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Biotransformation of letrozole in rat liver microsomes: Effects of gender and tamoxifen

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Abstract

The *in vitro* metabolic kinetics of letrozole were investigated by incubating letrozole (10–500 μM) in female or male rat liver microsomes to assess the effect of gender and to predict the *in vivo* biotransformation characteristics of letrozole in rats. The effects of tamoxifen (TAM) on the metabolic kinetics of letrozole were also examined by incubating letrozole in female rat liver microsomes in the presence or absence of TAM. The effects of chronic pretreatment of female rats with TAM (0.5, 1.0, 5.0 mg/kg/day, *i.p.* for 7 consecutive days) on liver microsomal protein content and metabolic activity were also examined.

The formation rate of the carbinol metabolite of letrozole, CGP44 645, was significantly higher ($p < 0.05$) in male rat liver microsomes in comparison to female. The V_{max}/K_m ratio for letrozole metabolism in female rat liver microsomes did not change significantly ($p > 0.05$) in the presence of TAM. After chronic pretreatment of female rats with TAM (up to a dose of 1.0 mg/kg/day), the hepatic microsomal protein content was significantly increased but the formation rate of CGP44 645, when normalized for protein content, did not change significantly.

These results suggest that there is a marked gender difference in letrozole metabolism in rats. It also appears that acute treatment of female rat liver microsomes with TAM produces negligible inhibitory effect on the CYP mediated metabolic clearance of letrozole. However, chronic pretreatment of female rats with TAM appear to induce CYPs, but does not significantly impact the metabolic activities of the enzymes associated with the formation of the carbinol metabolite of letrozole.

Keywords: Metabolism; Liver microsomes; Letrozole; Tamoxifen; Gender

1. Introduction

About 75% of breast cancers are positive for the estrogen receptor (ER) and/or progesterone receptor (PgR), and estrogen is the main stimulant in the development and growth of these tumors [1]. Therefore, estrogen deprivation is the primary mechanism of hormonal therapies for breast cancer.

There are two main ways in which estrogen deprivation may be achieved (Fig. 1). The most frequent approach is to use an antiestrogen such as tamoxifen (TAM, Fig. 2) to antagonize estrogens at the estrogen receptor (ER). TAM is now recommended for all pre-menopausal women with hormone-positive cancers, as well as for most postmenopausal women with breast cancer and/or a growing number of women with hormone-negative cancers [2,3]. However, the actions of TAM are complex. For example, it also has partial estrogen-agonist effects. These partial agonist effects can be beneficial, since they may help prevent bone demineralization in postmenopausal women [4,5], but also detrimental, since they are associated with increased risks of secondary cancer, such as uterine and/or liver cancer [6–8] and thromboembolism [9]. In addition, not all patients with advanced ER-positive disease respond to TAM and nearly all of those who do respond eventually relapse with resistant disease [1]. Studies over many years have indicated that the partial agonist effect of TAM may play a part in the development of TAM resistance [10].

An alternative approach is to use an aromatase inhibitor (AI) such as letrozole to reduce the synthesis of estrogens. The aromatase is a cytochrome P450 enzyme that converts adrenal androgens such as testosterone and androstenedione to estrogens such as estradiol and estrone, respectively. Letrozole (Fig. 2) is a

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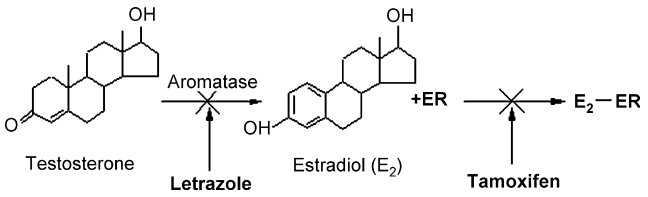

Fig. 1. Inhibition of aromatase by letrozole and antagonism of estrogen receptor (ER) binding by tamoxifen.

nonsteroidal, highly potent and competitive inhibitor of the aromatase enzyme system. It has been reported that the treatment of women with letrozole significantly lowers serum estrone (E_1), estradiol (E_2) and estrone sulfate (E_1S) by 88–98% [11], but produces no androgenic, progestrogenic or estrogenic effects, such as weight gain, acne, or hypertrichosis [12]. Therefore, it is used as a second-line agent (after TAM) and now challenging the place of TAM for the treatment of advanced breast cancer in postmenopausal women.

The differences in the mechanism of action between letrozole and tamoxifen have led to suggestions that a combination of the two agents may produce a better outcome in breast cancer treatment compared to either agent alone. However, a combination of letrozole and tamoxifen also raises the possibility of potential metabolic interaction between the two drugs since both compounds are substrates for cytochrome P450 (CYPs). It has been known that subfamilies of CYP3A4 and CYP2A6 are responsible for the metabolism of letrozole to its carbinol metabolite, CGP44 645, in humans [13]. However, no account of the metabolic kinetics of letrozole either in animal or human models has been reported. Therefore, our first goal was to obtain metabolic kinetic parameters of letrozole in rat liver microsomes, in an attempt to predict the *in vivo* clearance and other pharmacokinetic characteristics of letrozole in rats. Interestingly, hepatic CYP3A4 is the major subfamily responsible for the metabolism of TAM as well [14], indicating that letrozole and TAM are both substrates of CYP3A4. Therefore, there is a possibility of metabolic interaction between these two drugs as a result of enzyme inhibition or induction. Previous studies have shown that acute treatment of rats with TAM increased hepatic CYP2A1 activity and decrease CYP2C11 activity [15], while pretreatment with TAM induced CYP2B1 and CYP3A1 in rat liver microsomes [16]. However, it has also been observed that TAM and its metabolites reversibly inhibit CYP3A4 and CYP1B1 in human liver microsomes [17]. Nevertheless, there is little information regarding metabolic interaction between letrozole and TAM in the literature. Therefore, it is necessary to investigate the impact of TAM on the metabolic kinetics of letrozole.

The objective of this investigation was to evaluate the *in vitro* metabolic kinetics of letrozole and examine gender differences and the effect of TAM treatment on the biotransformation of letrozole in rat liver microsomes. The information generated may be useful to predict the *in vivo* intrinsic clearance of letrozole and metabolic interaction between letrozole and tamoxifen in animals.

2. Experimental

2.1. Materials

Letrozole was generously provided by Dr. A. Brodie (University of Maryland, School of Medicine, Baltimore, MD, USA), and its carbinol metabolite CGP44 645 was synthesized by Drs. Piao and Canny (Temple University School of Pharmacy, Philadelphia, PA). Methanol, acetonitrile, monobasic (and dibasic) sodium phosphate, and potassium chloride (KCl) were purchased from Fisher Scientific (Pittsburg, PA, USA). Tamoxifen, tamoxifen citrate salt, β-nicotinamide adenine dinucleotide (β-NADP), glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PD), bovine serum albumin, phosphate buffer saline tablets and hydroxypropyl-β-cyclodextrin (HPβCD) were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). BioRad reagent was ordered from BIO-RAD Laboratories (Richmond, CA, USA). All chemicals and solvents were of analytical or HPLC grade. Distilled water was obtained from an in-house Barnstead NANOpure® apparatus (Barnstead International, Dubuque, IA, USA). Bond Elut-C_8® extraction cartridges were ordered from Varian (Walnut Creek, CA, USA).

2.2. Preparation of rat liver microsomes

Male and Female Sprague–Dawley rats (300 ± 20 g) were obtained from Charles River Laboratories (Wilmington, MA, USA). The animals were maintained in a controlled environment of constant temperature (20 °C), 50% relative humidity and 12-h light:12-h dark cycles for 7 days prior to use. The rats were provided with food and water *ad libitum*. The health of all animals was monitored throughout the study by observing the changes in body weights.

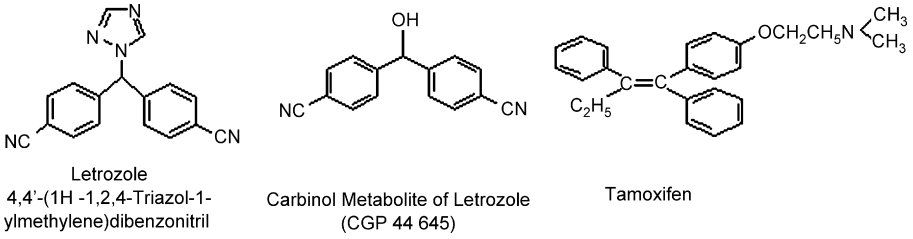

Fig. 2. Structures of letrozole, its carbinol metabolite, CGP 44645, and tamoxifen.

The rats were sacrificed using carbon dioxide (CO_2) and the livers were quickly removed, rinsed in ice-cold normal saline, weighed and immediately homogenized in ice-cold phosphate buffered saline (PBS, 67 mM, pH 7.4) containing 0.15 M KCL (three times of liver weight, v/w). The mixture was then centrifuged twice at 10,000 × *g* (Sorvall® RC-5B refrigerated superspeed centrifuge, Kendro Laboratory, Asheville, NC) for 20 min (4 °C). The supernatant was transferred to a pre-cooled ultra-centrifuge tube and centrifuged at 100,000 × *g* (Beckman L8-M ultracentrifuge, Beckman Coulter, Inc. Fullerton, CA) for 60 min (4 °C). The pellets were then re-suspended in PBS solution (67 mM, pH 7.4 with 0.15 M KCL) to obtain rat liver microsomes. All chemicals, solution and tubes were maintained on ice during the whole procedure. The microsomes were stored at −80 °C.

The protein content in rat liver microsomal fraction was determined by a Bio-Rad protein assay method [18].

2.3. Analytical methodology

2.3.1. Sample preparation

Accurately measured aliquots (100 μL) of microsomal incubation mixtures were diluted with 500 μL of phosphate buffer (500 mM, pH 6.8), and then extracted by solid-phase extraction using Bond Elut C_8® cartridge following the steps described below; at each step, the elution through the cartridge was performed by a vacuum manifold processing station to induce the desired flow-rate through the cartridge.

(1) Conditioning: 1.0 mL of methanol was dispensed onto the cartridge and then washed by 1.0 mL phosphate buffer (500 mM, pH 6.8), at a pressure of −70 kPa.
(2) Sample loading: the diluted sample was slowly loaded onto the cartridge and allowed to elute, at a pressure of −30 kPa.
(3) Column washing was carried out by 1.0 mL of phosphate buffer (0.01 M, pH 6.8) at −70 kPa, followed by 1.0 mL of buffer-ACN (80:20, v/v), at a pressure of −70 kPa.
(4) Elution: 500 mL of ACN was used to elute the analytes, at a pressure of −30 kPa. The eluate was then dried under nitrogen and reconstituted into 100 mL of mobile phase for HPLC analysis.

2.3.2. Identification of CGP44 645 in rat liver microsomal incubations by LC–MS

In a preliminary incubation experiment, the UV spectrum for a formed metabolite after incubate 100 nM of letrozole in female rat liver microsomes was obtained by diode array detector (DAD) and then compared with the UV spectrum of the synthesized metabolite. In order to further confirm the formation of CGP44 645, the Agilent® 1100 series LC/MSD system was used.

The separation of letrozole and its carbinol metabolite was carried out on a Zorbax C_{18} column (5 μM, 250 mm × 4.6 mm I.D. Agilent, Wilmington, DE). The mobile phase consisting of acetonitrile and 0.1% (v/v) formic acid in H_2O (60:40, v/v) was eluted in an isocratic mode for 25 min. An electron spray module (ESI) was used for ionization and the negative polarity was detected by the scan mode. Other conditions were fragmentor = 100 V, capillary voltage = 3500 V, dry gas flow = 10 L, dry gas temperature = 300 °C, and nebulizer pressure = 30 psig.

After incubation, the sample was prepared as described under Section 2.3.1, and analyzed by LC–MSD. A standard solution of CGP44 645 was also injected into the LC–MSD under the same conditions as described above. The MS spectrum, together with the UV spectrum from DAD, of the formed peak in the incubation sample was compared with the synthesized metabolite in order to confirm that CGP44 645 was formed after incubation of letrozole in rat liver microsomes.

2.3.3. Quantification of CGP44 645 by HPLC

The HPLC equipment used was an Agilent® 1100 series system with a diode array detector and a 1046 fluorescence detector (Agilent, Wilmington, DE). The separation of letrozole and its carbinol metabolite was carried out on a Zorbax C_{18} column (5 μM, 250 mm × 4.6 mm I.D., Agilent, Wilmington, DE). To protect the column, a Zorbax analytical guard-cartridge with same packing material (5 μM, 12.5 mm × 4.6 mm I.D., Agilent, Wilmington, DE) was installed. Agilent ChemStation® was used for data collection and integration.

The mobile phase used for analysis of microsomal samples consisted of acetonitrile and phosphate buffer (10 mM, pH 6.8) (35:65, v/v). The mobile phase was eluted in an isocratic mode at 1.0 mL/min for 25 min. The excitation (λ_{ex}) and emission wavelengths (λ_{em}) of the fluorescence detector were set at 230 and 295 nm, respectively.

2.3.4. Calibration curve and assay validation

The calibration curve for CGP44 645 in rat liver microsomal samples were constructed by spiking working solutions (10 μL) in controlled incubation medium (90 μL), which contained 4 mg/mL of denatured microsomal proteins (by preheating microsomes at 60 °C), 100 mM of phosphate buffer, 6.6 mM of $MgCl_2$, and an NADPH generating system, consisting of 2.6 mM of β-NADP, 6.6 mM of glucose-6-phosphate, and 0.8 U/mL of glucose-6-phosphate dehydrogenase. The final concentration of CGP44 645 in incubation mixture was 40 nM–10 μM. The calibration samples were taken through the sample preparation procedure as described above. Appropriate blank extraction was also prepared. The calibration curves were generated by plotting the peak area versus the concentration of CGP44 645 spiked in the samples. Linear regression analysis was performed using Microsoft® Excel. The limit of quantification (LOQ) was considered as the lowest concentration on the low range calibration curve that produced coefficient of variation (CV) of 15–20%. The recovery was calculated from the peak area of the CGP44 645 after extraction from rat liver microsomal incubation medium to the peak area of an equivalent amount of the standard solution. The quality control concentrations were 40 and 500 nM and 1, 10 and 20 μM. The calibration curves and assay validation studies were all performed in duplicate on three separate occasions ($n = 3$).

2.4. Hepatic microsomal incubation

2.4.1. Gender differences in letrozole metabolism in rat liver microsomes

A typical incubation mixture consisted of 100 mM of phosphate buffer and 6.6 mM of $MgCl_2$ under the optimal incubation conditions: NADPH generating system containing 1.3 mM of β-NADP, 3.3 mM of G6P, 0.4 U/mL of G6PD, and microsomal protein content of 4 mg/mL. The incubation mixture had a final volume of 1.0 mL. Letrozole (10–500 μM) was incubated in female rat liver microsomes and 3–500 μM of letrozole was incubated in male rat liver microsomes using a shaking water bath at 37 °C for 30 min. The metabolic reaction was initiated by the addition of microsomes and terminated by freezing on dry ice. The microsomal enzyme in control incubation was inactivated by heat treatment at 60 °C. Incubations were carried out in duplicated on three occasions ($n = 3$). The samples were prepared and analyzed as described above.

2.4.2. Effects of TAM on letrozole metabolism in female rat liver microsomes

Letrozole (10–500 μM) was incubated using the same conditions described above with female rat liver microsomes with or without the presence of TAM (25 or 250 μM). Incubations were carried out in duplicated on three occasions ($n = 3$). Control incubations were also performed by adding denatured microsomal protein. The samples were prepared and analyzed using the analytical procedures described above.

2.4.3. Effect of chronic pretreatment of female rats with tamoxifen on letrozole biotransformation

Tamoxifen citrate salt was dissolved in HPβCD (10% in saline) to produce equivalent TAM concentrations of 0.5, 1.0 and 5.0 mg/mL. Four groups ($n = 3$/group) of female Sprague–Dawley rats were all maintained in a controlled environment for 1 week prior to the treatment. Three groups received TAM formulations (0.5, 1.0, 5.0 mg/kg/day, respectively) and the control group received an equivalent volume of the vehicle (HPβCD, 10% in saline) by intraperitoneal injection for 7 consecutive days. The animals had free access to food and water during this period. The rats were sacrificed using carbon dioxide (CO_2) and liver microsomes were prepared and microsomal protein content was determined as described above.

The rat liver microsomes obtained after chronic pretreatment of rats with TAM were used to incubate letrozole under the same conditions described above in order to investigate the effect of pretreatment with TAM on letrozole biotransformation.

2.5. Microsomal enzyme kinetic data analysis

2.5.1. Parameter estimates

The formation rate of CGP44 645 was used to study the kinetics of letrozole biotransformation. The Michaelis–Menten equation was used to describe the relationship between the formation rate of CGP44 645 and letrozole concentration (C) (Eq. (1)):

$$\text{rate} = \frac{V_{\max} C}{K_m + C} \tag{1}$$

where $V_{\max}$ represents the maximum rate of formation of CGP44 645, which reflects the maximum metabolic capacity; The Michaelis–Menten constant, K_m, represents the concentration of letrozole at half maximum rate. The formation rate of CGP44 645 was calculated using Eq. (2):

$$\text{rate} = \frac{\text{amount of CGP44645 formed in incubation mixture (pmol)}}{\text{protein conctent (4 mg/mL)} \times \text{incubation time (30 min)}} \tag{2}$$

Lineweaver-Burke approach was used to estimate initial Michaelis–Menten parameters and the final parameter estimates were obtained by fitting the rate-concentration curve as in Eq. (1) using WinNonlin 4.1 (Pharsight Corporation, Inc., Mountain View, CA, USA) with Poisson error weighting scheme (weight = $1/C$).

The final estimates of the Michaels–Menten parameters ($V_{\max}$, K_m) were then used to calculate the *in vitro* intrinsic clearance, $CL_{int\,(in\ vitro)}$, based on Eq. (3):

$$CL_{int\,(in\ vitro)} = \frac{V_{\max}}{K_m + C} \tag{3}$$

Usually, plasma drug concentration (C) in the therapeutic range is relatively low compared to the K_m for the metabolizing enzyme, thus Eq. (3) approximates to Eq. (4):

$$CL_{int\,(in\ vitro)} = \frac{V_{\max}}{K_m} \tag{4}$$

The *in vitro* intrinsic clearance for letrozole in rat liver microsomes was scaled up to the *in vivo* intrinsic clearance, $CL_{int\,(in\ vivo)}$, for letrozole in an average adult rat using scaling factor of 1600 mg microsomal protein/kg in Eq. (5) (assuming that 1 g of rat liver contains approximately 50 mg of microsomal protein and the liver of a 250 g rat weighs approximately 8 g [19]. The hepatic clearance (CL_H) of letrozole in rats was then predicted from $CL_{int\,(in\ vivo)}$ using Eq. (6).

$$CL_{int\,(in\ vivo)} = \frac{1600\ \text{mg microsomal protein}}{\text{body weight (kg)}} \times \frac{V_{\max}}{K_m} \tag{5}$$

$$CL_H = \frac{Q_H \times f_u CL_{int\,(in\ vivo)}}{Q_H + f_u CL_{int\,(in\ vivo)}} Q_H \times E \tag{6}$$

where Q_H is the value of hepatic blood flow in rats, the average value of which is 65 mL/min/kg, and f_u is the unbound fraction of letrozole in blood. It has been reported that letrozole is weakly bound to proteins in human plasma (unpublished data by Novatis pharmaceuticals Corporation). As a result, we assumed that the bound fraction of letrozole to microsomal or plasma protein is negligible. Therefore, Eq. (6) was simplified to Eq. (7):

$$CL_H = \frac{Q_H \times CL_{int\,(in\ vivo)}}{Q_H + CL_{int\,(in\ vivo)}} Q_H \times E \tag{7}$$

E is defined as the extraction ratio and can be calculated by Eq. (8):

$$E = \frac{CL_{int\,(in\ vivo)}}{Q_H + CL_{int\,(in\ vivo)}} \quad (8)$$

The Student's t-test was used to examine gender difference in metabolic kinetics of letrozole in rat liver microsomes. The analysis of variance (ANOVA) module on SAS system for Windows Version 8 (SAS Institute Inc. Cary, NC, USA) was used to determine the statistical differences between metabolic kinetics of letrozole in female rat liver microsomes when incubated with or without TAM. A p-value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Confirmation of the structure of CGP44 645

A major new peak was detected after incubation of letrozole with rat liver microsomes compared with the control incubation, in which the microsomes were inactivated by heat treatment [20], and the new peak had the same retention time as the synthesized metabolite, CGP 44 645 (Fig. 3). The UV spectrum of the formed metabolite showed the same pattern as that of the synthesized standard with maximum absorbance wavelength (λ_{max}) at 246 nm (Fig. 4).

The mass spectra of the synthesized metabolite and that of the formed metabolite in incubation samples displayed a similar pattern. The m/z of the base peak in each spectrum was 269, representing the molecular weight of CGP44 645 (234) plus the molecular weight of formic acid (46) that was present in the mobile phase and minus 1 (*i.e.* m/z 234 + 46 − 1 = 269), in the negative polarity mode. Also the molecular ion (m/z 234 − 1 = 233) was also a major fragment found in both spectra (Fig. 5). The mass spectral data, along with the retention time and UV spectra demonstrated the formation of CGP44 645 in rat liver microsomal incubations.

3.2. Analytical method

The sample preparation and HPLC procedures were suitable for clean-up, separation and quantification of letrozole and its carbinol metabolite in rat liver microsomal incubations (Figs. 3 and 4). The extraction recoveries were greater than 84% over the entire concentration range examined. The method was fairly sensitive with a LOQ of 40 nM for CGP44 645. Linear calibration curves were obtained over a concentration range of 40 nM–20 μM ($y = 0.568 \times + 3.58$, $R^2 = 0.998$). The intra-day and inter-day (up to 5 days) precision, expressed as relative error, ranged from 0.6 to 2.0% (with CV% of 0.7–6.7%) and 3.5–7.2% (with CV% of 2.4–14.5%), respectively, within the entire range of the calibration curve.

3.3. Metabolic kinetics of letrozole in rat liver microsomes

3.3.1. Gender differences

The formation rate of CGP44 645 *versus* letrozole concentration curves after incubation in female and male rat liver microsomes are displayed in Fig. 6. The estimates of Michaels–Menten parameters (V_{max}, K_m) are listed in Table 1. Since the clinical dosage of letrozole is relatively low and letrozole concentrations *in vivo* are expected to be much smaller than K_m, $CL_{int\,(in\ vitro)}$, $CL_{int\,(in\ vivo)}$, CL_H and E were calculated according to Eqs. (4)–(8), respectively (Table 1). The rate of metabolism, based on values of $CL_{int\,(in\ vitro)}$, $CL_{int\,(in\ vivo)}$ and CL_H, is significantly higher in male rat liver microsomes compared to female (Table 1, $p < 0.05$), suggesting that there is a marked gender difference in the formation of CGP44 645 in rat liver microsomes. It would appear that CYP 450 enzymes, which catalyze letrozole biotransformation, are hormone-dependent.

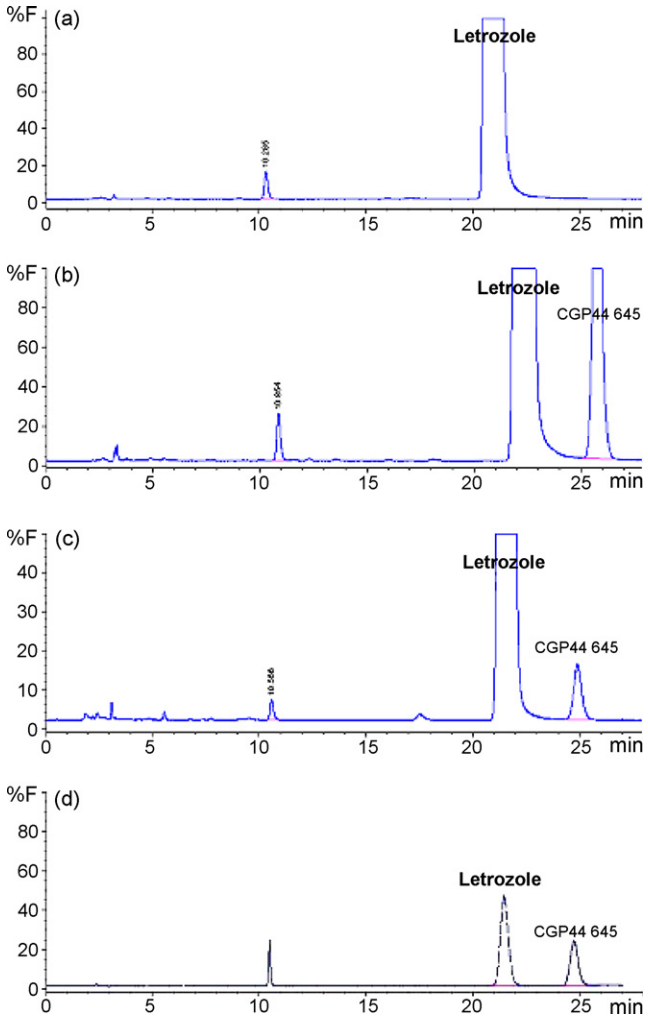

Fig. 3. Typical chromatograms of letrozole and its carbinol metabolite after incubation of 100 μM of letrozole in rat liver microsomes: (a) in denatured rat liver microsomes; (b) in female rat liver microsomes; (c) in male rat liver microsomes; (d) blank microsomal sample spiked with 250 nM of letrozole and its carbinol metabolite.

3.3.2. Effects of TAM on metabolic kinetics of letrozole in female rat liver microsomes

The formation rate of CGP44 645 *versus* letrozole concentration curves after incubation of letrozole in female rat liver microsomes in the absence or presence of TAM (20 and 250 μM) are displayed in Fig. 7. The V_{max}, K_m and other parameters,

Table 1
Gender differences in the biotransformation of letrozole in rat liver microsomes

	V_{max} (pmol/mg protein/min)	K_m (μM)	$CL_{int\ (in\ vitro)}$ (mL/mg protein/min × 10^{-3})	$CL_{int\ (in\ vivo)}$ (L/h/kg)	CL_H (L/h/kg)	Extraction ratio (%)
Male microsomes	69.6 ± 4.4	126.4 ± 14.1	0.55 ± 0.08	0.18 ± 0.03	0.17 ± 0.03	4.2 ± 0.7
Female microsomes	1.8 ± 0.3	48.8 ± 3.0	0.036 ± 0.003	0.012 ± 0.001	0.011 ± 0.001	0.3 ± 0.03
p-Value	0.001*	0.01*	0.008*	0.01*	0.01*	0.01*

* Significant difference based on two-tail *t*-test (p-value < 0.05).

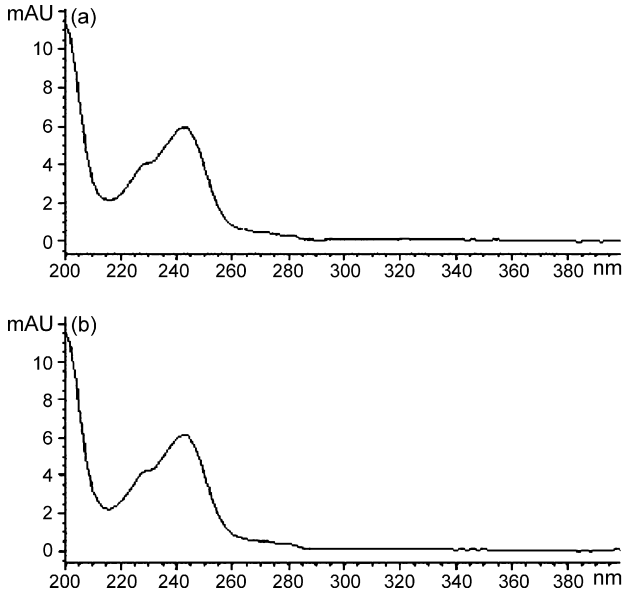

Fig. 4. UV spectra of CGP44 645 collected by diode array detector: (a) UV spectrum of standard and (b) UV spectrum of the formed peak in rat liver microsomal incubations.

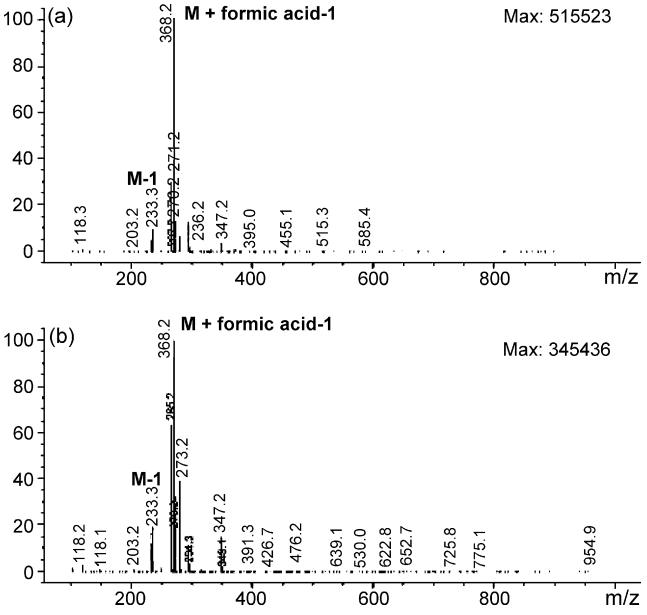

Fig. 5. Mass spectra of CGP44 645 after LC/MS analysis: (a) MS of standard and (b) MS of the formed peak in rat liver microsomal incubations.

such as $CL_{int\ (in\ vitro)}$, $CL_{int\ (in\ vivo)}$ and CL_H, which were calculated according to Eqs. (4), (5) and (7), respectively, are listed in Table 2. The metabolic parameters were not significantly altered in the presence of TAM in female rat liver microsomes (Table 2, $p > 0.05$), suggesting that TAM did not significantly inhibit the biotransformation of letrozole to CGP44 645 in female rats.

3.3.3. *Effects of chronic pretreatment of female rats with tamoxifen on letrozole metabolism in hepatic microsomes*

The protein contents in female rat liver microsomes after pretreatment of rats with TAM for 7 consecutive days are displayed in Fig. 8. The protein contents were significantly increased after pre-administration of TAM at 0.5 and 1.0 mg/kg/day (p-value < 0.05) compared with the control group, in which the rats were given the vehicle alone. However, the microsomal protein content did not increase further after pretreatment of rats with TAM (5.0 mg/kg/day) for 7 days. In fact, it decreased to some extent, although this was not statistically significant.

The formation rate of CGP44 645 *versus* letrozole concentration curves after incubation of letrozole in female rat liver microsomes prepared from rats pretreated with TAM (0.5, 1.0 and 5.0 mg/kg/day) for 7 consecutive days are displayed in Fig. 9. The V_{max}, K_m and $CL_{int\ (in\ vitro)}$, which were calculated according to Eq. (4), are listed in Table 3. Although the protein contents after pretreatment with TAM (0.5 and 1.0 mg/kg/day) were significantly increased, the formation rates of CGP44 645, when normalized for protein content, did not significantly increase (Fig. 9). Also, the parameters, including V_{max}, K_m and the *in vitro* intrinsic clearance were not significantly altered

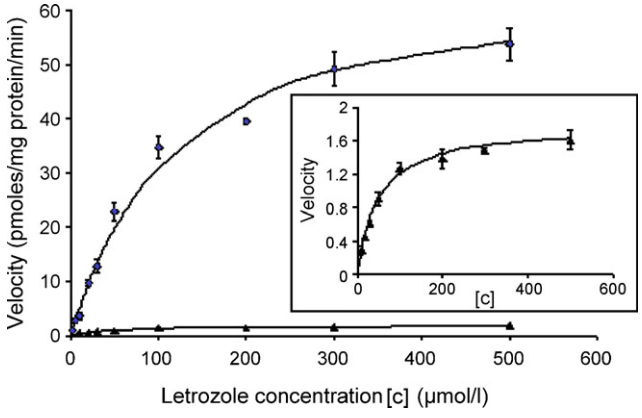

Fig. 6. The effect of gender on the microsomal catalyzed metabolism of letrozole to its carbinol metabolite in rat liver microsomes: (♦) incubated in male rat liver microsomes; (▲) incubated in female rat liver microsomes. Values represent means ± S.D. from three incubations.

Table 2
Effects of tamoxifen (TAM) on the metabolic kinetics of letrozole to its carbinol metabolite in female rat liver microsomes

	V_{max} (pmol/mg protein/min)	K_m ((M)	$CL_{int\ (in\ vitro)}$ (mL/mg protein/min × 10^{-3})	$CL_{int\ (in\ vivo)}$ (L/h/kg)	CL_H (L/h/kg)
Without TAM	1.8 ± 0.3	48.8 ± 3.0	0.036 ± 0.003	0.012 ± 0.001	0.011 ± 0.001
With 25 μM TAM	1.9 ± 0.4	59.9 ± 6.4	0.032 ± 0.005	0.010 ± 0.002	0.011 ± 0.002
With 250 μM TAM	1.8 ± 0.3	57.3 ± 4.6	0.032 ± 0.004	0.010 ± 0.002	0.010 ± 0.002
p-Value	0.89	0.09	0.43	0.33	0.73

p-value for comparison of metabolic kinetic parameters of letrozole using ANOVA.

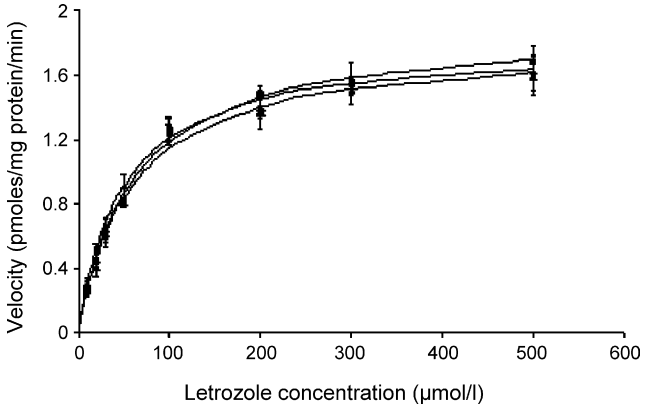

Fig. 7. The effect of tamoxifen (TAM) on the microsomal catalyzed metabolism of letrozole to its carbinol metabolite in female rat liver microsomes: (♦) incubation without TAM; (■) incubation with 25 μM TAM; (▲) incubation with 250 μM TAM. Values represent means ± S.D. from three incubations.

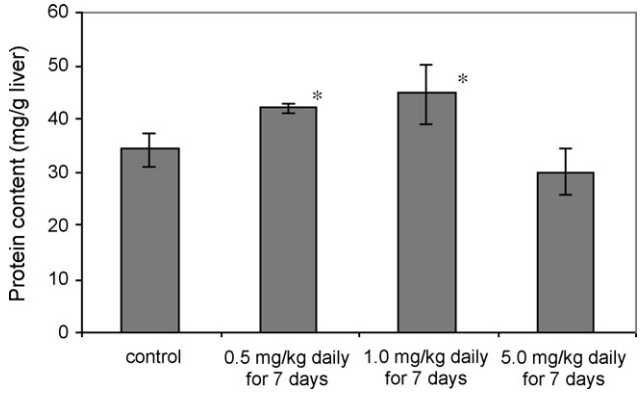

Fig. 8. The effects of chronic pretreatment of female rats with tamoxifen on protein content in female rat liver microsomes. * Significant difference compared with control group using *t*-test ($p < 0.05$).

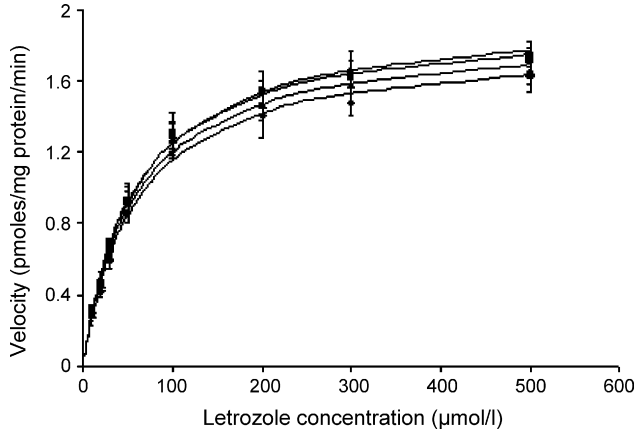

Fig. 9. The effect of chronic pretreatment of female rats with tamoxifen (TAM) on the microsomal catalyzed metabolism of letrozole to its carbinol metabolite in female rat liver microsomes: (♦) pretreatment with vehicle; (▲) pretreatment with 0.5 mg/kg/day of TAM; (●) pretreatment with 1.0 mg/kg/day of TAM; (■) pretreatment with 5.0 mg/kg/day of TAM. Values represent means ± S.D. from three incubations.

(Table 3, $p > 0.05$) by TAM pretreatment. These results suggest that although TAM tends to stimulate the production of some biotransformation enzymes, the metabolic activity of enzymes associated with the formation of CGP44 645 did not change significantly.

4. Discussion

The evaluation of drug metabolism kinetics in hepatic microsomes and prediction of intrinsic clearance of drugs based on biotransformation data obtained *in vitro* has important implications in the study of drug–drug interactions in animals. We used rat liver microsomes to evaluate the *in vitro* metabolic kinetics of letrozole in an attempt to predict the intrinsic clearance of letrozole, and examine the effects of gender and TAM on the

Table 3
Effects of chronic pretreatment of female rats with tamoxifen (TAM) on the metabolic kinetics of letrozole to its carbinol metabolite in female rat liver microsomes

	V_{max} (pmol/mg protein/min)	K_m (μM)	$CL_{int\ (in\ vitro)}$ (mL/mg protein/min × 10^{-3})
Pretreatment with vehicle	1.8 ± 0.1	57.7 ± 5.9	0.032 ± 0.003
0.5 mg/kg/day of TAM	1.9 ± 0.2	56.6 ± 3.7	0.033 ± 0.003
1.0 mg/kg/day of TAM	2.0 ± 0.3	58.2 ± 2.7	0.034 ± 0.004
5.0 mg/kg/day of TAM	1.9 ± 0.2	55.2 ± 3.4	0.035 ± 0.004
p-Value	0.73	0.81	0.32

p-Value for comparison of metabolic kinetic parameters of letrozole using ANOVA.

biotransformation of letrozole in rats. Although two unidentified metabolites of letrozole have been reported in humans [21], CGP44 645 was the only major metabolite detected in rats in our study. Therefore, the formation rate of CGP44 645 was used to investigate the biotransformation kinetics of letrozole in rat liver microsomes.

We observed marked gender differences in the microsomal catalyzed metabolic kinetics of letrozole in rats. The ratio of V_{max}/K_m of letrozole was significantly higher in male rat liver microsomes compared to female. The *in vivo* intrinsic clearance and hepatic clearance predicted from *in vitro* microsomal incubation were also significantly higher in male rats than in females. These results suggesting that the hepatic clearance of letrozole is significantly higher in intact male rats compared to females is supported by a previous *in vivo* study [22]. The low predicted *E* values for letrozole in rats are consistent with the characterization of letrozole as a low extraction ratio drug and suggest that first-pass metabolism of letrozole will not be extensive when given orally [23]. Also, the relatively small values for $CL_{int\ (in\ vivo)}$ and CL_H obtained from our microsomal studies indicate slow *in vivo* clearance and long elimination half-life for letrozole in female rats [22,23]. Incidentally, we also observed that the predicted CL_H (2.8 mL/min/kg in male rats and 0.2 mL/min/kg in female rats) was much smaller than hepatic blood flow (65 mL/min/kg). That is consistent with the argument that letrozole is cleared slowly in rat livers.

We also observed that acute treatment of female rat liver microsomes with TAM did not significantly alter the metabolic kinetics of letrozole, suggesting that TAM has negligible inhibitory effect on the CYP mediated intrinsic clearance of letrozole to its carbinol metabolite. However, it would appear that chronic exposure of the body to TAM stimulates the production of some biotransformation enzymes, which may be associated with letrozole metabolism. In fact, it has been known that CYP3A4 metabolized letrozole to the carbinol metabolite while CYP2A6 formed both the carbinol metabolite and its ketone analog in human liver microsomes, but the contribution of each isoenzyme in each metabolic pathway is still unknown [13]. Some investigators have also reported that TAM induces CYP2B1 and CYP3A1 in rats [16]. Although further work on induction of CYPs by TAM needs to be done, it is quite possible that enzymes homologous to human CYP3A family (CYP3A4) are induced by TAM. The lower microsomal protein content after pretreatment of rats with 5.0 mg/kg/day of TAM may be due to liver impairment resulting from a high dose of TAM for a relatively long period of time. This contention is supported by a 10–15% decrease in the body weight of rats after administration of 5 mg/kg/day of TAM for 7 consecutive days that was observed in these studies.

Although the formation rates of CGP44 645, when normalized for protein content, did not significantly increase after chronic pretreatment with TAM, it is still possible that other metabolic pathways of letrozole are altered by prior administration of TAM, since two unidentified metabolites of letrozole have been reported [21] although the contribution of different isoenzyme in each metabolic pathway is still unknown [13]. Chronic pretreatment of rats with TAM is expected to increase the hepatic clearance of letrozole *in vivo* since $CL_{int\ (in\ vivo)}$ and CL_H are increased with increasing microsomal protein content. An investigation of the pharmacokinetics of letrozole in female rats has been conducted to verify the conclusions reached from our *in vitro* data [23].

In conclusion, the present study demonstrates that there is a marked gender difference in the hepatic metabolism of letrozole in rats. Based on our microsomal studies, letrozole is considered a low extraction drug in rats. It also appears that acute treatment of female rat liver microsomes with TAM has a negligible inhibitory effect on CYP mediated clearance of letrozole. However, chronic pretreatment of female rats with TAM appears to induce microsomal CYPs, but does not significantly alter the conversion of letrozole to its carbinol metabolite.

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