

CYP19 (Aromatase): Characterization of the Recombinant Enzyme and Its Role in the Biotransformation of Xenobiotics

Joanne McNamara, Penny Stocker, Vaughn Miller, Christopher Patten, David Stresser, and Charles Crespi
GENTEST Corporation, 6 Henshaw Street, Woburn, MA 01801 USA

Abstract

Cytochromes P450 are the principle enzymes responsible for the metabolism of drugs, steroids and environmental pollutants. CYP19 (Aromatase) is the enzyme responsible for the conversion of androgens to estrogens. Due to the role of estrogen in many hormonal diseases, inhibitors to this enzyme have been developed, and more potent and specific ones are sought. CYP19 has also been shown to metabolize xenobiotics.

Heterologous expression can provide a means for producing P450 enzyme of high specific activity for studies of xenobiotic metabolism. The studies herein describe the cDNA expression of CYP19 using the baculovirus/insect cell expression system. The level of expression was 2.1 nmole/mg protein. A typical Cytochrome P450 CO-reduced difference spectra was seen with a λ_{max} at 449nm. A small shoulder was seen at about 420nm (P420). Microsomes prepared from infected cells contained high levels of CYP19 activity as measured by

Introduction

Cytochromes P450 are a superfamily of membrane bound enzymes that metabolize drugs, steroids and other xenobiotics. CYP19 (Aromatase) is the cytochrome P450 responsible for the conversion of androgens to estrogens. Estrogen synthesis is critical for normal physiological processes such as expression of secondary sexual characteristics, establishment and maintenance of pregnancy, and maintaining bone mineral homeostasis (1,2). CYP19 has attracted a lot of attention due to its unique reaction mechanism and the major role estrogens play in the growth of hormone sensitive cancers such as breast cancer. CYP19 is an extrahepatic P450 localized in the endoplasmic reticulum. Electrons for its catalytic reaction are supplied via NADPH and NADPH:P450 oxidoreductase. In humans, CYP19 is expressed in a number of tissues including placenta, ovary, muscle and adipose cells, Sertoli and Leydig cells of the testis and in various sites in the brain (1-4).

CYP19 converts testosterone to estradiol and androstenedione to estrone. The aromatase mechanism is unlike other typical P450 catalytic mechanisms; involving a unique 3-step process. The first two oxidative steps are thought to be typical cytochrome P450 hydroxylations. A theory originally proposed by M. Akhtar, suggests the third step to be a ferric peroxide attack to remove the aldehyde, followed by aromatization of the A ring. Kinetic

Materials and Methods

cDNA expression in Insect cells

Recombinant baculoviruses containing the cDNA for CYP19 were constructed using vectors and viral DNA from PharMingen (San Diego, CA) according to the manufacturer's instructions. The cDNA for human NADPH:P450 oxidoreductase was co-expressed with the CYP19 cDNA. Enzyme expression was carried out in spinner flasks of BTI-TN-5B1-4 cells (Hi5 cells). Infection time was 48 hours. The final enzyme preparation was designated CYP19+OR.

Microsome Preparation

Microsomes from insect cells were prepared according to the standard methods. (11) Protein concentrations were determined by the method of Lowry using BSA as standard. Cytochrome P450 content was determined by standard procedures (Omura and Sato) using an extinction coefficient of 91 (mM cm⁻¹).

Enzyme Assays

Aromatase Activity Assay

A 0.25 mL reaction mixture containing enzyme protein, 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 50uM testosterone in 0.1 M potassium phosphate (pH 7.4) was incubated at 37° for 20 minutes. After incubation the reaction was stopped with the addition of acetonitrile (ACN) and centrifuged to pellet the protein. "Blank" incubations were terminated with ACN prior to the addition of enzyme. A portion of the supernatant was injected directly into a Zorbax 4.6 mm X 250mm 5u C18 HPLC column. The column was kept at 45° C, and eluted isocratically with 40% acetonitrile and water at a flow rate of 1.5 mL/min. (12) The product was detected by its absorbance at 200nm and quantitated by comparison to the absorbance of a standard curve for β -estradiol. HPLC analysis was performed on a Waters 2690 Separations Module equipped with a Waters 2487 Dual Absorbance Detector.

For kinetic determinations the following testosterone concentrations were used: 0, 25, 50, 75, 100, 125, 150, 200, 250, 300, 400, 600 nM. The incubation time was 4 minutes. Apparent K_m and V_{max} values were calculated by non-linear regression using SigmaPlot software. Activity values are expressed as turnover number (pmol product/min pmol P450-min⁻¹).

Coumarin Assay

A 0.25mL reaction mixture containing enzyme protein, 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.2 mM coumarin in 0.1M Tris Buffer (pH 7.5) was incubated at 37°C for 30 min. The reaction was stopped with trichloroacetic acid and then centrifuged to pellet the protein. The fluorescence of the product was measured after dilution into 0.1 M Tris pH 9 in a spectrofluorometer with excitation at 368 nm and emission at 456 nm. The increase in fluorescence was quantitated by comparison to a standard curve of product 7-hydroxycoumarin.

Paclitaxel Assay

A 0.25 mL reaction mixture containing enzyme protein, 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.02 mM paclitaxel in 50 mM potassium phosphate (pH 7.4) was incubated at 37°C for 30 min. The reaction mixture was stopped by the addition of 75uL acetonitrile and centrifuged to pellet the protein. A portion was injected into a 4.6 x 250 mm 5u C18 HPLC column and separated at 45°C with a mobile phase of methanol/water at a flow rate of 1 mL per min. The product was detected by its absorbance at 230 nm and quantitated by comparison to the absorbance of a standard curve for 6 α -hydroxypaclitaxel.

Diclofenac Assay

A 0.25 mL reaction mixture containing enzyme protein, 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.1 mM diclofenac in 0.1M Tris buffer (pH 7.5) was incubated at 37°C for 30 min. After incubation, the reaction was stopped by the addition of 75 μ l 94% acetonitrile/6% glacial acetic acid and centrifuged to pellet the protein. A portion of the incubation was injected into a 4.6 x 250 mm 5u C18 HPLC column and separated at 45°C with a mobile phase of acetonitrile, methanol and 1 mM perchloric acid in water. The product was detected by its absorbance at 280 nm and quantitated by comparison to the absorbance of a standard curve for 4'-hydroxydiclofenac.

Bufuralol Assay

A 0.25 mL reaction mixture containing enzyme protein, 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.1 mM (+/-) bufuralol in 0.1M potassium phosphate (pH 7.4) was incubated at 37°C for

testosterone conversion to estradiol. Kinetic analysis determined the K_m value for estradiol formation to be 43 nM and V_{max} value to be 8.4 min⁻¹.

cDNA expressed CYP19 was further tested for its ability to metabolize xenobiotics. We found low but detectable activity for testosterone 6 β -hydroxylase and bufuralol. No activity was seen with diclofenac, coumarin, 7-ethoxyresorufin, paclitaxel, and (S)-mephenytoin. Various fluorescent high-throughput P450 substrates were also tested as potential CYP19 substrates. CYP19 metabolized 7-Ethoxy-3-cyanocoumarin (CEC), 7-Methoxy-4-trifluoromethylcoumarin (MFC), 7-Benzyloxy-4-trifluoromethylcoumarin (BFC), Dibenzyl fluorescein (DBF), and 7-Ethoxy-4-trifluoromethylcoumarin (7-EFC). By having high-throughput screening assays available, many new compounds can be quickly and easily examined as potential CYP19 inhibitors.

studies of the aromatase reaction have shown that the first oxidation step is rate limiting ($k_1 < k_2 < k_3$) (8).

CYP19 has been expressed using a variety of heterologous expression systems, including insect cells (Hi5 and Sf9), mammalian cells, yeast and E.Coli (5). Highest expression levels have been achieved in insect cells as compared to the other expression systems (1,5,6). Kinetic analysis of the conversion of testosterone to estradiol by reconstituted aromatase has been reported to have K_m values in the range 100-200 nM, while K_m values in tissue microsomes were lower in the 10-60 nM range (3,6,7). Although aromatase has long been considered specific for aromatization of androgens to estrogens, recent evidence suggests that it may also be capable of xenobiotic metabolism (9,10)

The focus of the current study was to express CYP19 in insect cells using the baculovirus expression system. The results show that CYP19 is efficiently expressed in Hi5 insect cells. The expressed enzyme is shown to possess high activity for the conversion of testosterone to β -estradiol. Also, various xenobiotics were demonstrated to be substrates for CYP19.

30 minutes. After incubation, 25 μ l of 70% perchloric acid was added and the mixture was centrifuged to pellet the protein. The supernatant was injected into a 4.6 x 250 mm 5u C18 HPLC column and separated at 45°C with a mobile phase of acetonitrile/water with perchloric acid. The fluorescence of the product was measured in the flow cell of a spectrofluorometer with excitation at 252 nm and emission at 302 nm. The response was quantitated by comparison to a standard curve of product 1'-hydroxybufuralol.

Testosterone Hydroxylase Assay

A 0.25 mL reaction mixture containing enzyme protein, 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.4 mM testosterone in 0.1 M potassium phosphate (pH 7.4) was incubated at 37°C for 30 min. The reaction mixture was stopped by the addition of 125 μ l acetonitrile and centrifuged to pellet the protein. A portion was injected into a 4.6 x 250 mm 5u C18 HPLC column and separated at 45°C with a mobile phase of methanol/water at a flow rate of 1 mL per min. The product was detected by its absorbance at 254 nm and quantitated by comparison to the absorbance of a standard curve for 6 β -hydroxytestosterone.

7-ER Assay

A 2.0 mL reaction mixture was made containing enzyme protein, 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 1 mg/mL 7-ER in 0.1M potassium phosphate (pH 7.4) The fluorescence of the product was measured in a spectrofluorometer with excitation at 550 nm and emission at 586 nm. The increase in fluorescence was quantitated by comparison to a standard curve of resorufin.

[¹⁴C]-[S]-mephenytoin Assay

A 0.1 mL reaction mixture containing enzyme protein, 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 0.1 mM [¹⁴C]-[S]-mephenytoin in 50 mM potassium phosphate (pH 7.4) was incubated at 37°C for 30 min. After incubation, the reaction was stopped by the addition of 25uL acetonitrile and centrifuged to pellet the protein. A portion of the incubation was injected into a 4.6 x 250 mm 5u C18 HPLC column and separated at 45°C with a mobile phase of methanol and water. The product was detected and quantitated by liquid scintillation counting.

High-throughput Fluoremetric Assays

Six-fluorescent substrates (7-Ethoxy-3-cyanocoumarin, CEC; 7-Methoxy-4-trifluoromethylcoumarin, MFC; 7-Benzyloxy-4-trifluoromethylcoumarin, BFC; 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin, AMMC; Dibenzyl fluorescein, DBF; 7-Ethoxy-4-trifluoromethylcoumarin, 7-EFC) were tested for their ability to be potential substrates for CYP19 by means of high-throughput fluoremetric assays conducted in 96-well (200uL volume) microtiter plates (Corning Costar, Cambridge, MA) (Table 1). The reaction mixture (0.2 mL volume) contained enzyme protein, 1.3 mM NADP⁺, 3.3 mM glucose-6-phosphate, 0.4 U/mL glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and substrate (concentrations shown in Table 1) in 50 mM potassium phosphate (pH 7.4). The plates were incubated for 45 minutes and the reaction was terminated by addition of 75 μ l of the appropriate stop solution; 80% Acetonitrile/20% 0.5 M Tris Base or 2N NaOH (Table 1). Fluorescence was measured using a FLUOstar model 403 fluorescence plate reader (BMG Lab Technologies, Inc., Durham, NC). For the DBF assay, the plate was read 2 hours after stopping the reaction. The increase in fluorescence was quantitated by comparison to a standard curve of the appropriate metabolite. Metabolite concentrations were measured using the excitation and emission wavelengths shown in Table 1. Data were exported and analyzed using an Excel spreadsheet.

Table 1

Substrate	Concentration	Excitation/Emission	Stop solution
7-EFC	200uM	410/510	ACN:0.5M Tris Base
7-MFC	200uM	410/510	ACN:0.5M Tris Base
7-BFC	200uM	410/510	ACN:0.5M Tris Base
AMMC	25uM	390/460	ACN:0.5M Tris Base
DBF	2uM	485/538	2N NaOH
CEC	50uM	410/460	ACN:0.5M Tris Base

Results

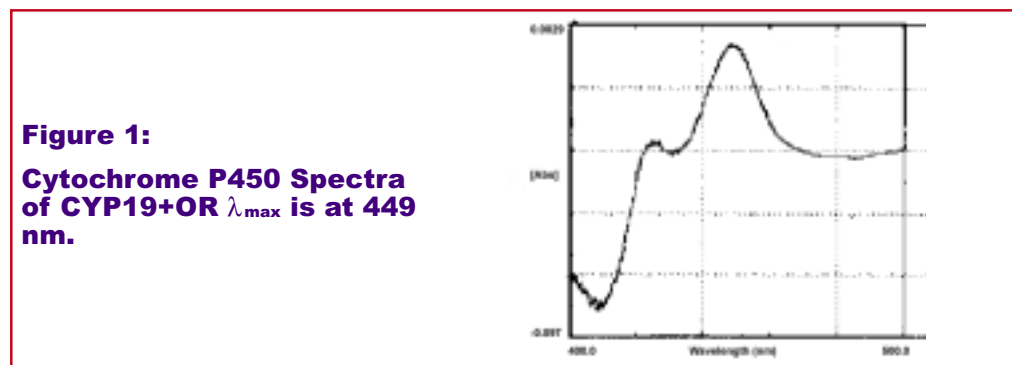
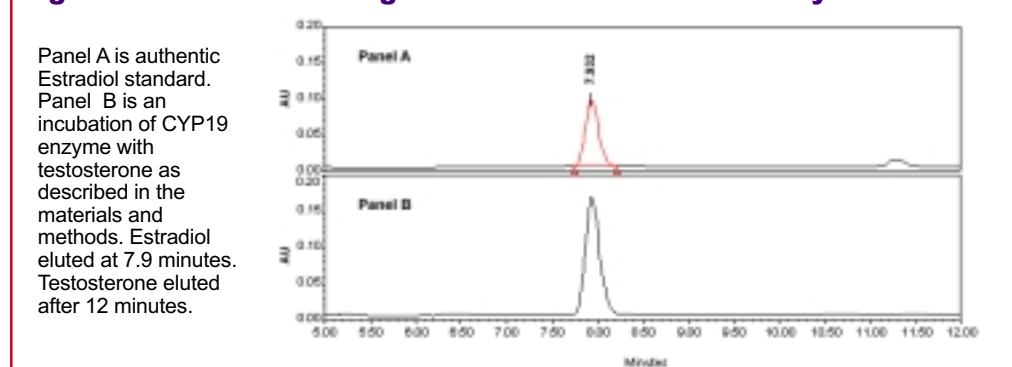


Figure 1:
Cytochrome P450 Spectra of CYP19+OR λ_{max} is at 449 nm.

Figure2: HPLC chromatogram of estradiol formation by CYP19+OR.



Panel A is authentic Estradiol standard. Panel B is an incubation of CYP19 enzyme with testosterone as described in the materials and methods. Estradiol eluted at 7.9 minutes. Testosterone eluted after 12 minutes.

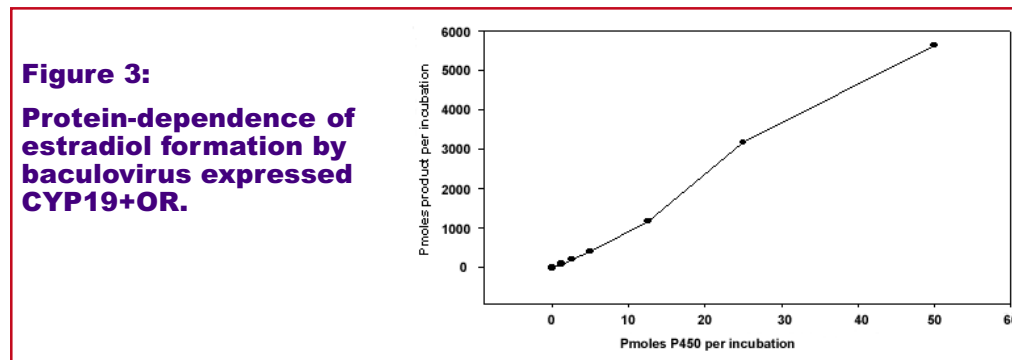


Figure 3:
Protein-dependence of estradiol formation by baculovirus expressed CYP19+OR.

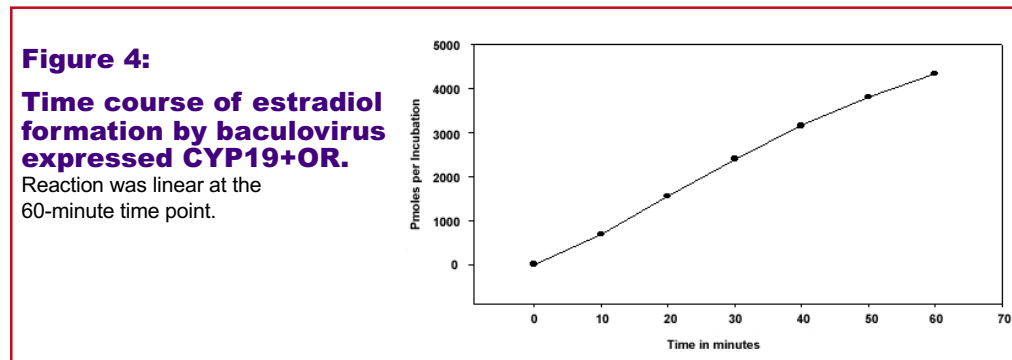


Figure 4:
Time course of estradiol formation by baculovirus expressed CYP19+OR. Reaction was linear at the 60-minute time point.

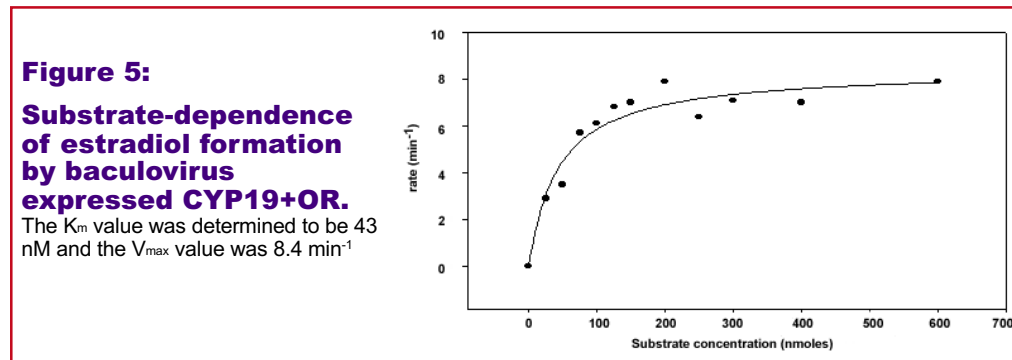


Figure 5:
Substrate-dependence of estradiol formation by baculovirus expressed CYP19+OR. The K_m value was determined to be 43 nM and the V_{max} value was 8.4 min⁻¹

Table 2 Metabolism of Testosterone to Estradiol by CYP19

Condition	Activity(min ⁻¹)
Blank	ND*
CYP19+OR(-)b5	4.4
CYP19+OR(+b5)	1.1

* ND- Metabolite was not detected

Table 3 Metabolism of P450-specific substrates by CYP19

Substrate	[Substrate]	Product formed	Activity(min ⁻¹)
Testosterone	400uM	6 β -hydroxytestosterone	0.5 *
Testosterone	400uM	Unknown metabolite	0.8 **
Diclofenac	100uM	4'-hydroxydiclofenac	ND
Bufuralol	100uM	1'-hydroxybufuralol	0.2 *
Paclitaxel	20uM	6 α -hydroxypaclitaxel	ND
Coumarin	200uM	7-hydroxycoumarin	ND
7-ER	1mg/mL	Resorufin	ND
(S)-Mephenytoin	100uM	Nirvanol	ND
(S)-Mephenytoin	100uM	4'-hydroxymephenytoin	ND

* Activity is the average of 4 preparations of enzyme whose activity was within 25 % of one another.

** 6 β -hydroxylase was used to quantitate the unknown metabolite.

Table 4 Metabolism of P450 High-Throughput Fluorescent Substrates by CYP19

Substrate	[Substrate]	Product Formed*	Activity(min ⁻¹)	S/N* ratio
7-EFC	200uM	7-HFC	1.0	10:1
7-MFC	200uM	7-HFC	1.1	12:1
7-BFC	200uM	7-HFC	0.1	2:1
AMMC	25uM	AHMC	ND	ND
DBF	2uM	Flourescein	0.070	20:1
CEC	50uM	CHC	0.006	4:1

* (7-Hydroxy-4-trifluoromethylcoumarin, 7-HFC; 3-[2-(N,N-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin hydrochloride, AHMC; 7-Hydroxy-3-cyanocoumarin, CHC)

*S/N=Signal to Noise Ratio

Summary and Conclusions

- CYP19 was expressed in insect cells using the baculovirus expression system. The level of expression was 2.1 nmole/mg protein.
- Stability tests (freeze/thaw) showed no loss of activity after 6 freeze/thaw cycles.
- Expressed CYP19 was able to convert testosterone to estradiol with a low K_m value of 43 nM, and a V_{max} of 8.4 min⁻¹.
- Estradiol formation was linear with time up to 60 minutes.
- Protein dependence was linear up to 20 pmol/P450 per incubation.
- CYP19 exhibited low, but detectable activity for xenobiotic substrates; i.e. testosterone 6 β -hydroxylase and bufuralol.
- CYP19 was also able to metabolize various fluorescent high-throughput P450 substrates (i.e. 7-EFC, 7-MFC, 7-BFC, DBF, CEC). AMMC, a CYP2D6 substrate, was not metabolized by CYP19.
- High-throughput assays can be used for rapid screening of potential aromatase inhibitors.

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