Rat and Human Liver Cytochrome P-450 Isoform Metabolism of Ecteinascidin 743 Does Not Predict Gender-dependent Toxicity in Humans[1]

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ABSTRACT

Ecteinascidin 743 (ET743, NSC648766) is a marine natural product with potent *in vivo* activity in human xenograft models. Hepatotoxicity was the most prominent toxicity in preclinical studies and was greater in female rats than in male rats. To assess the potential implications for human toxicities, the *in vitro* metabolism of ET743 was characterized using rat and human preparations. NADPH-dependent ET743 metabolism was greater with male rat liver microsomal preparations than with preparations from female rats and was induced by pretreatment of rats with phenobarbital and dexamethasone but not by pretreatment with 3-methylcholanthrene. Rat and human microsomal metabolism of ET743 was reduced in the presence of chemical CYP3A inhibitors or antirat CYP3A2 antiserum and to a much lesser extent by CYP2E, CYP2C, and CYP2A inhibitors. In human liver panel studies, ET743 disappearance was highly correlated with CYP3A activities and to a lesser extent with CYP2C activities. ET743 was metabolized by a number of cDNA-expressed rat P-450 isoforms, including male-predominant CYP2A2 and CYP3A2. ET743 was metabolized by cDNA-expressed human CYP3A4 and to a much lesser extent by CYP2C9, CYP2D6, and CYP2E1 preparations. Three oxidative metabolites were detected in cDNA-expressed isoform incubations, including the *N*-demethylated metabolite ET729 and two additional products characterized by laser capture-mass spectrometry analyses. The plasma pharmacokinetics and biliary excretion of ET743 were characterized in rats. There were no gender-dependent differences in half-life or total body clearance values. Although very modest, the biliary excretion of ET743 in male rats (0.48%) was greater than in female rats (0.28%). In contrast, the biliary excretion of the cytotoxic *N*-demethylated metabolite ET729 was 5-fold greater in the female rat (1.05% of dose) than in the male rat (0.19% of dose). Biliary excretion of ET729 may contribute to the hepatic toxicity in rats. These data are consistent with a major role for CYP3A isoforms in ET743 rat and human metabolism. Although there are conflicting data in the literature, expression of CYP3A isoforms in human tissues and elimination of CYP3A substrates have not been shown to vary substantially by gender. There are no indications that the other CYP isoforms implicated in ET743 metabolism are expressed differently in males and females. Thus, although it is not possible to rule out gender differences in ET743 human toxicities, our data do not predict major gender-dependent differences in the toxicity of ET743 based on metabolism.

INTRODUCTION

ETs[3] are marine natural products with potent cytotoxic activity isolated from extracts of the tunicate *Ecteinascidia turbinata* (1, 2). Structurally related to the safracin class of antitumor antibiotics, ETs form covalent adducts with the N2 amino group of guanine in the minor groove of DNA (3, 4). ET729 (NSC638718; Fig. 1) was the first of this potent series of molecules to complete preclinical investigations (5), but unacceptable toxicity in rodents and dogs (6) and limited availability of this agent led to investigation of other analogues. ET743 (NSC648766; Fig. 1) was selected for further study based on potent *in vivo* activity against human breast cancer xenograft models and on the availability of material (7). ET743 has a unique activity profile in the National Cancer Institute's panel of 60 human tumor cell lines that is most closely related to DNA-binding agents (8). ET743 forms DNA-protein cross-links that target transcription-coupled nucleotide excision repair (9) rather than topoisomerase I cleavage complexes agents (8). Consequences of such lesions may include inhibition of transcriptional activation by NF-Y and heat shock factor (10) and inhibition of multidrug resistance 1 activation (11).

ET743 is presently under investigation in Phase I/II clinical trials, where promising activity has been seen against soft tissue

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[3] The abbreviations used are: ET, ecteinascidin; CYP, cytochrome P-450; HPLC, high-pressure liquid chromatography; PB, phenobarbital; DEX, dexamethasone; 3-MC, 3-methylcholanthrene; AUC, area under the plasma concentration *versus* time curve; FAB, fast atom bombardment; MS, mass spectrometry; NMR, nuclear magnetic resonance.

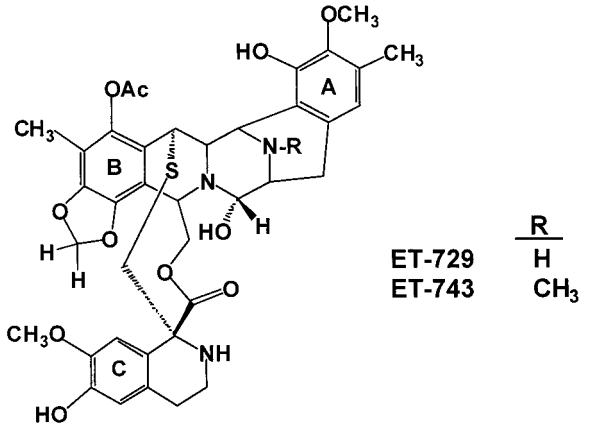

Fig. 1 Structures of ET729 and ET743.

sarcoma (12). The most prevalent toxicity observed in Phase I/II has been hepatotoxicity, defined as increases in serum transaminase enzymes, that was dose dependent, transient, and not cumulative when patients received multiple cycles (12–14).

In preclinical toxicity studies, rats were most sensitive to ET743 (15). Female Fisher 344 rats were more sensitive to ET743 than were male Fisher 344 rats, particularly with regard to hepatotoxicity consisting of multiple, irreversible hepatic and biliary lesions and to increases in plasma concentrations of liver enzymes, bilirubin, and bile acids (16). The biliary lesions consisted of inflamed bile duct epithelium, fibrosis, and an abnormal appearance consistent with cholangitis that was not observed in untreated rats. Differences in toxicity and other pharmacological responses to xenobiotics observed in male and female rats is often associated with gender-dependent expression of CYP isoforms (17). Although gender-dependent expression of P-450 isoforms and/or gender-dependent metabolism of P-450 substrate drugs have not been documented to significantly alter the pharmacology of such agents in humans, there are recent reports of modest differences in the metabolism and elimination of CYP3A substrates by males and females (reviewed in Ref. 18).

To address the potential role of CYP metabolism in gender-dependent hepatotoxicity, we characterized rat hepatic microsomal metabolism and ET743, pharmacokinetics, and biliary excretion by male and female rats. Because the rat is an important preclinical model in anticancer drug development and is often used to predict drug toxicity and disposition in humans, we also characterized P-450 metabolism of ET743 by human liver microsomes.

MATERIALS AND METHODS

Materials

ET743 was provided by the Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute. All reagents were analytical grade, and all solvents were of HPLC grade. Human liver microsomes were provided by Jerry M. Collins (Center for Drug Evaluation and Research, United States Food and Drug Administration, Rockville, MD). Human liver samples, medically unsuitable for transplantation, were acquired under the auspices of the Washington Regional Transplant Consortium (Washington, DC), and their preparation and characterization has been published previously (19). Rat liver microsomes were isolated by differential centrifugation of liver homogenates obtained from male and female Fisher 344 rats (20). For some experiments, CYPs were induced by pretreatment with DEX (300 mg/kg), phenobarbital (80 mg/kg), or 3-methylcholanthrene (20 mg/kg) i.p. for three consecutive days and sacrificed on the fourth day.

In Vitro Metabolism

Incubation Conditions. Microsomal suspensions were incubated in amber glass vials maintained at 37°C in a shaker bath. Each incubation mixture (0.1–0.5 ml) contained 50 μM ET743, human or rat liver microsomes (1 mg/ml protein), NADP+ (0.4 mM), glucose 6-phosphate (25 mM), glucose 6-phosphate dehydrogenase (0.7 units/ml), magnesium chloride (5 mM), and potassium phosphate (100 mM) buffer adjusted to pH 7.4. The following incubations served as controls: incubations with active microsomes in which $NADP^+$ was omitted or the atmosphere was replaced with nitrogen or a carbon monoxide:oxygen (80:20) mixture and incubations with heat-inactivated microsomes. In selected experiments, CYP-selective chemical inhibitors [coumarin, quinidine, erythromycin, quercitin, chlorzoxazone, phenacetin, and sulfenazole dissolved in methanol, and α-naphthoflavone dissolved in DMSO] (20 or 200 μM) were incubated with ET743 (50 μM) for 60 min in reaction buffer containing NADP+ (1.0 mM), glucose 6-phosphate (10 mM), and glucose 6-phosphate dehydrogenase (1.0 units/ml). Control incubations of DMSO or methanol were also run. The incubation mixtures were preincubated for 2 min before the initiation of the reaction upon the addition of ET743. Reactions were terminated by the addition of two volumes ice-cold methanol. The aqueous methanol supernatants obtained after centrifugation (10,000 × *g* for 2 min) were diluted 1:1 (v/v) with acidified 10 mM ammonium acetate buffer (prepared by mixing 200 μl of 10% phosphoric acid with 20 ml of 10 mM ammonium acetate, pH 5) and analyzed by HPLC.

Correlation of ET743 Metabolism with Marker Activities of Selected CYP Enzymes. Microsomal fractions from 10 individual human livers were obtained from Human Biologics, Inc. (Phoenix, AZ). Microsomal suspensions were prepared in the reaction buffer to achieve a final protein concentration of 1 mg/ml and incubated with ET743 (50 μM) for a 60-min reaction period as described above.

Metabolism by cDNA Expressed Human CYP Enzymes. Microsomal suspensions from the human B-lymphoblastoid cell line AHH-1 TK+/− expressing cDNA constructs for CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2E1, and CYP3A4 were obtained from Gentest Corporation (Woburn, MA). Microsomes from cells without the cDNA construct and from cells without the vector were used as controls. The microsomes were added to the reaction buffer to achieve a final concentration of 1 mg/ml and incubated with ET743 (50 μM) for a 30-min reaction period as described above.

Immunoinhibition by Antiserum. Antirat CYP3A2 antiserum (Gentest Corp.) was preincubated with rat and human liver microsomes at room temperature (on ice for cDNA expressed CYP3A4) for 30 min before adding reaction buffer and

ET743 (50 μM). Incubations were carried out for 30 min as described above.

Immunoblotting

Microsomal fractions (1–5 μg protein) were resolved on 8% polyacrylamide gels as described by Laemmli (21). Electrophoresis was carried out at 40 mA for 3–3.5 h. The proteins were then transferred to polyvinylidene difluoride membrane (0.45-μm pore size; Millipore, Bedford, MA) according to Towbin *et al.* (22) applying 90 V for 2 h. After blocking overnight with 7.5% milk in TBS-T (10 mM Tris, 137 mM NaCl, and 0.1% Tween 20, pH 7.6), the membranes were incubated for 2 h with primary antibodies diluted with 7.5% milk in TBS-T (polyclonal goat antirat-CYP1A1, antirat-CYP2B1, antirat-CYP2C11, and antirat-CYP3A2; Gentest), rabbit antirat-CYP 3A1, antirat-CYP3A4, or sheep antirat-CYP1A2 (Chemicon International, Inc., Temecula, CA). Membranes were washed in TBS-T for 1 h (2 × 15 min, 3 × 10 min) and then incubated in the appropriate secondary antibody (diluted with 3% milk in TBS-T):goat antirabbit IgG, rabbit antisheep IgG (Chemicon International, Inc.), or rabbit antigoat IgG (Sigma Chemical Co., St. Louis, MO) for 1 h. Polyvinylidene difluoride membranes were washed as before and then incubated for 2–3 min with Supersignal chemiluminescence (Pierce, Rockford, IL) diluted 1:2 with double-distilled water. Membranes were then exposed to Hyperfilm ECL (Amersham, Arlington Heights, IL).

Pharmacokinetics

ET743 (0.288 mg/ml) was dissolved in a 5% dextrose solution containing 50 mM monobasic potassium phosphate (pH 4), and 500 μg/kg was administered i.v. to male and female Fisher 344 rats (jugular or tail vein) using a 1-ml tuberculin syringe fitted with a 27-gauge needle. Rats were anesthetized under ether vapors at specified times. Blood samples were collected from the retroorbital sinus using heparinized capillary tubes and transferred to silanized microcentrifuge tubes containing 15 μl of heparin solution (1,000 units/ml). Plasma was separated by centrifugation (10,000 rpm × 3 min), transferred to microcentrifuge tubes, and immediately frozen. Plasma samples were prepared for analysis by protein precipitation with methanol.

Biliary Excretion of ET743

Male (340–400 g) and female (180–210 g) Fisher 344 rats were administered drug (250 or 500 μg/kg) dissolved in 5% dextrose solution containing 50 mM monobasic potassium phosphate, pH 4 (0.288 mg/ml ET743) over 30 s into the femoral vein via an Injection Site septum (Baxter) attached to PE-10 polyethylene tubing. After drug administration, the line was rinsed with 0.5 ml of NaCl solution. Blood samples (0.3–0.5 ml) were removed from the opposite femoral vein 4, 6, 12, 30, 60, and 120 min after drug administration. Heparin (1,000 units/ml) was added to blood (10 μl/0.1 ml of whole blood), and samples were placed on ice until the completion of the experiment. Plasma was separated by centrifugation (10,000 rpm × 3 min) transferred to silanized microcentrifuge tubes and frozen immediately. Approximately 2–3 ml of whole blood were removed from each rat.

Rats were prepared with bile fistulae (23). Bile ducts were cannulated with PE-10 polyethylene tubing. The proximal end of tubing was tied below the hepatic duct bifurcation and above the entrance of the pancreatic ducts. Bile was collected every 10 min into preweighed silanized microcentrifuge tubes. The volumes of collected samples were determined by weight assuming a bile density of 1.0 g/ml. Bile was placed on ice until the end of the experiment and was frozen for later analysis. Before chromatography, the bile was diluted 1:1 with acetonitrile and chromatographed via the gradient HPLC method.

HPLC Assays

***In Vitro* Metabolism.** ET743 and its metabolites were separated on a Whatman Partisil 10 ODS3 (250 mm × 4.6 mm inside diameter) analytical column eluted with a mobile phase consisting of methanol:10 mM ammonium acetate buffer, pH 5 (60:40). The column temperature was maintained at 40°C. The flow rate, injection volume, and detector wavelength were 1 ml/min, 50 μl, and 254 nm, respectively.

Pharmacokinetics. Separations were achieved on an Inertsil C_{18} column with gradient elution from solvent A (acetonitrile:50 mM monobasic potassium phosphate, pH 4, 20:80, v/v) to solvent B (acetonitrile:50 mM monobasic potassium phosphate, pH 4.0, 55:45, v/v) over a 7-min period after elution with solvent A for 3 min. The flow rate, detection wavelength, and column temperature were 0.7 ml/min, 210 nm, and 40°C, respectively. Plasma samples were prepared for HPLC analysis by solid phase extraction using a modification of the method of Rosing *et al.* (24). Bond Elut CN solid phase extraction columns were preconditioned with 2 ml of methanol and 2 ml of water. The rat plasma samples (500 μl) were diluted with an equal volume of 0.2 M ammonium acetate, pH 5.0. The sample was loaded, rinsed with 1 ml of 0.1 M ammonium acetate (pH 5) and 1 ml of methanol:water (10:90, v/v), and eluted with 1 ml of 0.1 N hydrochloric acid in methanol. The eluate was evaporated to dryness under nitrogen and reconstituted in 100 μl of mobile phase (20:80 acetonitrile:50 mM monobasic potassium phosphate, pH 4.0). The lower limit of sensitivity and linear range of this HPLC method were 10 ng/ml and 10–200 ng/ml, respectively.

The HPLC assays met published quality assurance criteria for between-day accuracy and precision (25). SDs of the back-calculated standards and the slope of the standard curves were <4 and $<15\%$ for the *in vitro* metabolism and pharmacokinetics assays, respectively.

Structure Identification

^{1}H-NMR spectra of M1 and M2 were recorded on 750 MHz UNITY INOVA 750 NMR and 500 MHz General Electric GN-500 spectrometers, respectively. Both spectra were referenced to the residual solvent resonances of deuterated methanol (3.34 ppm). High-resolution fast atom bombardment mass spectra (HRFABMS and FABMS/MS) were recorded on a VG ZAB-SE mass spectrometer using DTT-dithioerythritol as the matrix. Electrospray ionization mass spectra (ESIMS and ESIMS/MS) were acquired on a VG Quattro mass spectrometer.

Table 1 Comparison of P-450 content and enzyme activity in male and female rat and human liver microsomes

	Female	Male
Rat livers	$n = 4$	$n = 4$
ET743 disappearance[a]	472 ± 40	658 ± 86[b]
Human livers[c]	$n = 6$	$n = 4$
ET743 disappearance[a]	350 ± 220	220 ± 120
Testosterone 6β-hydroxylation[a] (CYP3A)	4960 ± 5040	2800 ± 2460
Tolbutamide hydroxylation[a] (CYP2C)	172 ± 66	136 ± 35

[a] pmol/mg protein/30 min.
[b] $P = 0.0078$.
[c] Cytochrome P-450 concentrations were similar for male and female preparations (0.33–0.35 nmol/mg protein).

Analysis of ET743 Plasma Concentration-Time Data

Data were fitted by nonlinear least squares regression to a two-compartmental open model using the program WINNONLIN (Version 1.5; Statistical Consultants, Lexington, KY). AUCs were determined by linear trapezoidal approximation.

RESULTS

CYP-dependent Metabolism of ET743. NADPH-dependent metabolism was observed when ET743 was incubated with rat and human liver microsomes as summarized in Table 1. ET743 disappearance was almost completely abolished (>85%) by an atmosphere of nitrogen, by a mixture of $CO:O_2$ (80:20), and by heat inactivation of microsomal protein. The modest ET743 disappearance observed in the absence of NADPH (not shown) most likely was attributable to adsorption to incubation vials. ET743 metabolism by male rat liver microsomes was significantly greater ($P = 0.0078$) than by female microsomes (Table 1). In studies with a panel of the human liver microsomal preparations, 6 female livers metabolized ET743 more extensively than did 4 male livers; however, the differences were not statistically significant (Table 1).

ET743 metabolism in male rat liver microsomes was increased by 3-fold after pretreatment with PB and DEX, whereas 3-MC increased ET743 metabolism by <0.5-fold. ET743 metabolism by female rat liver microsomes was increased 2.4-fold by DEX, increased 1.5-fold by PB, and reduced 0.35-fold by 3-MC (Fig. 2*A*). Increased ET743 metabolism after pretreatment of animals with PB and DEX was associated with increased expression of CYP3A1 and CYP3A2 isozymes in those same preparations (Fig. 2*B*). CYP2B1 expression was also increased after PB but not after DEX treatment (Fig. 2*B*). Although 3-MC treatment substantially increased CYP1A1 expression (Fig. 2*B*), no increase in ET743 metabolism nor in CYP3A expression was observed in those microsomes. CYP2C11 is a male-specific rat isoform known to metabolize a number of drugs (26). Treatment of rats with 3-MC, PB, and DEX had no effect on expression of this CYP2C11 isoform (Fig. 2*B*).

Among a series of CYP-selective inhibitors, the greatest inhibition of ET743 metabolism by male and female rat and human liver microsomes was achieved with α-naphthoflavone and quercitin, inhibitors of both CYP2C and CYP3A (Fig. 3). Little inhibition of ET743 metabolism was observed in incubations containing chlorzoxazone (CYP2E) and coumarin (CYP2A; Fig. 3). Consistent with cDNA and inhibitor studies, antirat CYP3A2 antiserum reduced ET743 oxidation by rat liver microsomal preparations 50–90%. Similarly, antirat CYP3A2 antiserum (which is cross-reactive with human CYP3A4) also reduced ET743 metabolism in human liver microsome incubations 60–90%.

Correlation Analysis of Individual Variability in Human Liver Microsomal CYP Activities with ET743 Oxidation. When ET743 metabolism by a panel of human liver microsomes screened for selective P-450 marker activities was compared with marker P-450 isoform activities in those same lines, the highest correlation was with the CYP3A marker activity testosterone 6β-hydroxylation ($r = 0.98$) and the CYP2C marker activity tolbutamide methyl-hydroxylation ($r = 0.80$; Table 2). There were no significant correlations with other CYP marker activities. In this panel, testosterone 6β-hydroxylation (CYP3A) and tolbutamide methyl-hydroxylation (CYP2C) were greater in the 6 female livers than in the 4 male livers, but those differences were not statistically significant (Table 1), consistent with the previously noted differences in ET743 metabolism (Table 2).

ET743 Metabolism by cDNA-expressed Rat and Human P-450 Isoforms. When ET743 was incubated with cDNA-expressed rat P-450 isoform preparations, male-predominant CYP2A2 and CYP3A2 had the greatest activity for ET743 metabolism. Substantial ET743 metabolism was also observed for other rat CYP2 isoforms (CYP2C6, CYP2D1, and CYP2E1) with much less metabolism by CYP2C13, CYP1A1, and CYP1A2 (Table 3). On the basis of CYP isoform concentrations, ET743 metabolism by human recombinant isoforms was substantially less than found with rat isoforms. CYP3A4 had the greatest activity for ET743 metabolism. Metabolism of ET743 was also observed with CYP2C9, CYP2D6, and CYP2E1 (Table 3).

Identification of ET743 Metabolites. HPLC analysis with UV absorbance detection at 254 nm revealed accumulation of three ET743 metabolites, designated as M1, M2, and ET729, in incubations with rat and human liver microsomes. M1 and M2 were detected in all recombinant P-450 preparations that metabolized ET743 as summarized in Table 3. A representative chromatogram for an incubation of ET743 with CYP3A4 is illustrated in Fig. 4. ET729 was only detected in incubation with human cDNA-expressed CYP3A4 and CYP2E1 microsomes. Occasionally, trace amounts of M1 were detected in preparations absent the cofactor NADPH.

Comparison of ^{1}H-NMR (750 MHz) resonances of M1 (Fig. 5*A*) with those of ET743 and relevant model compounds indicated that M1 was a metabolite containing the aromatic unit B of ET743 (as in Fig. 1) connected to a vinyl-formamide unit and a methyl ketone. Resonances at δ 2.04, 2.28, and 6.09 were almost identical to those reported for $-CH_3$ (δ 2.03), $-OCOCH_3$ (δ 2.29), and the dioxymethylene protons (δ 6.11 and 6.01) in ET743, respectively (2). In addition, the ^{1}H-NMR spectrum revealed the partial structure –CH = CH-NHCHO (trans, δ 7.09, d, 1H, $J = 15$ Hz; δ 6.19, d, 1H, $J = 15$ Hz; δ 8.04, s, 1H) and the presence of an additional methyl group (δ 2.52, s, 3H). The chemical shift of this methyl group matched well with that of acetophenone (δ 2.55; Ref. 27). It is interesting to note that the

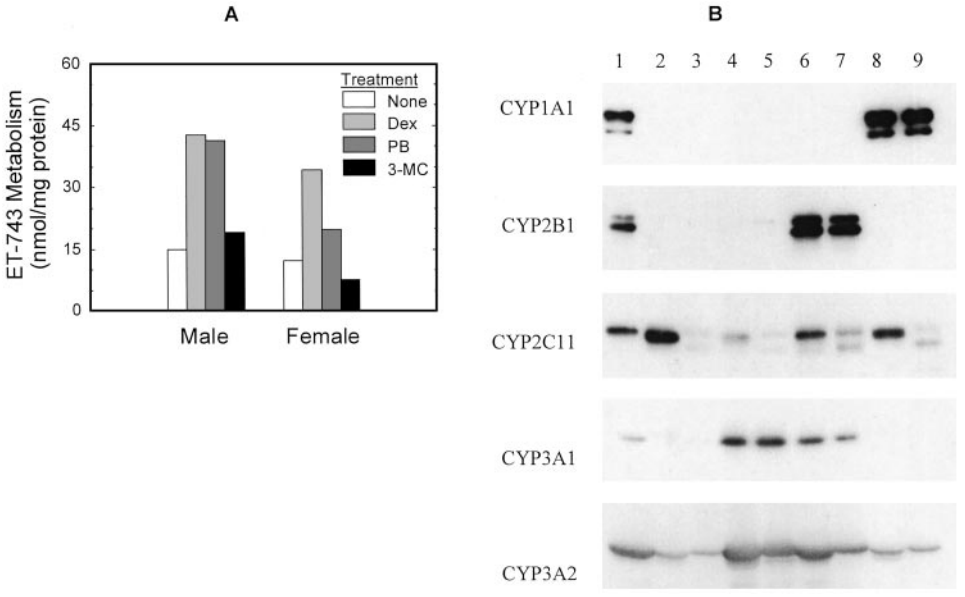

Fig. 2 *A,* NADPH-dependent metabolism of ET743 during 30-min incubations with microsomal suspensions from male or female rat livers untreated or treated with P-450 inducers. Each *column* represents the average of two experiments. *B,* Western blots of P-450s expressed in liver microsomes from male rats (*Lanes 2*, *4*, *6*, and *8*) and female rats (*Lanes 3*, *5*, *7*, and *9*) following no treatment (*Lanes 2* and *3*), DEX (*Lanes 4* and *5*), PB (*Lanes 6* and *7*), and 3-MC (*Lanes 8* and *9*). The positive control for each P-450 protein is illustrated in *Lane 1*.

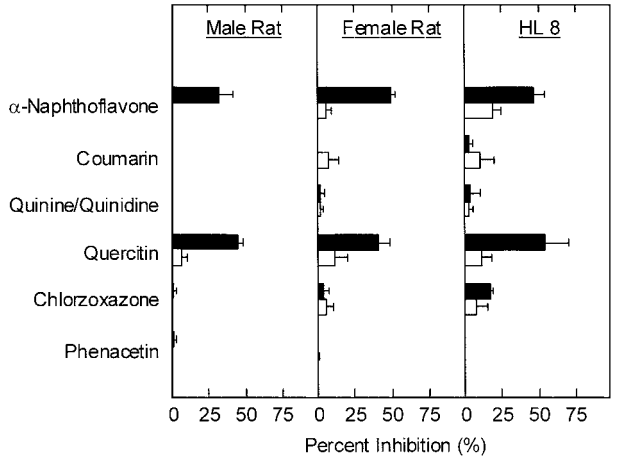

Fig. 3 Chemical inhibition of ET743 metabolism in male rat, female rat, and human liver microsomal suspensions. The percentage of inhibition of ET743 metabolism was determined by comparison of the NADPH-dependent ET743 loss in incubations containing vehicle only (methanol or DMSO) with the NADPH-dependent ET743 loss in incubations containing 20 (□) or 200 μM (■) inhibitor ($n = 3$).

Table 2 Correlation coefficients for the relationship between the extent of ET743 metabolism and marker activities for selected P-450 forms in a panel of 10 human liver microsomal preparations

Marker activity	CYP subfamily	Correlation coefficient[a]
Ethoxyresorufin 7-*O*-dealkylation	1A	0.143
Caffeine N3 demethylation	1A	0.214
Coumarin 7-hydroxylation	2A	0.189
Tolbutamide methyl-hydroxylation	2C	0.802
S-Mephenytoin 4′-hydroxylation	2C	−0.017
Dextromethorphan *O*-demethylation	2D	−0.006
Chlorzoxazone 6-hydroxylation	2E	0.544
Testosterone 6β-hydroxylation	3A	0.975
Lauric acid 12-hydroxylation	4A	0.531

[a] Mean of three experiments.

^{1}H-NMR spectrum of M1 consisted of two sets of resonances (4:1) because of rotational conformers around the –NH-CHO bond (28). High resolution FAB/MS analysis of HPLC-purified M1 yielded a MH^+ peak at *m/z* 306.0977 consistent with the M1 molecular formula $C_{15}H_{16}NO_6$. Tandem MS/MS analysis on parent ion (*m/z* 306, MH^+) yielded major ions at *m/z* 288 (H_2O loss), 246 (O-acetyl loss), 219 (loss of formamide and -$COCH_3$), and 43 (formamide fragment; data not shown).

Comparison of key ^{1}H-NMR (500 MHz) resonances of M2 (Fig. 5*B*) with those of ET 743 indicated that this metabolite was an *N*-methylisoquinolinium derivative. Singlets at δ 9.2 (H-1) and 4.2 (N-CH_3) and doublets at δ 7.8 (H-3, $J = 5$ Hz) and 7.7 (H-4, $J = 5$ Hz) readily confirmed the identity of the *N*-methylisoquinolinium skeleton (29). As in ET743, M2 showed resonances corresponding to an isolated aromatic proton (δ 6.8, s, H-5), a methoxy group (δ 3.9, s, 7-OCH_3), and an aromatic methyl group (δ 2.4, s, 6-CH_3). The chemical shifts of those signals were almost identical to those reported for H-15 (δ 6.6), 17-O-CH3 (δ 3.7), and 16-CH_3 (δ 2.3) in ET743 (2). High-resolution FAB/MS analysis of M2 yielded a MH^+ at *m/z* 204.1024, consistent with the M2 molecular formula $C_{12}H_{14}NO_2$. Tandem MS/MS analysis on parent ion (*m/z* 204, MH^+) yielded major ions at *m/z* 189 and 161, corresponding to loss of the quaternary nitrogen methyl moiety and ring cleavage loss of CHN^+-CH_3, respectively (data not shown). The site of initial CYP-catalyzed oxidation leading to formation of M1 and M2 is not obvious.

The third metabolite was tentatively identified as ET729 based on an identical retention time of the peak in incubation mixtures with that of authentic ET729 when each was monitored by UV absorbance detection at 254 nm. In addition, electrospray ionization mass spectrometry of the HPLC effluent detected a

Table 3 Metabolism of ET743 by human and rat CYPs expressed in B-lymphocyte[a] or insect cell lines[b] after 30-min incubation

CYP cDNA	ET743 metabolism (pmol/pmol P-450/30 min)
Rat	
1A1[b]	120 ± 17
1A2[b]	593 ± 63
2A1[a]	0
2A2[b]	2534 ± 31
2B1[b]	0
2C6[b]	1018 ± 46
2C13[b]	86 ± 36
2D1[b]	1600 ± 158
2E1[a]	1165 ± 227
3A1[b]	431 ± 50
3A2[b]	2143 ± 357
Human	
1A1[a]	0
1A2[a]	0
1B1[a]	0
2A6[a]	0
2B6[a]	0
2C8[a]	0
2C9[a]	107 ± 21
2C19[a]	0
2D6[a]	14 ± 9
2E1[a]	46 ± 12
3A4[a]	179 ± 10

[a] Expressed in B-lymphocyte cell lines.
[b] Expressed in insect cell lines.

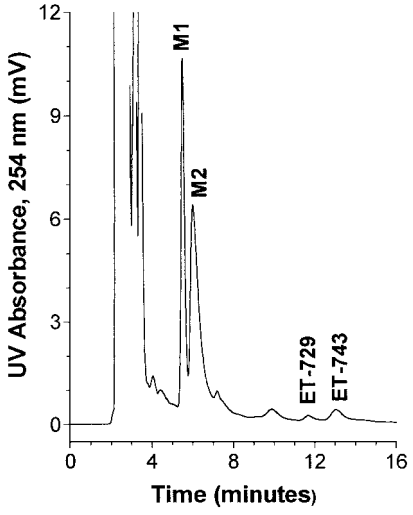

Fig. 4 Reverse-phase HPLC chromatograms of ET743 metabolites after incubations with cDNA-expressed CYP3A4. Chromatographic conditions and sample work-up are described in "Materials and Methods." UV absorbance was monitored at 254 nm. Retention times of M1, M2, ET729, and ET743 were 5.4, 6.3, 11.8, and 13.1 min, respectively.

peak with *m/z* 730 (M + H -H_2O) as with authentic standard (data not shown). The daughter ion spectra obtained by HPLC/MS/MS analysis were also identical for metabolite and authentic material (data not shown).

Pharmacokinetics and Biliary Excretion of ET743 in Male and Female Rats. ET743 pharmacokinetics were characterized in male and female Fisher 344 rats to determine whether gender differences in microsomal oxidative metabolism were reflected in differences in ET743 plasma disposition. Total body clearance and terminal elimination half-life values were similar in male rats and female rats (Table 4), although plasma concentrations were more variable for male rats than for female rats.

On the basis of the observed gender differences in dose-limiting hepatocellular and biliary toxicity, the biliary excretion of ET743 was examined in male and female rats (Table 4). ET743 and the demethylated metabolite ET729, but neither M1 nor M2, were detected in HPLC chromatograms of bile samples collected for 60 min after drug administration. ET729 was also detected in bile by HPLC/MS analysis (data not shown). Although the combined recoveries of ET743 and ET729 in male and female rat bile were <2%, the biliary excretion of ET729 by female rats (1.05% of dose) was 5-fold greater than for male rats (0.19% of dose; Table 4).

DISCUSSION

The goals of this study were to characterize the oxidative metabolism of ET743 by rat and human liver microsomal preparations with an emphasis on gender-dependent features, to characterize plasma and biliary excretion of ET743 in male and female rats, and to consider our findings in the context of potential metabolism and toxicity of ET743 in cancer patients. The predominant P-450s that catalyze ET743 metabolism are those of the CYP3A subfamily. The key observations in support of a major role for rat CYP3A isoforms include induction of ET743 metabolism by the CYP3A inducers PB and DEX (with corresponding increased expression of CYP3A-related enzymes), selective inhibition of ET743 metabolism by the CYP3A inhibitors α-naphthoflavone and quercitin and by anti-rat CYP3A2 antiserum, and metabolism by cDNA-expressed rat CYP3A1 and CYP3A2. Furthermore, liver microsomes from male rat catalyzed metabolism of ET743 to a greater extent than did liver microsomes from female rats, consistent with greater expression of CYP3A isoforms in males than females (30, 31). Likewise, a major role for human CYP3A isoforms in ET743 metabolism was determined by correlation with testosterone 6β-hydroxylation in the human liver panel, selective inhibition by α-naphthoflavone and quercitin and by the antirat CYP3A2 antiserum (cross-reactive with CYP3A4), and metabolism by cDNA-expressed human CYP3A4.

The gender-dependent toxicity of ET743 in rats observed during preclinical development was consistent with metabolism by certain CYPs with documented gender-dependent expression in rats. The CYP3A subfamily enzymes are an abundant source of CYPs in rats. In this species, they play a major role in drug metabolism, are highly inducible by classical inducing agents such as the glucocorticoids and anticonvulsant drugs, and they exhibit gender differences in expression (26). Metabolism by CYP3A enzymes in rats often predicts CYP3A-catalyzed metabolism in humans. Of note, there are many similarities between rat and human CYP3A enzymes. As a group, CYP3A enzymes are the most abundant source of CYPs in human liver, catalyze metabolism of many drugs, and are highly inducible by the classical enzyme-inducing agents

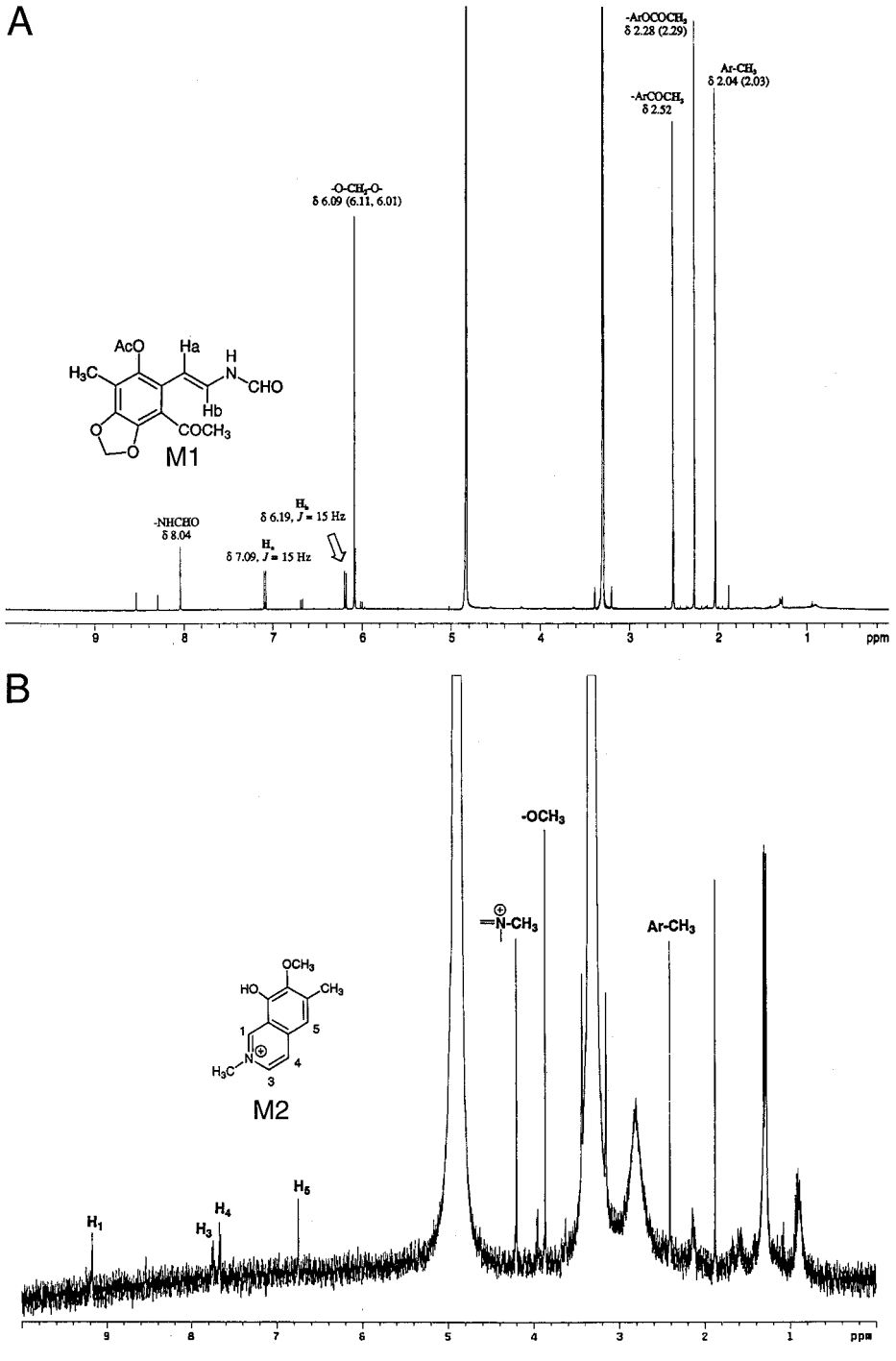

Fig. 5 ^{1}H-NMR spectra of ET743 metabolites M1 (*A*) and M2 (*B*). Number in parenthesis corresponds to δ in ET743.

(32). However, CYP3A enzymes do not exhibit gender differences in expression of humans.

Rat CYP3A enzyme activity reflects the combined expression of at least five family members, CYP3A1, CYP3A2, CYP3A9, CYP3A18, and CYP3A23. A distinct gender difference in the expression and activity for each of these enzymes has been reported (31, 33–35). In aggregate, the mRNA expression of CYP3A genes in adult male rats is ~50% greater than that in adult female rats, and CYP3A activities are substantially higher in male than in female rats (30, 31), consistent with our findings for ET743 metabolism.

Human CYP3A enzyme activity reflects the combined expression of at least four family members, CYP3A4, CYP3A5, CYP3A7, and CYP3A43 (36–42). Almost all adults express CYP3A4, but substantial variability (40-fold) in expression has been observed (43–45). Recently, functional polymorphisms for

Table 4 Summary of ET743 pharmacokinetics and biliary excretion after i.v. administration to Fisher 344 rats[a]

	Male	Female
$t_{1/2\alpha}$ (min)	6.4 (2.5)	3.0 (1.0)
$t_{1/2\beta}$ (min)	48.8 (66.6)	30.3 (8.6)
AUC (ng/ml·min)	1094 (380)	1123 (98)
V_{ss} (l/kg)	20.0 (17.7)	14.5 (2.9)
Cl_{TB} (ml/min/kg)	457 (159)	445 (39)
Biliary excretion (% of administered dose)[b]		
ET743	0.48	0.28
ET729	0.19	1.05

[a] Values represent estimates with SE in parentheses obtained by PCNONLIN analysis of pooled data from two experiments (13 animals) with male rats and three experiments (20 animals) with female rats after administration of 500 μg/kg ET743.

[b] Values represent the mean of two experiments for male rats and the mean of three experiments for female rats after administration of 250 μg/kg ET743.

CYP3A5 have been reported in some individuals. Individuals with at least one *CYP3A5*1* allele have CYP3A5 enzyme activity that is at least 50% of the total CYP3A enzyme activity (40). The CYP3A5 polymorphism may represent a substantial portion of the variable activity previously attributed to CYP3A4, given overlapping substrate specificity and high homology. Although individual genes are differentially regulated, gender-dependent expression of CYP3A has not been consistently observed at the mRNA or protein level (42, 45).

In humans, gender differences in the pharmacokinetics of CYP3A drug substrates, including parent drug or metabolite plasma clearance, have been modest, not statistically significant, and often in conflict with other studies with the same drug reporting no gender differences (see the recent review by Wilkinson and references therein, Ref. 46) For example, two studies on erythromycin pharmacokinetics found a gender difference in expired $^{14}CO_2$ after a dose of ^{14}C-labeled erythromycin (47, 48), whereas another study found no gender differences in expired $^{14}CO_2$ (43). In other studies, cyclosporine blood levels (43) and dose (49) correlated with $^{14}CO_2$ expired in the breath after a dose of ^{14}C-labeled erythromycin but did not correlate with gender. There have been two recent reports that midazolam plasma clearance was more rapid in young adult women than in young adult men (50, 51). However, two other reports did not find any differences in midazolam clearance between women and men (52, 53). The human female liver microsomes in our small panel exhibited greater ET743 metabolism and testosterone 6β-hydroxylation than did human male liver microsomes, but these differences were not statistically significant. The variability of CYP3A4 expression among humans, up to 50–100 fold (44), is much greater than the recently reported differences in plasma elimination or systemic exposure of CYP3A4 substrate drugs in humans. Thus, we would not anticipate substantial gender-dependent ET743 pharmacokinetics or toxicity in cancer patients.

Studies of microsomal preparations containing cDNA-expressed rat CYPs suggest other rat CYPs, most notably CYP2 isoforms, may contribute to ET743 metabolism detected in rat liver microsomal preparations. Male rat predominant CYP2A2 (54) and CYP2D1 (26) catalyzed ET743 metabolism with enzyme activities that were similar to CYP3A2. However, hepatic expression of CYP2A2 and CYP2D1 proteins are much lower than expression of CYP3A2, ET743 metabolism by rat liver microsomes was not increased after pretreatment with 3-MC, which induces CYP2D1 expression, and little residual metabolism was observed after addition of a selective CYP3A2 antibody. Thus, CYP2A2 and CYP2D1 most likely do not contribute to gender differences in enzymes that catalyzed ET743 metabolism. Likewise, absence of increased ET743 metabolism in liver microsomes from 3-MC-pretreated rats is consistent with a limited role for CYP1A1 and CYP1A2.

Human cDNA-expressed CYP2C9, CYP2D6, and CYP2E1 had moderate catalytic activity for ET743 metabolism. None of these human P-450s have been reported to exhibit differential expression by gender. CYP2C9 likely contributed moderately to ET743 metabolism based on a correlation with tolbutamide methyl-hydroxylation and inhibition by α-naphthoflavone and quercitin. CYP2E1 and CYP2D6 probably have a minor role in ET743 metabolism, based on the absence of a correlation with chlorzoxazone 6-hydroxylation and dextromethorphan *O*-demethylation, as well as absence of inhibition by quinidine and chlorzoxazone.

We identified three metabolites in liver microsomal preparations after incubation with ET743. All cDNA-expressed CYPs that metabolized ET743 produced metabolites M1 and M2. Although the initial sites of oxidation are unclear, these oxidations led to major cleavage of the parent structures based on interpretation of the HPLC/MS/MS and ^1H-NMR data. ET729, the demethylated ET743 metabolite, was detected by HPLC/MS analysis of cDNA-expressed CYP3A4 incubations. Absence of ET729 in rat and human liver microsomal preparations may be the result of subsequent metabolism by other P-450s not found in microsomes for the individual cDNA-expressed enzymes.

In a study to screen for potential *in vivo* metabolites of ET743, Sparidans *et al.* (55) reported the appearance of small amounts of products with structures identical to those of M1 and M2 in HPLC tracings from incubations in the absence of microsomes, raising the possibility of formation by degradation rather than metabolism. In our studies, trace quantities of M1 were detected in microsomal preparations in the absence of NADPH, but formation was greatly enhanced in the presence of cofactor. M2 formation was observed only in the presence of cofactor. Sparidans *et al.* (55) also detected small amounts of other oxidative metabolites and a glucuronide conjugate in microsomal incubations and deacetylated ET743 in a plasma incubation. The much longer incubation period used in their studies (6 h *versus* 2 h) may account for detection of small amounts of other oxidative metabolites. We did not evaluate the formation of glucuronide conjugates or hydrolysis products. The *in vivo* contribution of conjugation and hydrolysis pathways is unclear because these metabolites were not detected in patient urine after treatment with ET743 (55). Because radiolabeled drug was not available for our studies or for those of Sparidans *et al.* (55), mass balance could not be determined.

Despite the differences observed in ET743 metabolism by male and female rat preparations, there were no gender differences in ET743 plasma disposition, as measured by AUC and plasma clearance, at the dose administered in the pharmacokinetic study. That dose (500 μg/kg) was 5.5–7.1-fold greater than doses that were hepatotoxic to male (90 μg/kg) and female (50–70 μg/kg) rats. The higher doses were necessary for pharmacokinetic evaluation because of the limited sensitivity of the HPLC assay with UV detection. The higher dose may have concealed the effect of a potential gender difference in metabolism on plasma clearance. Gender-dependent aspects of ET743 elimination in our study were limited to biliary excretion of parent drug and the cytotoxic metabolite ET729. Biliary excretion of ET729 was very low (<1.3%) but was ~5-fold greater in female rats when compared with male rats. Because ET729 has 17-fold greater cytotoxicity than ET743 (1, 5), the greater biliary excretion of this cytotoxic metabolite may contribute to the greater hepato- and biliary toxicity of ET743 in female rats.

Results of several Phase I trials (13, 14, 56) and one Phase II trial (12) of ET743 have been reported recently. The dose-limiting toxicity was neutropenia. Although hepatotoxicity was observed, it was usually mild and limited to transient elevations of liver enzymes that returned to baseline before subsequent treatment cycles, diminished in intensity with subsequent treatment, and were similar in males and females. Thus, consistent with our *in vitro* data, where gender-dependent metabolism was observed for rat, but not human, preparations, the gender difference in ET743 hepatotoxicity observed in rats has not been observed in early clinical trials.

In conclusion, rat and human liver microsomes catalyzed NADPH-dependent metabolism of ET743, consistent with a role for cytochromes P-450. The predominant CYPs that catalyzed rat and human ET743 metabolism were those in the CYP3A subfamily, which exhibit gender differences in expression in rats but not humans. The gender difference in rat liver microsomal metabolism of ET743 was consistent with known differences in CYP3A expression, which is greater in male rats but was not associated with altered ET743 plasma clearance. Human CYP3A, implicated by the rat metabolism data, were the predominant CYPs that catalyzed ET743 in human liver microsomes. CYP3A enzymes catalyzed formation of at least three metabolites, including ET729, which was excreted to a greater extent in female rats. Although these findings suggest that ET729 may be responsible for the transient hepatotoxicity seen in early clinical trials of ET743, it is unlikely that ET743 will exhibit gender differences in toxicity toward humans.

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