poorer inhibitors of aromatase than flavones.

While it is exciting to find that isolated phytochemicals like flavones can suppress aromatase activity, it is critical to determine whether plant extracts actually contain sufficient amounts of phytochemicals that can inhibit aromatase activity. Our laboratory has initiated an investigation to explore whether certain kinds of fruits and vegetables contain anti-aromatase chemicals. Among the fruit group, grapes were found to contain chemicals that act as potent inhibitors of aromatase [39]. The oral ingestion of grape juice was found to be able to suppress the MCF-7aro-derived tumor formation in nude mice. MCF-7aro cells are MCF-7 cells that over-express aromatase [7,25]. The nude mouse studies are very important in demonstrating the in vivo effects of phytochemicals. We have also demonstrated by both in vitro and in vivo experiments that red wine extract also contains potent aromatase inhibitors [40]. Red wine was shown to be much more effective than white wine in the suppression of aromatase activity. Whole wine, lyophilized wine, and heat-treated extracts were examined for aromatase inhibition in a human placenta microsomal assay. C18 Sep-Pak cartridge (Waters Co.) separation of red wine extracts under an increasing acetonitrile (ACN) gradient found that the most active components were in the 20% ACN fraction, in that they inhibited the wild-type human placenta aromatase, wild-type porcine placenta and blastocyst aromatase in a dose-dependent fashion. The 20% ACN active fraction was heat stable and inhibited aromatase in a non-competitive manner. The aromatase-inhibitory action of red wine extracts was also examined with a transgenic mouse model in which aromatase is over-expressed in the mammary tissues [40]. It was found that the intake of the 20% ACN fraction by gavage completely abrogated aromatase-induced hyperplasia and other changes in the mammary tissue. This has been the first report demonstrating that wine, especially red wine, contains phytochemicals that can inhibit aromatase. Recently, the intake of anti-aromatase chemicals from red wine was also able to suppress MCF-7aro-mediated tumor formation in nude mice (unpublished results).

Among the vegetable group, mushrooms were found to contain chemicals that can suppress aromatase activity [41]. The white button mushroom (species *Agaricus bisporus*) was found to suppress aromatase activity in a dose dependent fashion. Enzyme kinetics demonstrated mixed inhibition suggesting the presence of multiple inhibitors or more than one inhibitory mechanism. “in-cell” aromatase activity and cell proliferation were measured using MCF-7aro, an aromatase-transfected breast cancer cell line. Phytochemicals in the mushroom aqueous extract inhibited aromatase activity and proliferation of MCF-7aro cells. These results suggest that diets high in mushroom may modulate the aromatase activity and function in chemoprevention in postmenopausal women by reducing the in situ production of estrogen. Current efforts are devoted toward the isolation and characterization of the anti-aromatase chemicals in grapes, red wine, and mushrooms. These results indicate that some fruits and vegetables in our diet contain adequate amounts of anti-aromatase chemicals. Lee et al. [42] have isolated several anti-aromatase chemicals from *Broussonetia papyrifera* and found that the most potent chemicals (such as isolicoflavonol) suppress aromatase with IC_{50} values near 0.1 μM. Filleur et al. [43] have isolated four lignans with anti-aromatase activity from the petrol extract of *Myristica argentea* mace (Myristicaceae). Weber et al. [44] and Lund et al. [45] have reported that dietary soy-phytoestrogens decrease testosterone levels, aromatase activity and prostate weight in rats.

5. MCF-7aro and T-47Daro

Stable aromatase-expressing estrogen receptor-positive MCF-7 and T-47D cell lines (i.e. MCF-7aro and T-47Daro) have been prepared in our laboratory by aromatase cDNA transfection and G418 (neomycin) selection [25]. Aromatase activity in the transfected MCF-7 and T-47D cell lines was determined to be $73 \pm 6\,\text{pmol}\,(\text{mg}\,\text{h}^{-1})$ and $48 \pm 4\,\text{pmol}\,(\text{mg}\,\text{h}^{-1})$, respectively (see Table 1). It is thought that these cell lines express aromatase in a stable manner, as demonstrated by a steady expression of the enzyme during culture in the absence of G418. These aromatase-expressing cell lines have shown to be very useful to evaluate the growth suppressive effect of aromatase inhibitors. The growth of these cells could be stimulated by androgens (1–10 nM) as demonstrated through monolayer as well as spheroid three-dimensional culture method. In

order to test the hypothesis that tumor aromatase can affect breast tumor growth in a paracrine manner, we have carried out cell culture experiments by co-culturing MCF-7 cells with either MCF-7aro or T-47Daro cells. Testosterone (1 nM) increased cell growth to a similar degree for MCF-7/MCF-7aro co-culture (0.75 million cells each type) as with MCF-7aro only (two to threefold). In addition, the enzyme specific activities remained unchanged for MCF-7/MCF-7aro co-culture samples with and without androgen treatment, indicating that estrogen produced by transfected cells can also stimulate the growth of untransfected cells. The androgen response could be inhibited by an addition of 4-OHA (0.01–0.1 mM). For MCF-7/T-47Daro co-culture experiments, a clear induction of cell growth by androgen was observed and the level of the increase was similar to that on T-47Daro only. However, for either culture with T-47D only or with MCF-7/T-47Daro co-culture, the aromatase activity was found to increase significantly after testosterone treatment. Since T-47Daro cells were not subjected to a clonal purification, it is thought that the androgen treatment may selectively stimulate the growth of high aromatase-expressing T-47Daro cells. These results indicate that estrogen synthesized by the tumor aromatase can stimulate breast tumor growth in both an autocrine and a paracrine manner.

Recently, we have evaluated the activity of letrozole and tamoxifen using three-dimensional breast cancer cell culture models [46]. Two types of three-dimensional cell culture model (a spheroid culture model and a Matrigel thread model) have been used to evaluate the activities of letrozole and tamoxifen on the proliferation of ER-positive and aromatase-positive breast cancer cells. For the spheroid culture, MCF-7aro and T-47Daro) were cultured over a 1 mm layer of 1.5% agarose in a 100 mm Petri dish. After 48–72 h, cell spheroids were formed, and vigorous pipetting could not separate the cells. For the Matrigel thread culture, the cells were homogeneously mixed with Matrigel solution for 10 min in ice. The cell–Matrigel mixture was delivered into a pre-chilled and sterilized 6 cm × 0.8 mm (i.d.) Teflon tube with the aid of a 35 ml syringe. Upon exposing the Matrigel filled Teflon tube at room temperature for about 2–3 min, the congealed Matrigel was extruded out by the pressure created from the syringe. The cell spheroids or Matrigel threads were cultured in phenol red-free media containing 10% dextran-coated charcoal-treated fetal calf serum. While the two types of three-dimensional cell cultures exhibit their own unique features, the proliferation of MCF-7aro and T-47Daro, under both of the above described culture conditions, was stimulated by 1 nM 17β-estradiol or 1 nM testosterone. Letrozole, an aromatase inhibitor, could suppress the testosterone-dependent proliferation, in a dose-dependent manner. In contrast to the suppressive effect of letrozole, tamoxifen was found to act as a weak agonist of the estrogen receptor leading to a stimulation of the cell proliferation. These results demonstrate that letrozole is a more effective agent than tamoxifen thereby inhibiting the growth of hormone-responsive breast cancer cells even when grown as three-dimensional cell cultures.

The MCF-7aro cell line has also been used to induce tumor formation in nude mouse. This animal model is used in this and other laboratories to evaluate the in vivo action of aromatase inhibitors (e.g. [39,47]).

6. Conclusion

In summary, aromatase inhibitors are important drugs to treat breast cancer. Our structure–function studies involve computer modeling, site-directed mutagenesis, inhibition kinetic analysis and reaction metabolite analysis. A large number of aromatase mutants have been generated to help us understand the binding nature of existing aromatase inhibitors. The computer model and mutants are found to be very useful to characterize the anti-aromatase action of natural products or environmental chemicals. Up to now, our mutagenesis experiments are mainly performed using the CHO cell-expression method. CHO cell-expressed aromatase has kinetic properties comparable to those of the enzyme purified from human placenta. The enzyme assay can be conveniently performed using an “in-cell” method utilizing the endogenous NADPH-cytochrome P450 reductase as the source of the reductase. In addition, the level of aromatase activity was well maintained in the stable aromatase-expressing CHO cells, allowing us to perform multiple analyses of expressed aromatase and its mutants. An important progress for our structure–function studies of aromatase is the success in the expression and purification of functionally active human aromatase in *E. coli*. It is now possible to produce a significant quantity of recombinant aromatase for X-ray structural analysis. Besides, our laboratory has prepared stable aromatase-expressing MCF-7 and T-47D cell lines (i.e. MCF-7aro and T-47Daro). These cell lines are demonstrated to be very useful to evaluate the anti-aromatase and growth-inhibitory activity of aromatase inhibitors by monolayer and three-dimensional cell cultures, as well as tumor induction model in nude mice.

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