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In vitro characterization of the human biotransformation and CYP reaction phenotype of ET-743 (Yondelis®, Trabectidin®), a novel marine anti-cancer drug

Esther F.A. Brandon[1], Rolf W. Sparidans[1], Kees-Jan Guijt[1], Sjoerd Löwenthal[1], Irma Meijerman[1], Jos H. Beijnen[1,2] and Jan H.M. Schellens[1,3]
[1]*Faculty of Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands;* [2]*Slotervaart Hospital, Amsterdam, The Netherlands;* [3]*The Netherlands Cancer Institute, Amsterdam, The Netherlands*

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Summary

ET-743 is a potent marine anti-cancer drug and is currently being investigated in phase I and II clinical trials, e.g. in combination with other anti-cancer agents. To assess the biotransformation and CYP reaction phenotype and their potential implications for human pharmacology and toxicology, the *in vitro* metabolism of ET-743 was characterized using incubations with human liver preparations, cytochrome P450 (CYP) and uridine diphosphoglucuronosyl transferase (UGT) supersomes.

CYP supersomes and liver microsomes showed that ET-743 was metabolized mainly by CYP3A4, but also by CYP2C9, 2C19, 2D6, and 2E1. ET-743 showed the highest affinity for CYP3A4 and the highest maximal metabolic rate for CYP2D6 among the CYPs shown to metabolize ET-743. In addition, the K_m value of ET-743 in female microsomes was significantly lower compared to male microsomes, while the $V_{\max}$ values did not differ. ET-743 glucuronidation, catalyzed by UGT2B15, was observed in microsomes and S9 fraction. In addition, conjugation by glutathione-S-transferase and no sulphation was observed for ET-743 in cytosol and S9 fraction. ET-743 was more extensively metabolized when CYP activity was combined with phase II enzymes UGT and glutathione-S-transferase (GST), indicating that CYP, UGT, and GST simultaneously metabolize ET-743 in the S9 fraction.

These results provide evidence that CYP3A4 has a major role in the metabolism of ET-743 *in vitro* with additional involvement of CYP2C9, 2C19, 2D6, and 2E1. Furthermore, ET-743 is conjugated by UGT and GST. This information could be important for interpretation of the pharmacokinetic data of clinical trials and prediction of drug-drug interactions.

Introduction

Ecteinascidin-743 (ET-743, Yondelis®, Trabectedin®) (Figure 1) is a tetrahydroisoquinoline isolated from the Caribbean tunicate *Ecteinascidia turbinata* [1]. The compound exhibited *in vitro* activity at nanomolar concentrations against various solid tumor cell lines, including melanoma and ovarian, renal, prostate, breast, and non-small cell lung cancer cell lines [2]. In addition, ET-743 appears effective against human xenografts of non-small cell lung, melanoma and breast tumors *in vivo* [2, 3]. The mode of action of ET-743 has not been completely elucidated, but several mechanisms have been proposed. It is believed to involve binding to the minor groove of the DNA, interactions with transcription factors and DNA binding proteins, disorganization of the microtubule network, inhibition of topoisomerase I, pertubation of the cell cycle, and interference with DNA repair mechanisms [4, 5].

Multiple infusion schedules were investigated in phase I clinical trials and studies investigating the effects of ET-743 combined with cisplatin, carboplatin, and doxorubicin are currently in progress or preparation [5]. From phase I trials, a treatment schedule was chosen for phase II clinical trials. In these studies, ET-743 was administered as 3 or 24 h continuous i.v. infusion [6]. Phase II clinical trials are still ongoing, but activities against soft-tissue sarcomas, breast tumors, endometrial cancer, and ovarian cancer have already been shown [1, 6–8].

Reid et al. [9] investigated the biotransformation of ET-743 and showed that ET-743 was metabolized by microsomes from cytochrome P450 (CYP) 2C9, 2D6, 2E1, and 3A4 transfected human B-lymphocyte cell lines [9]. Further, studies by Sparidans et al. [10] showed that ET-743 was metabolized by human liver microsomes and was conjugated by rabbit UGT [10]. However, the enzyme kinetics of

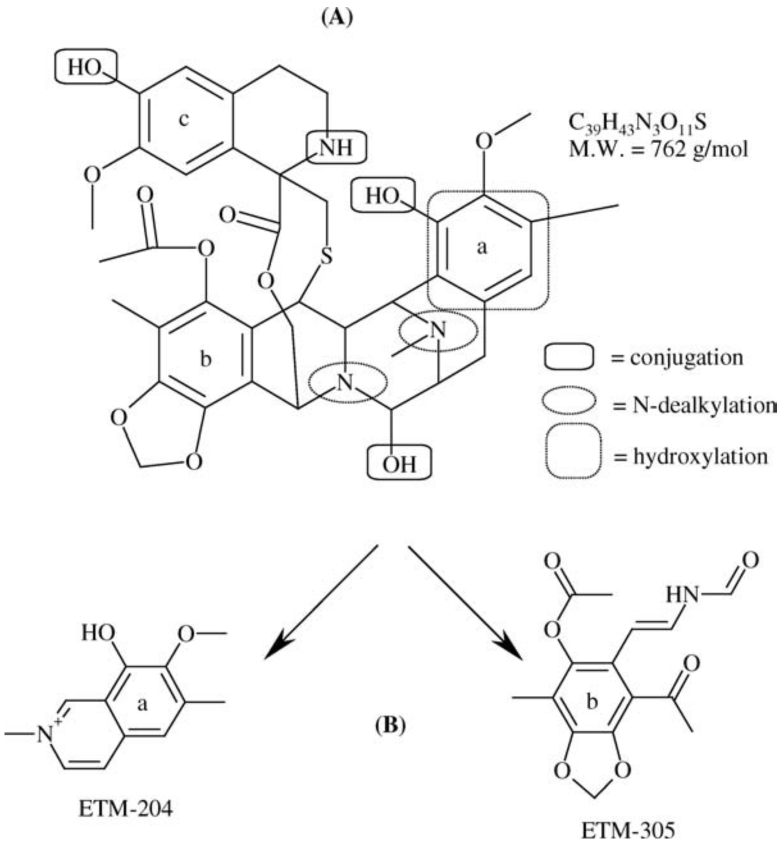

Figure 1 Chemical structure of ET-743 (A) and its degradation products ETM-204 and ETM-305 (B) [10]. The different squares and ovals indicate potential sites for biotransformation [11, 12]. In addition, all the ester bonds are potential sites for hydrolysis.

ET-743 and the relative contribution (%) of each CYP (CYP reaction phenotype) have not yet been identified. Knowledge about enzyme kinetics and CYP reaction phenotype is important in order to interpret the pharmacological properties found in clinical trials and to predict possible drug-drug interactions with other (anti-cancer) drugs. Furthermore, the biotransformation of ET-743 by human phase II enzymes and phase I in combination with phase II enzymes has not yet been reported. The elucidation of the biotransformation products of ET-743 may be complicated because of the presence of several potential sites for phase I and II reactions and the formation of degradation products (Figure 1) [11, 12].

Different *in vitro* methods were therefore used in this explorative investigation, including pooled human liver microsomes, cytosol, and S9 fraction in combination with HPLC-UV analysis. The contribution of various isoforms of CYP and uridine diphosphoglucuronosyl transferase (UGT) to the biotransformation was investigated using pooled human liver microsomes in combination with specific CYP inhibitors and CYP and UGT supersomes.

Materials and methods

Materials. ET-743 was kindly donated by PharmaMar (Tres Cantos, Madrid, Spain). Methanol (HPLC grade) and acetonitrile (gradient grade) were purchased from Biosolve (Valkenswaard, The Netherlands) and formic acid (p.a.), ammonium acetate (p.a.), $MgCl_2 \cdot 6H_2O$ (p.a.), and dimethylsulfoxide (DMSO, synthesis grade) from Merck (Darmstadt, Germany). Water was purified on a multi-laboratory scale by reversed osmosis. Pooled human liver microsomes (mixed gender, male, and female), pooled human liver cytosol, pooled human liver S9 fraction, and human CYP and UGT supersomes (Baculovirus-insect-cell expressed) were provided by Gentest (Becton Dickinson, Woburn, MA, USA). Ritonavir was provided by Abbott (Chicago, IL, USA) and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) and were of analytical grade.

ET-743 incubations with pooled human liver microsomes (mixed gender, male, and female). The incubation procedure of ET-743 with human liver microsomes was a modification of the method described by Sparidans et al. [10]. Twenty-five μl of 0.5 M potassium phosphate buffer (pH 7.4) were pipetted into a polypropylene micro tube on ice and 50 μl NADP regenerating system (NRS: 1.5 U/ml glucose-6-phosphate dehydrogenase, 0.5 mg/ml β-NADP, 4.0 mg/ml D-glucose-6-phosphate in 0.6% $NaHCO_3$), 7.5 μl of 20 mg/ml $MgCl_2 \cdot 6H_2O$ solution, and 50 μl of an aqueous ET-743 solution (1% DMSO, final concentration of 50 μg/ml in the microsomes suspension) were added. After vortex-mixing briefly, the tubes were incubated for 2 min at 37°C in a shaking water bath. Next, 5 μl of mixed gender (lot number 18), male (lot number 2), or female (lot number 1) pooled human liver microsomes were added. The tube was vortex-mixed briefly again and the mixture was incubated for 4 h at 37°C in a shaking water bath. The reaction was terminated by adding 125 μl ice-cold methanol and vortex-mixing. The sample was centrifuged at approximately 15,000 g and 4°C for 1 min to remove proteins and the supernatant was injected for gradient chromatographic analysis. Control experiments were performed without ET-743 and without liver microsomes, respectively.

ET-743 incubated with pooled human liver microsomes in the absence and presence of CYP inhibitors. ET-743 incubations with liver microsomes in the absence and presence of CYP inhibitors were performed according to the method described for liver microsomes with slight modifications. Twelve and a half μl of 1 M potassium phosphate buffer (pH 7.4) were pipetted into a polypropylene micro tube on ice and 50 μl NRS, 7.5 μl of 20 mg/ml $MgCl_2 \cdot 6H_2O$ solution, and 10 μl of an aqueous CYP inhibitor solution (1% (v/v) DMSO) were added. ET-743 was incubated with microsomes and the following inhibitors: 50 μM sulfaphenazole (CYP2C9), 200 μM (S)-(+)-mephenytoin (CYP2C19), 50 μM quinidine (CYP2D6), 200 μM chlorzoxazone (CYP2E1), and 100 μM ritonavir (CYP3A4). After vortex-mixing briefly, the tubes were incubated at 37°C in a shaking water bath for 2 min. Next, 5 μl of pooled human liver microsomes (mixed gender, lot number 21) were added. The tube was vortex-mixed briefly again and the mixture was then incubated at 37°C in a shaking water bath for 5 min. Fifty μl of an aqueous ET-743 solution (1% (v/v) DMSO, final concentration of 50 μg/ml) were added and vortex-mixed briefly. The tube was incubated further at 37°C in a shaking water bath for 4 h. The reaction was terminated and proteins were removed as previously described. The supernatant was injected for gradient chromatographic analysis. Control experiments were performed without ET-743 and without liver microsomes, respectively.

ET-743 incubated with human CYP supersomes. Incubations with human CYP supersomes were performed as with the liver microsomes. Instead of liver microsomes, 5 μl of the CYP supersomes suspension were added. The following human CYP supersomes were tested: CYP1A1 (lot number 15), CYP1A2 (lot number 20), CYP2A6 (lot number 6), CYP2B6 (lot number 8), CYP2C8 (lot number 11), CYP2C9 $*$ 1(Arg_{144}) (lot number 17), CYP2C19 (lot number 12), CYP2D6 $*$ 1 (lot number 27), CYP2E1 (lot number 9), CYP3A4 (lot number 40), and CYP4A11 (lot number 7). All CYPs were co-expressed with P450 reductase and CYP2A6, 2B6, 2C8, 2C9, 2C19, 2E1, and 3A4 were also co-expressed with cytochrome b_5 in the insect cells. A concentration of 50 μg/ml ET-743 was incubated with the human CYP supersomes. The incubation was terminated as described previously after 4 h. The proteins were removed as previously described and the supernatant was injected for gradient chromatographic analysis. Control experiments were performed without substrate or with insect cell control supersomes (lot number 22).

Glucuronidation of ET-743 by pooled human liver microsomes. Thirty μl of 0.1 M $MgCl_2 \cdot 6H_2O$ solution, 10 μl of 0.5 mg/ml alamethicin, 50 μl ET-743 in water (1% (v/v) DMSO, final concentration of 50 μg/ml), 25 μl of 1 M potassium phosphate buffer (pH 7.4), 50 μl of 15 mg/ml uridine diphosphoglucuronic acid (UDPGA), and 25 μl water were pipetted into a polypropylene micro tube on ice. After vortex-mixing briefly, the tube was incubated at 37°C in a shaking water bath for 2 min. Next, 10 μl of pooled human liver microsomes (lot number 21) were added. The tube was vortex-mixed briefly again and the mixture was then incubated at 37°C in a shaking water bath for 5 h. The reaction was terminated by adding 200 μl ice-cold methanol and vortex-mixing briefly. Proteins were removed and the supernatant was injected for gradient chromatographic analysis. Individual control experiments were performed without ET-743 and without pooled human liver microsomes, respectively.

ET-743 incubated with human UGT supersomes. The incubation of ET-743 with human UGT supersomes was a modification of the method described by Gentest [13]. Twenty μl of 1 M potassium phosphate buffer (pH 7.4) were pipetted into a polypropylene micro tube on ice and 10 μl of 0.5 mg/ml alamethicin, 20 μl of 0.1 M $MgCl_2 \cdot 6H_2O$, 20 μl of 20 mM UDPGA, 50 μl ET-743 in water with 1% (v/v) DMSO (final concentration of 50 μg/ml in the supersomes suspension), 70 μl H_2O, and 10 μl of the supersomes suspension were added. The following human UGT supersomes were tested: UGT1A1 (lot number 8), UGT1A3 (lot number 8), UGT1A9 (lot number 6), and UGT2B15 (lot number 5). After vortex-mixing briefly, the mixture incubated for 5 h at 37°C in a shaking water bath. The reaction was terminated and proteins removed as previously described. The supernatant was injected for gradient chromatographic analysis. Control experiments were performed without substrate and without UDPGA or with UGT insect cell control supersomes (lot number 5).

Conjugation of ET-743 by N-acetyl transferase, sulfotransferase and glutathione-S-transferase in pooled human liver cytosol. The incubation of ET-743 with pooled human liver cytosol was a modification of the method described by Gentest [13]. Equal volumes (20 μl) of 1 M potassium phosphate buffer (pH 7.4), 10 mM dithiotreitol (DTT), 1 mM acetyl-coenzyme A (acetyl-CoA), 45 mM acetyl-DL-carnitine, 80 units/ml carnitine acetyl transferase (from pigeon breast muscle), 1 mM adenosine 3′-phosphate 5′-phosphosulfate (PAPS), and 10 mM glutathione were pipetted into a polypropylene micro tube on ice. Six μl H_2O and 50 μl of an aqueous dilution of ET-743 (1% (v/v) DMSO, final concentration of 50 μg/ml in the cytosol suspension) were added and vortex-mixed briefly. Next, 4 μl human liver cytosol (lot number 2) were added and, after vortex-mixing briefly, the mixture was incubated at 37°C in a shaking water bath for 5 h. The reaction was terminated by adding 200 μl acetonitrile and vortex-mixing. The proteins were removed as previously described and the supernatant was injected for gradient chromatographic analysis. Individual control experiments were performed without ET-743, only DTT, acetyl-CoA, acetyl-DL-carnitine, and carnitine acetyl transferase (only N-acetyltransferase (NAT) activity), only PAPS (only sulfotransferase (SULT) activity), only glutathione (only glutathione-S-transferase (GST) activity), and without all co-factors for enzyme activity. In addition, all four substrates of NAT were individually tested as controls.

ET-743 incubations with pooled human liver S9 fraction. The incubation of ET-743 with pooled human liver S9 fraction was a modification of the method described by Gentest [13]. Equal volumes (10 μl) of 75 mg/ml UDPGA, 10 mM DTT, 1 mM acetyl-CoA, 45 mM acetyl-DL-carnitine, 80 units/ml carnitine acetyl transferase, 1 mM PAPS, and 10 mM glutathione were pipetted into a polypropylene micro tube on ice. Twenty-four μl NRS (5 U/ml glucose-6-phosphate dehydrogenase, 1.67 mg/ml β-NADP, and 13.33 mg/ml D-glucose-6-phosphate in 2% (w/v) $NaHCO_3$), 50 μl of an aqueous dilution of ET-743 (1% (v/v) DMSO, final concentration of 50 μg/ml in the S9 suspension), 12 μl of 20 mg/ml $MgCl_2 \cdot 6H_2O$, 20 μl of 1 M potassium phosphate buffer (pH 7.4), and 14 μl H_2O were added and vortex-mixed briefly. Subsequently, the tubes were incubated at 37°C in a shaking water bath for 2 min. Next, 10 μl pooled human liver S9 fraction (lot number 5) were added and vortex-mixed. The mixture was incubated for 4 h at 37°C in a shaking water bath and the reaction was terminated by adding 200 μl ice-cold methanol and vortex-mixing. The sample was centrifuged at approximately 15,000 g and 4°C for 1 min and the supernatant was then injected for gradient chromatographic analysis. Individual control experiments were performed without substrate, without all co-factors for enzyme activity and with co-factors present for only one or two enzymes (only one enzyme or a combination of CYP with a phase II enzyme were active), respectively.

Lineweaver-Burke plot of ET-743 in mixed gender, male, and female pooled human liver microsomes. The incubation procedure of ET-743 with pooled human liver microsomes to obtain a Lineweaver-Burke plot was a modification of the method described previously for microsomes. Seven different concentrations of ET-743 (concentration range of 0.33–10 μg/ml) were incubated with human liver microsomes to generate one Lineweaver-Burke plot. The following human liver microsomes were tested: mixed gender (lot number 21), male (lot number 3), and female (lot number 2). Each ET-743 concentration was incubated for 7 different time points at 37°C in a shaking water bath and the length of the incubation depended on the substrate concentration (Table 1). The incubation was terminated by adding 125 μl acetonitrile and vortex-mixing. The supernatant was injected for isocratic chromatographic analysis. The determination of the V_{max} and K_m was based on the disapearance of ET-743 from the incubation mixture.

Table 1. ET-743 incubation times with pooled human liver microsomes. A concentration range of 0.33–10 μg/ml ET-743 were incubated with mixed gender, female, and male human liver microsomes

	ET-743 conc. (μg/ml) in incubation mixture					
Incubation times	0.33	0.50	0.67	1.0	2.0	10
0-10-20-30-40-50-60 s	X					
0-0.5-1-1.5-2-2.5-3 min		X	X	X	X	
0-5-10-15-20-25-30 min						X

Lineweaver-Burke plot of ET-743 in CYP supersomes. Incubations with human CYP supersomes were performed according to the incubation method as described in the previous paragraph. The following CYP supersomes were tested: CYP2C9 ∗ 1 (lot number 22), CYP2C19 (lot number 17), CYP2D6 ∗ 1 (lot number 35), CYP2E1 (lot number 12), and CYP3A4 (lot number 50). Each ET-743 concentration was incubated for 7 different time points at 37°C in a shaking water bath (Table 2). The supernatant was injected for isocratic liquid chromatographic analysis. The determination of the V_{max} and K_m was based on the disapearance of ET-743 from the incubation mixture.

Determination of the protein-binding of ET-743 in pooled human liver microsomes. ET-743 (final concentration range of 2–50 μg/ml) was pre-incubated with human microsomes for 15 min on ice. The reaction was terminated by removing proteins using ultra-centrifugation with Micronon YM-10 ultra-centrifuge tubes (cut-off filter of 10 kDa) (Millipore, Bedford, MA, USA) for 120 min at 14,000 g and 4°C. The protein binding was estimated by quantification of ET-743 in the ultra-filtrate. The samples were diluted 1:1 (v/v) with methanol and analyzed with gradient liquid chromatographic assay. Calibration was performed using the same concentration range of ET-743 incubated in phosphate buffered saline.

Analysis of ET-743 and possible metabolites by gradient HPLC. The chromatographic assay was a modification of the method described by Sparidans et al. [10]. The supernatants of the incubated mixtures were analyzed on an HPLC system consisting of two LC-10AT_{VP} pumps, a SIL-10AD_{VP} autoinjector (equipped with a 500 μl sample loop), a SCL-10A_{VP} system controller, and a SPD-

Table 2. ET-743 incubation times with CYP supersomes

CYP2C9 $*$ 1	ET-743 conc. (μg/ml) in incubation mixture							
Incubation times	0.20		0.80		1.33	2.0	4.0	10
0-2.5-5-7.5-10-12.5-15 min	X							
0-5-10-15-20-25-30 min			X		X	X	X	X
CYP2C19								
Incubation times	1.0	1.6	2.0	3.0	4.0	10	20	40
0-5-10-15-20-25-30 min	X	X	X					
0-10-20-30-40-50-60 min				X	X	X	X	X
CYP2D6 $*$ 1								
Incubation times	1.33	5	7.5	10	12.5		25	50
0-2.5-5-7.5-10-12.5-15 min		X	X					
0-2-4-8-12-16-20 min					X			
0-5-10-15-20-25-30 min	X			X			X	X
CYP2E1								
Incubation times	1.33		2.0		4.0		10	
0-5-10-15-20-25-30 min	X		X		X			
0-10-20-30-40-50-60 min							X	
CYP3A4								
Incubation times	0.33	0.40	0.50	0.67	1.0	2.0	4.0	10
0-05-1-1.5-2-2.5-3 min	X	X	X	X	X	X		
0-2-4-5-6-8-10 min							X	
0-5-10-15-20-25-30 min								X

M10A$_V$P photodiode array detector (all from Shimadzu, Kyoto, Japan). The column was thermostated by a Waters temperature control module and a Waters column heater module (Milford, MA, USA). Data were recorded on a Hermac Pentium 440, 122 MB personal computer (Scherpenzeel, The Netherlands) equipped with the Class-VP 5.032 software (Shimadzu). Injections (50 μl) were made on a Symmetry C18 column (4.6 $\times$ 100 mm, d_p = 3.5 μm, Waters) with a Sentry Guard Symmetry C18 pre-column (3.9 $\times$ 20 mm, $d_p = 5$ μm, Waters). The column temperature was maintained at 40°C. A gradient program was used with eluent A comprising 10 mM formic acid in water and eluent B comprising 10 mM formic acid in acetonitrile. After injection, elution started with 45% B and the eluent composition was raised linearly to 75% B during 20 min. This percentage was maintained for 2 min before conditioning with 45% B for 8 min. The eluent flow rate was 1.0 ml/min, the UV detection array was used between 190 and 300 nm and the peak areas were determined at 225 nm.

Analysis of ET-743 by isocratic HPLC. The system consisted of a P100 pump, an AS300 autoinjector (equipped with a 100 μl sample loop), and a UV100 detector (all from ThermoSeparation Products, Fremont, CA, USA). The column was thermostated by a Waters temperature control module and a Waters column heater module. Data analysis, column temperature and injections were performed as described previously. The eluent comprised of 65% (v/v) 25 mM phosphate buffer and 35% (v/v) acetonitrile. The eluent flow rate was 1.0 ml/min and the UV detection wavelength was set at 225 nm.

The CYP reaction phenotype. The relative contribution of each individual CYP to the biotransformation of ET-743 (CYP reaction phenotype) was calculated by dividing the individual CYP contribution by the total contribution of all five CYPs investigated and multiplying by 100. Each individual CYP contribution, which is an estimation of the relative rate of metabolism attributed to the CYP isozyme, was calculated by multiplying the metabolism rate of ET-743, calculated from the Michaelis-Menten kinetics determined in this study, in CYP supersomes with the relative activity factor (RAF) value [14–16]. The RAF indicates the relative activity of a respective isoform in human liver microsomes [14–16].

$$RAF = \textit{formation rate in microsomes}/\ \textit{formation rate in supersomes}$$

Both formation rates were calculated in nmol/(mg protein $*$ min) [14] and were based on the activity data for a standard substrate for each CYP provided by Gentest with each lot number [13]. The individual CYP contributions and CYP reaction phenotypes for the five CYPs investigated were calculated for two microsomal preparations (female (lot number 2) and male (lot number 3)) and for two concentrations of ET-743: 50 μg/ml (concentration used for microsomal incubations) and 1 ng/ml (plasma concentration near $C_{\max}$ in patients after 24 h infusion [17, 18]).

Intrinsic metabolic clearance of ET-743 from microsomes and CYP supersomes. The intrinsic metabolic clearance (CL_{int}) is the elimination of a compound by biotransformation at concentrations well below K_m and can be calculated using the following equation [19]:

$$CL_{\text{int}} = V_{\max}/K_m$$

The CL_{int} was calculated for mixed gender, female, and male human liver microsomes and for CYP supersomes.
Data analysis. The results are expressed as mean ± standard deviation (SD). Differences between the results were analyzed by the student *t*-test for unpaired observations.

Results

For all experiments control incubations were performed, incubations without substrate, without microsomes, without co-factors of phase II enzymes and with control supersomes, respectively. None of these control incubations showed any metabolic conversion of ET-743.

Comparison of the ET-743 biotransformation in male, female, and mixed gender pooled human liver microsomes. Incubation of ET-743 with mixed gender human liver microsomes at 37°C for 4 h reduced the amount of ET-743 with 68.1 ± 6.0%. Incubations of ET-743 with female human liver microsomes showed a small, statistically not significant, difference in percentage ET-743 metabolized (61.8 ± 4.0%) compared to mixed gender (8.1 ± 6.0%). Male human liver microsomes resulted in a significant decrease ($p < 0.05$) of the percentage ET-743 metabolized compared to mixed gender (50.3 ± 4.9%). Some possible ET-743 metabolites were observed, but could not be identified due to the impurity of the supplied ET-743 (the impurity was mild (<1%), but resulted in intervening peaks in the chromatogram (results not shown)). Further, two degradation products of ET-743 were observed, namely ETM-204 and ETM-305 (Figure 1B, results not shown).

ET-743 biotransformation by human CYP supersomes. ET-743 is significantly metabolized by CYP2C9, 2C19, 2D6, 2E1, and 3A4 supersomes during 4 h at 37°C; 91.0 ± 2.5%, 93.1 ± 4.3%, 95.6 ± 2.7%, 95.6 ± 3.0%, and 90.9 ± 0.8% of the initial amount of ET-743 was metabolized, respectively. The other CYP supersomes did not significantly metabolize ET-743.

ET-743 biotransformation by pooled human liver microsomes in the absence and presence of CYP inhibitors. To confirm the results found with CYP supersomes, ET-743 was incubated with human liver microsomes (mixed gender) in the presence of CYP inhibitors. Figure 2 shows that the CYP2D6, 2E1, and 3A4 inhibitors could significantly decrease the ET-743 biotransformation by pooled human liver microsomes. CYP3A4 is the main CYP isozyme responsible for the conversion of ET-743 in pooled human liver microsomes; the percentage ET-743 metabolized decreased in the presence of the CYP3A4 inhibitor ritonavir from 66.6 ± 1.3% to 17.8 ± 5.8%. Quinidine (CYP2D6) and chlorzoxazone (CYP2E1) reduced the ET-743 percentage metabolized to 55.9 ± 7.2% and 54.1 ± 10.5% respectively. The CYP2C9 and 2C19 inhibitors, sulfaphenazole and (S)-(+)-mephenytoin, had no influence on the biotransformation of ET-743 by human liver microsomes.

Glucuronidation of ET-743 by pooled human liver microsomes and UGT supersomes. Significant glucuronidation was observed for ET-743 by pooled human liver microsomes (HLM). After 5 h, 80.1 ± 2.2% and 46.7 ± 6.3% of the ET-743 was recovered, respectively. UGT2B15 supersomes significantly glucuronidated ET-743 (24.9 ± 6.0% decrease in ET-743). UGT1A1, 1A3, and 1A9 did not metabolize ET-743.
ET-743 conjugation by pooled human liver cytosol. After 5 h, ET-743 was significantly conjugated by the phase II enzyme GST present in pooled human liver cytosol (81.4 ± 2.2% of the ET-743 was recovered). SULT did not conjugate ET-743. The metabolism of ET-743 by NAT could not be studied due to degradation of ET-743 in the presence of the NAT cofactors (results not shown).

Biotransformation of ET-743 by pooled human liver S9 fraction. Cytochrome P450, UGT, and GST in pooled human liver S9 fraction significantly metabolized ET-743 (Figure 3). The CYPs present in the S9 fraction metabolized 25.0 ± 7.9% of the ET-743 during 5 h at 37°C and UGT and GST conjugation resulted in 31.2 ± 10.1% and 29.1 ± 7.7% ET-743 metabolized, respectively. CYP activity in combination with the individual phase II enzymes UGT and GST resulted in a further reduction of ET-743 compared to CYP, UGT, or GST alone (46.5 ± 0.4% and 47.3 ± 7.5%, respectively). When all the enzyme substrates were present, 64.2 ± 11.2% of the ET-743 is converted by pooled human liver S9 fraction. The phase II enzyme SULT did not metabolize ET-743.

Protein binding of ET-743 in human liver microsomes. ET-743 has a protein binding of 38.4 ± 7.4% in human liver microsomes in the concentration range of 2–50 ng/ml (results not shown). The free fraction (*fu*) value was used

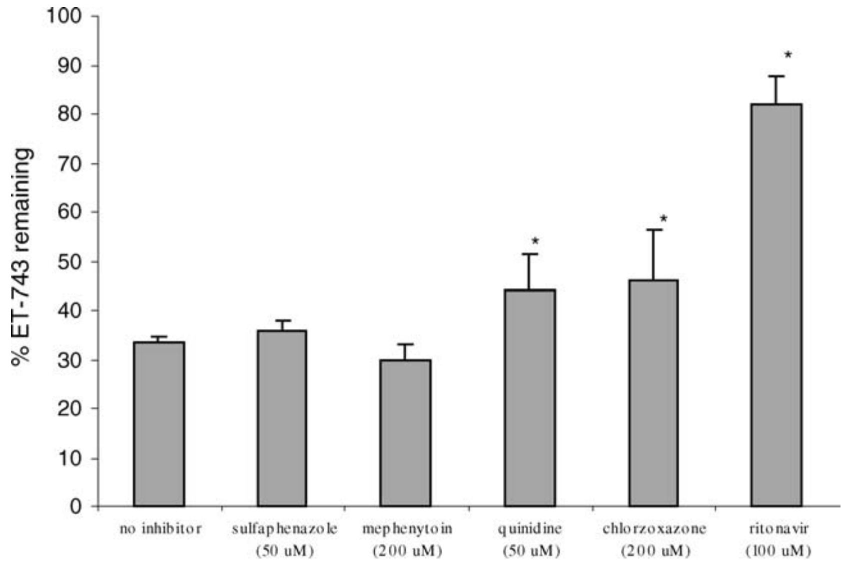

Figure 2 Percentage ET-743 remaining after incubation of 50 μg/ml ET-743 with pooled mixed gender human liver microsomes in the presence of the CYP inhibitors sulfaphenazole (CYP2C9), (S)-(+)-mephenytoin (CYP2C19), quinidine (CYP2D6), chlorzoxazone (CYP2E1), and ritonavir (CYP3A4). The percentage remaining was determined using an ET-743 incubation without pooled human liver microsomes as control. Each column is the mean of 3 replicates; bars indicate the SD. * significantly different ($p < 0.05$) compared to no inhibitor.

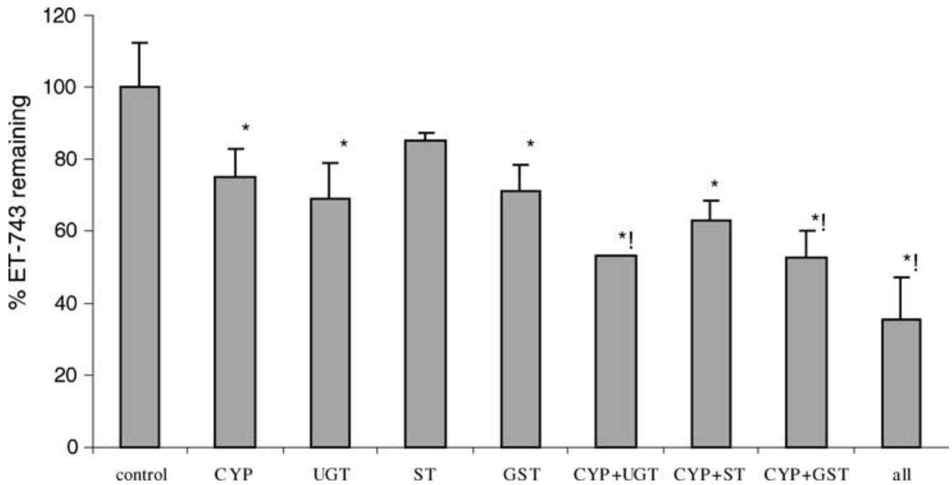

Figure 3 Comparison of the biotransformation of ET-743 in human liver S9 fraction by CYP and by CYP and phase II enzymes (UGT, SULT, and GST). The percentage ET-743 remaining was determined using an ET-743 incubation without S9 fraction as control. Each column is the mean of 3 replicates; bars indicate SD. * significantly different ($p < 0.05$) compared to control and ! significantly different ($p < 0.05$) compared to CYP.

to calculate the K_m value from the $K_{m(\mathrm{app})}$ determined from the Lineweaver-Burke plot.

Enzyme kinetics and intrinsic clearance of ET-743. The $V_{\max}$ and K_m values of ET-743 in human liver microsomes and CYP supersomes were calculated from Lineweaver-Burke plots (not shown) using weighed regression (1/x) and are shown in Table 3. The $V_{\max}$ values are not significantly different in mixed gender, female, and male microsomes. However, the K_m value of ET-743 in female liver microsomes is significantly lower compared to male microsomes, the K_m decreased from 0.366 ± 0.067 μM to 0.118 ± 0.046 μM. CYP3A4 has the highest K_m value (2.27 ± 0.67 μM) and CYP2D6 has the highest $V_{\max}$ value for ET-743 (86 ± 22 min^{-1}). The intrinsic clearance was higher in female human liver microsomes (13 ± 5 ml/(nmol CYP $*$ min)) compared to male microsomes (3.7 ± 0.7 ml/(nmol CYP $*$ min)) ($p<0.05$). CYP3A4 has the highest intrinsic clearance for ET-743 for all CYP supersomes tested.

CYP reaction phenotype of ET-743. According to the calculations, CYP3A4 is the major isozyme involved in the biotransformation of 50 μg/ml ET-743 (<70%) and 1 ng/ml ET-743 (<94%) (Figures 4 and 5 respectively). The contribution of the other CYPs involved in the biotransformation of 50 μg/ml ET-743 in human liver microsomes are in the

Table 3. The K_m, V_{max}, and Cl_{int} values of ET-743 in mixed gender, female, and male human liver microsomes and in human CYP supersomes calculated from the Lineweaver-Burke plots

	$K_m \pm$ SD (μM)	$V_{max} \pm$ SD (min^{-1})	CL_{int}[ml/(nmol CYP * min)]
Human liver microsomes			
Mixed gender	0.304 ± 0.038	0.95 ± 0.03	3.1 ± 0.4
Female	0.118 ± 0.046*	1.57 ± 0.06	13 ± 5*
Male	0.366 ± 0.067	1.36 ± 0.08	3.7 ± 0.7
Supersomes			
CYP2C9 * 1	7.8 ± 3.3	5.0 ± 2.2	0.64 ± 0.28
CYP2C19	38 ± 17	33 ± 15	0.85 ± 0.40
CYP2D6 * 1	31 ± 8.3	86 ± 22	2.8 ± 0.7
CYP2E1	38 ± 17	35 ± 15	0.91 ± 0.41
CYP3A4	2.3 ± 0.67	32 ± 8	14 ± 4

*Significantly different ($p < 0.05$) compared to mixed gender and male human liver microsomes.

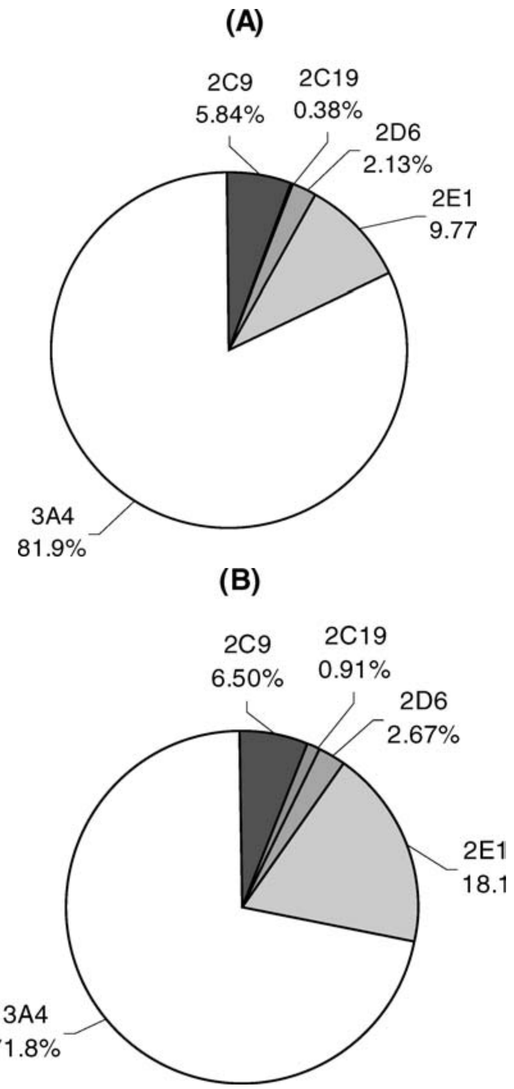

Figure 4 CYP reaction phenotype of 50 μg/ml ET-743 in female (A) and male (B) human liver microsomes, lot number 2 and 3 respectively.

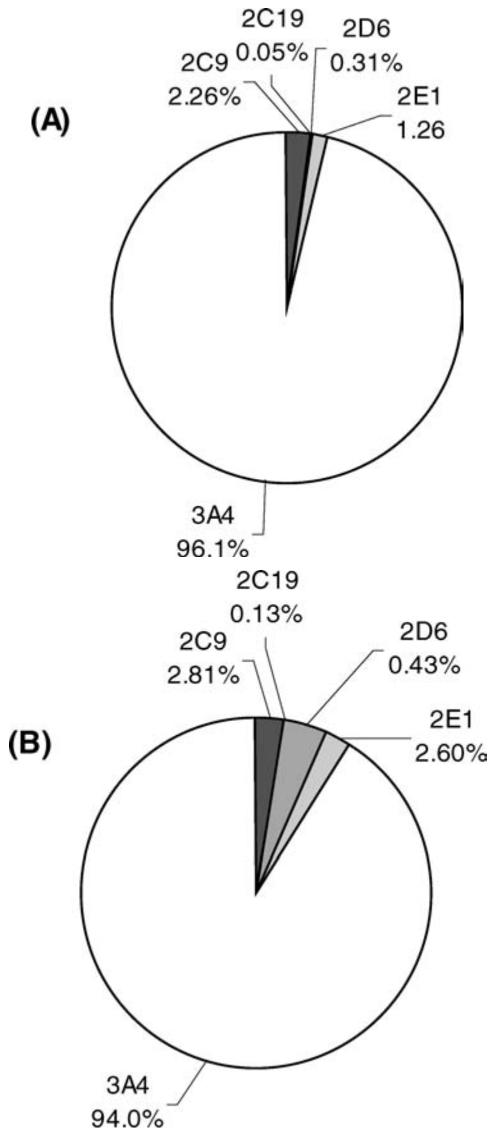

Figure 5 CYP reaction phenotype of 1 ng/ml ET-743 in female (A) and male (B) human liver microsomes, lot number 2 and 3 respectively.

order 2E1 > 2C9 > 2D6 > 2C19. One ng/ml ET-743 showed a slightly different order in the contribution of the other CYPs, namely 2C9 > 2E1 > 2D6 > 2C19.

Discussion and conclusion

ET-743 is a promising new anti-cancer agent in clinical trials. The biotransformation and CYP reaction phenotype of ET-743 in humans was investigated to support the pharmacokinetic findings of clinical studies and make predictions on drug-drug interactions for future co-treatment with other anti-cancer drugs.

Results from the CYP supersomes and inhibition experiments with human liver microsomes, indicate that CYP2C9, 2C19, 2D6, 2E1, and 3A4 may be involved in the biotransformation of ET-743 in the liver. The K_m values of ET-743 in the different CYP supersomes and the effects of the different CYP inhibitors on the biotransformation in human liver microsomes, reveal that CYP3A4 is the main CYP responsible for the biotransformation of ET-743 *in vitro*.

The validity of the method using CYP inhibitors combined with microsomes was already proved by others. The CYP inhibitors tested were selected using the human cytochrome P450 database from Gentest [20]. Sulfaphenazole, (S)-mephenytoin, and quinidine concentrations previously used were 5, 200, and 5 μM respectively [21]. Chlorzoxazone is a selective substrate for CYP2E1. However, it can also be used as a selective inhibitor for CYP2E1 and is used at concentrations between 10 and 500 μM [22, 23]. Furthermore, ritonavir is a potent CYP3A inhibitor at concentrations as low as 0.1 μM [24]. The inhibitor concentrations used in this study were within the range or above the concentrations used to inhibit CYPs by others. The results showed that quinidine, chlorzoxazone and ritonavir were able to inhibit the metabolism of ET-743 by human liver microsomes, which indicates that CYP2D6, 2E1, and 3A4 are involved in the biotransformation of ET-743. The lack of effect of the CYP2C9 and 2C19 inhibitors, indicates that both CYPs are hardly involved in the biotransformation of ET-743.

The pooled mixed gender human liver microsomes were formulated from material derived from at least 21 individuals and the single gender pools were derived from 5 or more male or female donors [13]. The pooled microsomes have been designed for a profile of catalytic activities representative for many individuals and for minimal lot-to-lot variability, however, significant variation between the different lot numbers may occur [13]. The V_{max} and K_m of ET-743 in female and male human microsomes were calculated for other lot numbers than those used to determine the percentage biotransformation after 4 h and thus the results of both experiments can not be compared without taking into account the differences in CYP activity levels. The biotransformation of ET-743 was not significantly different for mixed gender and female microsomes during the initial microsomal experiments, but male microsomes showed a significant lower ET-743 biotransformation rate. This small difference as well as the variation in V_{max} values (Table 3) were probably caused by the varying amounts of CYP3A4 and possibly other CYPs in the microsomal preparations. Typically, higher amounts of CYP3A4 and faster conversion of ET-743 were observed for female microsomes compared to male microsomes in both experiments. If the preparations are representative for the whole population, this indicates that on average the amount of CYP3A4 in the male liver is lower compared to female liver. Furthermore, a significantly lower K_m was observed for the female microsomes compared to the other preparations (Table 3). Unfortunately, it was not possible to explain this observation by differences in the activity of the individual CYPs in these female microsomes, only a polymorphism in one of the donors can be suggested as an explanation. The CYP reaction phenotyping allows the assessment of the relative contribution of the CYP forms to metabolic pathways [25, 26]. The data retrieved from the human liver fractions microsomes and CYP supersomes, widely used to characterize the metabolic profile of drugs, were used to identify CYP reaction phenotypes [27]. Useful predictions on the *in vivo* pharmacokinetics can be made by assessment of RAF from the results obtained with subcellular fractions [28, 29]. However, the interpretation of microsomal data is difficult because of the complex factors involved, like phase II reactions following phase I metabolism, and the number of different hepatic enzymes involved in the biotransformation of ET-743 [28, 29]. Furthermore, biotransformation is not influenced by drug transporters as these are lacking in microsomes and supersomes [29]. The lack of drug transporters could result in higher biotransformation rates in sub-cellular fractions compared to the human *in vivo* situation [27]. Despite this, the information obtained with CYP reaction phenotyping can be indicative for which *in vivo* drug interaction studies are required and can alert clinicians for the need of genotyped patients, when polymorphically expressed enzymes are involved in the biotransformation [25, 26, 30, 31]. The CYP reaction phenotype of 1 ng/ml ET-743 showed that CYP3A4 was the major isozyme involved in the biotransformation of ET-743 (~95%). The contribution of the other CYPs involved in the biotransformation were in the order 2C9 > 2E1 > 2D6 > 2C19. However, the contribution of CYP2D6 and 2C19 to the CYP reaction phenotype was less than 0.5%, thus *in vivo*, it is most likely that both isozymes are not significantly involved in the biotransformation of ET-743. According to the CYP reaction phenotype, it is expected that CYP2C9 and 2E1 will only slightly be involved in the biotransformation of ET-743 *in vivo*.

RAF can also be used for calculating the *in vitro* intrinsic clearance into *in vivo* pharmacokinetic clearance. The *in vitro* intrinsic clearance was shown to be comparable to the *in vivo* hepatic clearance for other compounds when scaling factors were applied [19, 29]. However, the human hepatic clearance for ET-743 has not decribed in literature. Therefore, no correlation can be made to the *in vivo* human situation. The *in vitro* intrinsic clearance by human liver microsomes was higher in female than in male microsomes and this may also be the case *in vivo* in patients treated with ET-743, the clinical relevance will discussed later.

The results indicate that CYP3A4 has an important role in the metabolism of ET-743. Therefore, the risk of *in vivo* drug-drug interaction, when ET-743 is combined with other CYP3A4 substrates, is present [32, 33]. Consideration is warranted when ET-743 treatment is given in combination with other anti-cancer drugs that are metabolized by CYP3A4 or drugs that influence its activity, e.g. doxorubicin [18, 34, 35]. Combination therapy of ET-743 with cisplatin, doxorubicin, and paclitaxel *in vitro* has shown sequence-dependent synergistic effects in combination with ET-743 in human breast, ovarian, and soft tissue cancer cell lines and a human rhabdomyosarcoma cell line [11, 21–24]. In a clinical trial, it was shown that combination therapy with dexamethasone, a known inducer of CYP-enzymes, increased hepatic clearance and reduced hepatotoxicity of ET-743 [27]. Furthermore, rats treated with ET-743 and the dietary agent indole-3-carbinol showed increased plasma clearance of ET-743 [36]. The same reduction was also observed in rats with other modulators of metabolism like β-naphtoflavone and phenobarbitone [37]. However, most of these data were obtained in rats, in which metabolism differs significantly compared to humans. D'Incalci and Jimeno [38] described that initial clinical results appeared to confirm that ET-743 in combination with other anticancer drugs showed more than additive effect. However, no pharmacokinetic studies in animals or humans have been described with ET-743 in combination with other cytostatics.

Furthermore, the intrinsic clearance and data obtained with the microsomes indicated that gender can play a role in the biotransformation and metabolic clearance in patients. However, gender differences are not always of clinical importance, due to high within-gender differences existing in CYP3A4 activity [39, 40]. Thus far, no gender differences in pharmacokinetics have been described for patients treated with ET-743. This emphasizes the influence of the high inter-individual variance in CYP3A4 activity on the pharmacokinetics of ET-743. The individual CYP isozyme activity is also influenced by food components, aging, disease and genetic polymorphisms [39, 40]. The genetic component in the inter-individual variability in CYP3A4 activity has been estimated to be between 60 and 90%, but the underlying genetic factors are largely unknown [41]. Furthermore, it is most likely that CYP3A5 (same substrates as CYP3A4) is capable of metabolizing ET-743. In less than 9% of the Caucasians, CYP3A5 is functional [42]. Patients with functional CYP3A5, may show a higher metabolic clearance of ET-743. It is of interest to explore whether genotyping the patients for CYP3A5 may contribute to the safety of the patients treated with ET-743 [25, 26].

ET-743 was glucuronidated *in vitro* by UGT2B15 in human UGT supersomes and by the UGT isozymes present in pooled human liver microsomes and S9 fraction. The tested UGT isozymes (UGT1A1, 1A3, 1A9, and 2B15) were chosen, because ET-743 is a large molecule and these enzymes are known to conjugate large molecules. The other UGT isozymes are known only to metabolize small molecules and are thus unlikely to glucuronidate ET-743. In addition, GST conjugated ET-743 in pooled human liver cytosol and S9 fraction. The other phase II enzyme studied, SULT, did not metabolize ET-743 in human cytosol and S9 fraction. Gender differences have been observed in humans with glucuronidation activity being higher in men than in women [39]. Inter-individual variability in GST activity in patients has also been observed. Furthermore, the individual UGT and GST activity is also influenced by aging, disease, food or drug intake, and genetic polymorphisms [40, 43]. The pharmacokinetics and toxicity of ET-743 in cancer patients caused by variation in UGT and GST activity should be taken into account. However, depending on the rate limiting step in the ET-743 metabolism (CYP, UGT or GST mediated), the inter-individual variance in activity of the enzyme of the rate limiting step is of clinical importance [39].

In conclusion, ET-743 metabolism in human liver microsomes, cytosol and S9 fraction was catalyzed by cytochrome P450 and the phase II enzymes UGT and GST. CYP3A4 was the predominant CYP metabolizing ET-743 and CYP2C9, 2C19, 2D6 and 2E1 play a minor role in the applied test systems. The role of these liver enzymes, especially CYP3A4 and UGT2B15, indicates that special care should be taken in patients with compromised liver functions and or liver metastasis due to a higher risk for hepatic toxicity. These findings can help to interpret the pharmacokinetic data obtained from the clinical trials with ET-743. In this respect it may be of interest to explore the usefulness of genotyping (CYP3A5) and the determination of the *in vivo* activity of CYP3A4.

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Address for offprints: Rolf W. Sparidans PhD, Pharmaceutical Sciences-Biomedical Analysis—Drug Toxicology, Utrecht University, Sorbonnelaan 16 3584, CA Utrecht, The Netherlands. Tel.: +31 (0)30-2537391, Fax: +31 (0)30-2535180; E-mail: R.W.Sparidans@pharm.uu.nl