In-vitro cytotoxicity of ET-743 (Trabectedin, Yondelis), a marine anti-cancer drug, in the Hep G2 cell line: influence of cytochrome P450 and phase II inhibition, and cytochrome P450 induction

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ET-743 is a marine anti-cancer drug and is currently in phase I trials in which the effect of combination therapies will be investigated. Its dose-limiting toxicity in patients is hepatotoxicity. In-vitro studies have shown that ET-743 is mainly metabolized by cytochrome P450 (CYP) 3A4, but also by 2C9, 2C19, 2D6 and 2E1, and the phase II enzymes uridine diphosphoglucuronosyl transferase and glutathione-S-transferase. Based on this metabolic profile, there is a risk of drug–drug interactions possibly influencing the hepatotoxicity of ET-743. Therefore, the effect of CYP and phase II activity on the cytotoxicity of ET–743 was investigated *in vitro* in a human cell line model system. The effect of different CYP and phase II inhibitors and CYP inducers on ET–743 cytotoxicity was studied after 48 and 120 h of treatment in Hep G2 cells using different assays. Furthermore, the toxicity of ET–743 metabolites was investigated. Potent cytotoxic activity of ET-743 after 120 h treatment was observed, which could be increased in combination with the CYP inhibitors metyrapone (3A4), phenanthrene (substrate for 2E1, 3A4), piperonyl butoxide (3A), proadifen (2C9, 2E1, 3A4), ritonavir (3A4), and warfarin (2C9, 2C19). No effect on the cytotoxicity of ET–743 was observed in combination with phase II enzyme inhibition and CYP induction. CYP metabolites of ET-743 were less toxic compared with ET–743. These findings indicate that combination therapy of ET-743 with CYP inhibitors, e.g. other anti-cancer drugs, could lead to changes in the hepatotoxicity of ET–743 and are therefore of clinical importance. *Anti-Cancer Drugs* 16:935–943

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Introduction

The Ecteinascidins are a group of tetrahydroisoquinolines isolated from the Caribbean tunicate *Ecteinascidia turbinata* [1]. The potent cytotoxicity of extracts of this tunicate were first discovered in the late 1960s and were identified 12 years ago. Ecteinascidin–743 (ET–743, Trabectedin, Yondelis) (Fig. 1) was selected for further development, based on its promising cytotoxic activity and relative abundance in the tunicate [2].

In-vitro studies with ET–743 in cell lines of human origin exhibited activity at nanomolar concentrations against various solid tumor cell lines, including melanoma, ovarian, renal, prostate, breast and non-small cell lung cancer cell lines. In addition, in-vivo ET–743 was effective against human xenografts of non–small cell lung cancer, melanoma and breast tumors [2,3]. In the clinic, activity against soft-tissue sarcomas, and breast, eudiometrical and ovarian cancer has already been shown in phase II trials [1,4–8].

Reid *et al.* [9] investigated the biotransformation of ET-743, and showed that ET–743 was metabolized by cytochrome P450 (CYP) 2C9, 2D6, 2E1 and 3A4 microsomes from transfected human B lymphocyte cell lines. Enzyme kinetic studies with human liver microsomes and CYP supersomes at our laboratory showed that ET-743 is metabolized primarily by CYP3A4. 2C9, 2C19 and 2D6, and 2E1 can also metabolize ET-743, but to a minor extent [10]. Sparidans *et al.* [11] also showed that ET–743 is conjugated by rabbit uridine diphosphoglucuronosyl transferase (UGT). In other studies at our

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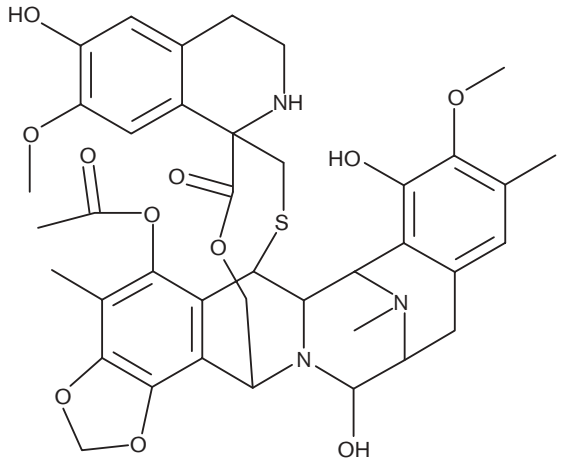
Fig. 1



Chemical structure of ET-743 [11]. $C_{39}H_{43}N_3O_{11}S$. Molecular weight = 769.1 g/mol.

laboratory, we showed that in-vitro human UGT2B15 is responsible for the glucuronidation of ET-743. In-vitro, ET–743 is also conjugated by glutathione-S-transferase (GST) [10]. These studies all unequivocally prove the importance of CYP enzymes and phase II enzymes for the metabolism of ET-743.

Combination therapy of ET-743 with cisplatin, doxorubicin and paclitaxel has already 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 [12–16]. It therefore seems very likely that patients are going to be treated with combinations of ET-743 and other anti-cancer drugs, of which several are known inhibitors or inducers of CYP and phase II enzymes [17]. As the role of these enzymes in the metabolism of ET-743 was unequivocally proven, it might be expected that these combinations will lead to clinically relevant drug interactions. At this moment, however, it is not clear whether the metabolism of ET-743 will increase or decrease the cytotoxicity of this drug in humans. Therefore, in this study the effect of CYP and phase II enzyme activities on the cytotoxicity of ET-743 *in vitro* in a human cell line was investigated in order to be able to predict drug–drug interactions in patients.

Materials and methods

Materials

ET-743 was kindly donated by PharmaMar (Tres Cantos, Madrid, Spain). Methanol (HPLC grade) and dichloromethane were purchased from Biosolve (Valkenswaard, The Netherlands) and formic acid (p.a.), $MgCl_26H_2O$ (p.a.) and DMSO (synthesis grade) from Merck (Darmstadt, Germany). Water was purified on a multi–laboratory scale by reversed osmosis. Mixed-gender pooled human liver microsomes, human CYP3A4 microsome standard and the WB-MAB-3A antibody were provided by Gentest (Becton Dickinson, Woburn, Massachusetts). Bovine anti-mouse IgG horseradish peroxidase (HRP) secondary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). RPMI–1640 medium (with L–glutamine and 25 mmol/l HEPES), MEM (with Earle's salt, without L–glutamine and phenol red), heat-inactivated FCS, penicillin/streptomycin, L–glutamine and HBSS (pH 7.4) were all obtained from Gibco/BRL (Breda, The Netherlands). Ritonavir was provided by Abbott (Chicago, Illinois, USA), and the lactate dehydrogenase kit (LDH), WST-1 assay kit and protease inhibitor cocktail tablets were obtained from Roche (Basel, Switzerland). All other chemicals were purchased from Sigma (St Louis, Missouri, USA) and were of analytical grade.

Cell culture growth

The human hepatic carcinoma cell line (Hep G2) was obtained form ATCC (Manassas, Virginia, USA). Routine cultivation of the monolayer cells was performed in RPMI–1640 medium (with L–glutamine and 25 mmol/l HEPES) supplemented with 10% (v/v) heat–inactivated FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were subcultured weekly [ratio of 1:5 (v:v)] and the medium was refreshed after 3 days.

Cytotoxicity of ET-743 in the absence and presence of cytochrome P450 inhibitors

Hep G2 cells (passage 89–110 or for the dexamethasone experiments 107–110) were seeded onto 96-well microtiter plates at a concentration of 8000 cells/well. The cells were cultured at 37°C, 5% CO_2 and 95% humidity. After 48 h, the cells were exposed to the inhibitors. Then, 20 µl medium per well was replaced by 20 µl medium with inhibitor. The cells were incubated for 1 h at 37°C, 5% CO_2 and 95% humidity. Next, the cells were exposed to ET–743 at concentrations of 0.001–500 ng/ml. Therefore, a concentration range of ET–743 with inhibitor (same concentration as in the wells) in medium was made and 20 µl was added to the wells, giving a final dilution of 1:10. In every experiment, every exposure was performed in triplicate. Cell growth was determined after 48 h using the LDH, WST-1 and SRB assay, and after 120 h using the LDH and SRB assay. Cell survival (%) was calculated relative to control cells and 100% killed cells (killed with 1% tributyltinchloride 1 h prior to the assay). Concentration–viability curves were constructed with this data and the IC_{50} (concentration of compound giving 50% cell death) was calculated by Softmax Pro 3.1 software (Molecular Devices, Sunnyvale, California, USA). Control experiments were performed without ET–743 and without inhibitor.

Effect of phase II inhibitors on the cytotoxicity of ET-743

The Hep G2 cells (passage 86–86) were seeded as described for CYP inhibitors and after 48 h the cells were

pre-incubated with the phase II inhibitors for 1 h. After addition of ET–743, the cytotoxicity was determined after 48 and 120 h of treatment with ET-743 as previously described.

ET-743 cytotoxicity after CYP induction with rifampicin or dexamethasone

The cells (passage 87–91) were seeded onto 96–well microtiter plates at a concentration of 8000 cells/well in the presence of 10 µmol/l rifampicin, 10 µmol/l dexamethasone or 0.1% DMSO (control) (concentrations were below the IC_5 value). After 2 days, the cells were exposed to ET–743 at concentrations of 0.001–500 ng/ml by replacing the medium with medium containing ET–743 and inducer. The IC_{50} values were determined after 48 and 120 h of treatment with ET-743 as previously described.

Cytotoxicity of ET-743 and metabolites formed by pooled human liver microsomes

The incubation procedure of ET-743 with human liver microsomes was a modification of the method described by Sparidans *et al.* [11]. Aliquots of 25 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 [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% (w/v) $NaHCO_3$], 7.5 µl of 20 mg/ml $MgCl_26H_2O$ solution and 50 µl of an aqueous ET-743 solution [1% (v/v) 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 pooled human liver microsomes (mixed gender, lot no. 21) was added. The tube was vortex mixed briefly again and the mixture was incubated at 37°C in a shaking water bath for 3 h (about 50% of the ET–743 was metabolized). The reaction was terminated by removing proteins using ultra–centrifugation with Micronon YM-10 ultra–centrifuge tubes (cutoff filter of 10 kDa) (Millipore, Bedford, Massachusetts, USA) for 90 min at 14 000 *g*. The polypropylene microtubes used to collect the filtrate were sterile and the filtrate was handled aseptically. The ultra–filtrate was injected for liquid chromatographic analysis to determine the ET-743 concentration. Control experiments were performed incubating for 3 h at 4°C, without liver microsomes and without ET–743. The ultra-filtrate was diluted in RPMI–1640 medium to an ET–743 concentration range of 0.001–500 ng/ml. The Hep G2 cells (passage 96–98) were seeded and treated as previously described for CYP inhibitors. The IC_{50} values were determined as previously described.

Analysis of ET-743 by gradient HPLC

The chromatographic assay was a modification of the method described by Sparidans *et al.* [11]. 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–$M10A_{VP}$ photodiode array detector (all from Shimadzu, Kyoto, Japan). Data were recorded on a Hermac Pentium 440, 122 MB personal computer (Scherpenzeel, The Netherlands) equipped with Class–VP 5.032 software (Shimadzu). Injections (50 µl) were made on a Symmetry C_{18} column (4.6 × 100 mm, d_p = 3.5 µmol/L Waters Chromatography, Milford, Massachusetts) with a Sentry Guard Symmetry C_{18} pre-column (3.9 × 20 mm, d_p = 5 µmol/l 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 and the peak areas were determined at 225 nm.

LDH assay

The LDH assay was performed as described in the kit protocol (Roche). Absorption was measured using a Versamax microtiter plate reader (Molecular Devices, Sunnyvale, California, USA). Data were recorded and analyzed on a Hermac Pentium 440, 122 MB personal computer equipped with the Softmax Pro 3.1 software (Molecular Devices).

WST-1 assay

The WST-1 assay was performed according to the protocol provided with the kit (Roche). Data were recorded and analyzed as previously described for the LDH assay.

SRB assay

The SRB assay was a modification of the method described by Higgins *et al.* [18]. The cell culture medium was removed and the cells were fixed in 100 µl of 10% (w/v) TCA for 60 min at 4°C. The wells were rinsed 3 times with tap water to remove solutes and cells were stained with 50 µl of 0.4% (w/v) SRB in 1% (v/v) acetic acid for 15 min. The cells were washed 3 times with 1% (v/v) acetic acid and air-dried. After drying, 120 µl of 10 M Tris in HBSS (pH 7.4) were added to solubilize the protein bound SRB. After mixing, the absorbance was measured at 540 nm using a Versamax microtiter plate reader. Data were recorded and analyzed as described for the LDH assay.

CYP3A4 protein levels

Cells (2.5×10^6) were plated in T25 culture bottles in the presence of 10 µmol/l rifampicin, 10 µmol/l dexamethasone or 0.1% DMSO (control). After 48 h, protein was isolated by scraping the cells and lysing them in PBS

containing 0.1% Triton X-100, 0.01% SDS, 0.1 mmol/l DTT and one protease inhibitor cocktail tablet. Total protein amounts were determined with a BCA protein assay kit according to the instructions of the manufacturer (Pierce, Rockford, Illinois). Total protein (25 μg) was separated by SDS–PAGE (10%) and then electroblotted onto Imobilon P (Millipore). After blocking overnight at 4°C with 3% BSA the membranes were incubated with anti-human CYP3A antibody WB-MAB-3A, followed by a bovine anti-mouse IgG HRP secondary antibody. The blots were developed with ECL reagents and the relative amount of CYP3A4 was calculated using a Gel Doc imaging system and Quantity One analysis software (Bio-Rad, Hercules, California). Human CYP3A4 standard (Gentest; Becton Dickinson) was used as a standard reference for these experiments.

Data analysis

The results are expressed as mean ± SD. Differences between the results were analyzed by Student's *t*-test for unpaired observations.

Results

IC_5 values of the inhibitors and inducers

Before using the different CYP and phase II inhibitors in combination with ET-743, the cytotoxicity of these compounds for the Hep G2 cell line was determined. Table 1 shows the IC_5 values of the used CYP and phase II enzyme inhibitors and the CYP inducers after 120 h, using the SRB assay. Berberine was shown to be the most cytotoxic in Hep G2 cells, with an IC_5 value of 1.2 μmol/l, while acetaminophen was the least toxic ($IC_5 > 500$ μmol/l).

Table 1 IC_5 values of CYP and phase II inhibitors and CYP inducers

Compound	Inhibition of	IC5 value (μmol/l)
CYP inhibitors		
chlorzoxazone	2E1	61
ketoconazole	1A1, 2A6, 2C8, 2C19, 2D6, 3A4	5
metyrapone	2A6, 3A4	>200
phenanthrene	2B6	60
piperonyl butoxide	3A	27
proadifen	2A6, 2B6, 2C9, 2E1, 3A4	10
ritonavir	3A4	18
sulfaphenazole	2C9	100
warfarin	2C9, 2C19	87
CYP inducers		
dexamethasone	CYP2A6, 2B6, 3A4, 4A11	80
rifampicin	1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2E1, 3A4	50
Phase II inhibitors		
acetaminophen	UGT	>500
berberine	NAT	1.2
2,6-dichloro-4-nitrophenol	SULT	20
S-hexylglutathione	GST	>200

Hep G2 cells were exposed to a concentration range of CYP and phase II inhibitors or inducers in triplicate for 120 h. Cytotoxicity was measured using the SRB assay and IC_5 values were determined using Softmax Pro 3.1 software.

Most of the inhibitors and inducers were used at a concentration below their IC_5 value, with the exception of ketoconazole, proadifen, sulfaphenazole and 2,6-dichloro-4-nitrophenol (DNP), which were used at their IC_5 concentration.

Cytotoxicity of ET-743 in combination with CYP inhibitors

The cytotoxicity of ET-743 in Hep G2 cells in combination with different specific inhibitors was determined by pre-incubating the cells for 1 h with the relevant inhibitor, followed by adding ET-743 in different concentrations. Using the SRB assay, it was shown that 10 μmol/l ritonavir (CYP3A4 inhibitor) significantly increased the cytotoxicity of ET-743 after 48 and 120 h (Fig. 2). Also, 200 μM metyrapone (2A6 and 3A4), 50 μmol/l phenanthrene (2B6), 10 μmol/l piperonyl butoxide (3A), 10 μmol/l proadifen (2A6, 2B6, 2C9, 2E1 and 3A4) and 50 μmol/l warfarin (2C9 and 2C19) significantly decreased the IC_{50} value of ET–743 (Fig. 2 and Table 2). The same pattern was observed after 48 h with the LDH and WST-1 assays, and after 120 h with the LDH assay; however, not all decreases were significant (Table 2). These results indicate that metabolism of ET-743 decreases the cytotoxicity of this anti-cancer drug. The direct effect of dexamethasone was studied by adding 50 μmol/l dexamethasone together with ET-743 using the same procedure as with the other inhibitors. Co-administration of dexamethasone and ET-743, however, had no statistically significant effect on the cytotoxicity of ET-743.

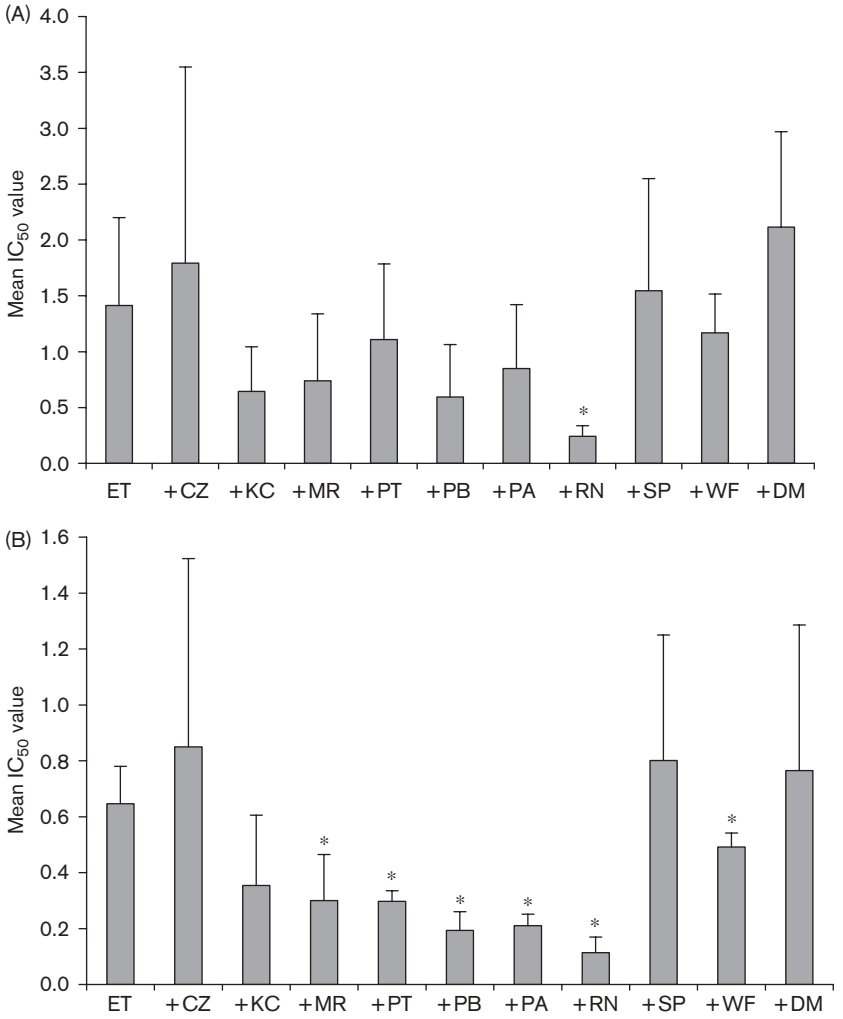
ET-743 cytotoxicity in the presence of phase II inhibitors

The effect of phase II inhibition on the cytotoxicity of ET-743 was tested using four different inhibitors: 200 μmol/l acetaminophen which inhibits UGT enzymes, 1 μmol/l berberine to inhibit *N*–acetyltransferase (NAT), 20 μmol/l of the sulfotransferase (SULT) inhibitor DNP or 200 μmol/l *S*–hexylglutathione to diminish GST activity. The observed IC_{50} values of ET–743 in the Hep G2 cells in these experiments after 48 and 120 h, using the SRB assay, were respectively 0.64 ± 0.33 and 0.41 ± 0.17 μg/ml (Table 3). No significant effect on the IC_{50} values, and therefore the cytotoxicity of ET-743, could be observed in the presence of any of these phase II inhibitors tested. The direct metabolism of ET-743 by phase II inhibitors therefore has no effect on the cytotoxicity of ET-743 in Hep G2 cells.

IC_{50} values of ET-743 after CYP induction

To examine the effect of induction of CYP enzymes on ET-743 cytotoxicity, Hep G2 cells were pre-incubated for 2 days in the presence of 10 μmol/l rifampicin or 10 μmol/l dexamethasone, before exposure to ET-743. These inducers are both well-known inducers of CYP enzymes, also in the Hep G2 cell line. Rifampicin induces CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2E1 and 3A4, while

Fig. 2



ET-743 cytotoxicity (IC_{50} values) in the presence of CYP inhibitors. Hep G2 cells [passage 89–110 or 107–110 (dexamethasone experiments)] were pre-incubated in triplicate with 50 μmol/l chlorzoxazone, 5 μmol/l ketoconazole, 200 μmol/l metyrapone, 50 μmol/l phenanthrene, 10 μmol/l piperonyl butoxide, 10 μmol/l proadifen, 10 μmol/l ritonavir, 100 μmol/l sulfaphenazole, 50 μmol/l warfarin or 50 μmol/l dexamethasone for 1 h. After addition of a concentration range of 0.001–500 ng/ml of ET-743, the cells were grown for (A) 48 or (B) 120 h. IC_{50} values (ng/ml) were determined using the SRB assay and Softmax Pro 3.1 software. Data are the means ± SE obtained from three experiments, with the exception of ET-743, which is the mean of 12 independent experiments. ET, ET-743; CZ, chlorzoxazone; KC, ketoconazole; MR, metyrapone; PT, phenanthrene; PB, piperonyl butoxide; PA, proadifen; RN, ritonavir; SP, sulfaphenazole; WF, warfarin and DM, dexamethasone. *Significantly different (*$P < 0.05$) compared with ET–743.

dexamethasone is able to increase the expression and activity of CYP2A6, 2B6, 3A4 and 4A11. In this experiment, no significant effect of treatment of the cells with these inducers on the cytotoxicity of ET–743 could be observed (Table 4).

CYP3A4 protein levels over the 2 days of induction were verified with Western blotting, clearly showed a significant, although modest increase, in CYP3A4 expression compared with control cells after exposure to both rifampicin or dexamethasone in this experimental set-up (Fig. 3). Human CYP3A4 microsomes were used as a standard reference in this experiment.

The results of these experiments indicate that the induction of CYP enzymes did not influence cytotoxicity in the used cell model, i.e. Hep G2 cells.

Cytotoxicity of ET-743 and its metabolites formed by cytochrome P450 in human liver microsomes

A direct way to measure the difference in cytotoxicity between ET-743 itself and its metabolites is to incubate the Hep G2 cells with the metabolites. Therefore mixed pooled human liver microsomes (Gentest; Becton Dickinson) were incubated for 3 h with 50 μl of aqueous ET-743 solution. After this incubation period about 50% of the ET-743 was metabolized (results not shown). The

Table 2 ET-743 cytotoxicity (IC_{50} values) in the presence of CYP inhibitors

Inhibitor	48 h			120 h	
	LDH	WST	SRB	LDH	SRB
None	3.34 ± 1.45	1.07 ± 0.72	1.41 ± 0.79	0.91 ± 0.22	0.65 ± 0.13
Chlorzoxazone	3.25 ± 1.09	1.36 ± 1.37	1.79 ± 1.76	0.92 ± 0.12	0.85 ± 0.67
Ketoconazole	1.61 ± 1.02	1.01 ± 0.95	0.64 ± 0.40	0.80 ± 0.21	0.35 ± 0.25
Metyrapone	3.13 ± 0.43	0.53 ± 0.16[a]	0.74 ± 0.60	0.75 ± 0.66	0.30 ± 0.16[a]
Phenanthrene	1.69 ± 1.21	0.49 ± 0.59	1.11 ± 0.68	1.20 ± 0.27	0.30 ± 0.04[a]
Piperonyl butoxide	0.62 ± 0.20[a]	0.17 ± 0.05[a]	0.60 ± 0.47	0.49 ± 0.11[a]	0.19 ± 0.07[a]
Proadifen	0.73 ± 0.28[a]	0.26 ± 0.11[a]	0.85 ± 0.57	1.26 ± 0.69	0.21 ± 0.04[a]
Ritonavir	0.99 ± 0.51[a]	0.40 ± 0.50	0.24 ± 0.09[a]	0.49 ± 0.11[a]	0.11 ± 0.06[a]
Sulfaphenazole	1.88 ± 0.30[a]	1.19 ± 0.72	1.55 ± 1.00	1.22 ± 0.33	0.80 ± 0.45
Warfarin	3.24 ± 0.77	0.25 ± 0.16[a]	1.17 ± 0.35	0.83 ± 0.06	0.49 ± 0.05[a]
Dexamethasone	5.50 ± 1.63	1.25 ± 0.39	2.11 ± 0.85	1.20 ± 0.55	0.76 ± 0.52

[a]Significantly different ($P < 0.05$) compared with no inhibitor. Hep G2 cells [passage 89–110 or 107–110 (dexamethasone experiments)] were pre-incubated in triplicate with 50 μmol/l chlorzoxazone, 5 μmol/l ketoconazole, 200 μmol/l metyrapone, 50 μmol/l phenanthrene, 10 μmol/l piperonyl butoxide, 10 μmol/l proadifen, 10 μmol/l ritonavir, 100 μmol/l sulfaphenazole, 50 μmol/l warfarin or 50 μmol/l dexamethasone for 1 h. After addition of a concentration range of 0.001–500 ng/ml ET-743, the cells were grown for 48 or 120 h. IC_{50} values (ng/ml) were determined using the SRB, LDH or WST-1 assay and Softmax Pro 3.1 software. Data are the means ± SE obtained from five experiments.

Table 3 ET-743 cytotoxicity (IC_{50} values) in the presence of phase II inhibitors

Inhibitor	48 h			120 h	
	LDH	WST	SRB	LDH	SRB
None	2.20 ± 0.59	0.36 ± 0.32	0.64 ± 0.33	1.03 ± 0.90	0.41 ± 0.17
Acetaminophen	1.56 ± 0.45	0.32 ± 0.27	0.82 ± 0.21	1.27 ± 0.68	0.22 ± 0.09
Berberine	2.07 ± 0.64	0.33 ± 0.08	0.67 ± 0.25	0.99 ± 0.42	0.32 ± 0.11
DNP	2.00 ± 0.69	0.34 ± 0.27	1.34 ± 0.54	0.74 ± 0.34	0.39 ± 0.12
S-hexylglutathione	2.32 ± 1.52	0.47 ± 0.08	0.80 ± 0.29	0.84 ± 0.56	0.41 ± 0.17

Hep G2 cells (passage 86–110) were pre-incubated in triplicate with 200 μmol/l acetaminophen (UGT), 1 μmol/l berberine (NAT), 20 μmol/l DNP (SULT), 200 μmol/l S–hexylglutathione (GST) or 0.1% DMSO (control) for 1 h. After addition of a concentration range of 0.001–500 ng/ml ET-743 the cells were grown for 48 or 120 h. IC_{50} values (ng/ml) were determined using the SRB, LDH or WST-1 assay and Softmax Pro 3.1 software. Data are the means ± SE obtained from five experiments.

Table 4 ET-743 cytotoxicity (IC_{50} values) after pre-incubation with CYP inducers

Inducer	48 h			120 h	
	LDH	WST	SRB	LDH	SRB
None	1.26 ± 0.59	0.52 ± 0.41	1.33 ± 0.72	0.80 ± 0.29	0.27 ± 0.18
Rifampicin	1.03 ± 0.37	0.54 ± 0.46	1.30 ± 0.98	0.85 ± 0.72	0.18 ± 0.11
Dexamethasone	1.72 ± 0.79	0.53 ± 0.39	1.34 ± 0.85	1.21 ± 0.46	0.27 ± 0.13

Hep G2 cells (passage 87–91) were seeded in triplicate in the presence of 10 μmol/l rifampicin or 10 μmol/l dexamethasone and grown for 48 h. After 2 days, medium was replaced with medium containing 0.001–500 ng/ml ET-743 and the inducers and after 48 or 120 h IC_{50} values (ng/ml) were determined using the SRB, LDH or WST-1 assay and Softmax Pro 3.1 software. Data are the means ± SE obtained from five experiments.

reaction was stopped by removing the proteins by centrifugation, and the cells were exposed to the solution containing ET-743 metabolites and which was corrected for ET-743 content. The results showed that the cytotoxicity of CYP metabolites of ET-743, formed by human liver microsomes, is most likely less toxic compared to ET–743 (Table 5). However, only a slight decrease in the IC_{50} value could be observed for ET-743 with its CYP metabolites after 48 h compared with ET–743 alone, which was not significant ($P > 0.05$) and not observed after 120 h.

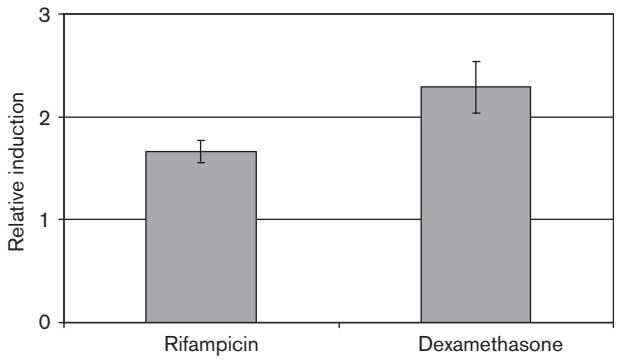
Discussion and conclusions

ET-743 has shown activity as a single agent in clinical trials in patients with advanced cancer. The clinical efficacy of ET-743 in combination with other anti-cancer drugs is now under investigation. However, the metabolic profile of ET-743 indicates that drug–drug interactions could be a risk factor in the application of combination therapy [9], especially because of the small therapeutic index of ET-743 which makes small changes in ET-743 concentration clinically important. Therefore, the effects of CYP and phase II inhibition and CYP induction on the cytotoxicity of ET-743 were investigated.

An increase of the cytotoxicity of ET-743 (IC_{50} decrease) could be observed in the Hep G2 cell line after incubation with the CYP inhibitors metyrapone (2A6 and 3A4), phenanthrene (2B6), piperonyl butoxide (3A), proadifen (2A6, 2B6, 2C9, 2E1 and 3A4), ritonavir (3A4) and warfarin (2C9 and 2C19). This indicates that the inhibited CYPs are involved in the biotransformation of ET–743 in humans and that the metabolites formed are

less toxic compared to ET-743. The role of CYP2C9, 2C19, 2E1 and 3A4 in the metabolism of ET-743 was previously confirmed by in-vitro enzyme kinetic studies, which showed that ET-743 is metabolized by these enzymes and CYP2D6, but not by other CYPs [9]. However, the major part of ET-743, an estimated amount of about 95%, will be metabolized by CYP3A4 [10]. Phenanthrene is an inhibitor of CYP2B6, but it is also a substrate for CYP2E1 and 3A4 [19]. As it was previously shown in our laboratory that ET–743 is not metabolized by CYP2B6, most likely competitive inhibition of either CYP2E1, 3A4 or both is involved in the inhibiting effect of phenanthrene. Dexamethasone is an inhibitor and inducer of CYP3A4 [17], but under the inhibition conditions tested (no pre–incubation) dexamethasone had no significant direct effect on the cytotoxicity of ET–743. The lack of a significant effect of chlorzoxazone (2E1), ketoconazole (1A1, 2A6, 2C8, 2C19, 2D6 and 3A4), sulfaphenazole (2C9) and dexamethasone (3A4) may be caused by incomplete inhibition of the isozyme(s) due to the practical limitation that the concentration had to be below the IC_5 value to prevent a direct cytotoxic effect of the inhibitors on the viability of the cells.

Fig. 3



Relative induction of CYP3A4 protein in Hep G2 cells. Hep G2 cells were seeded in the presence of 10 μmol/l rifampicin, 10 μmol/l dexamethasone or 0.1% DMSO (control). After 48 h, protein was isolated and immunoblotted with a CYP3A4-specific antibody, using a CYP3A4 protein standard as a reference. Relative inductions compared to the control were determined using Quantity One software. Data are the means ± SE obtained from three experiments. *Significantly different ($P<0.05$) compared to control.

Inhibition of phase II enzymes by acetaminophen (UGT), berberine (NAT), DNP (SULT) and S–hexylglutathione (GST) showed no significant influence on the cytotoxicity of ET-743. Previous in-vitro studies showed that ET-743 can be conjugated by UGT and GST, and phase II metabolism in general was already successfully studied by others in the Hep G2 cell line [10,20–24]. The lack of effect in this study indicates that in Hep G2 cells, phase II enzymes are not significantly involved in the biotransformation and detoxification of ET–743, and that CYP metabolism may be the rate-limiting step. This was confirmed in *in vitro* studies in pooled human liver S9 fractions, where it was shown that ET–743 was preferably metabolized by CYPs compared with UGTs or GSTs [10].

Based on the human CYP database, different CYP inhibitors were selected [19]. The applied concentrations were selected from results obtained in other studies in which the effect of CYP inhibitors on the biotransformation in human primary cells or cell lines was investigated. The phase II enzyme inhibitors tested were also selected based on other studies [25–28]. Acetaminophen is normally used as a substrate for UGT, mainly UGT1A1, 1A6 and 2B7, but in this study it was used as a competitive inhibitor for UGT [28]. As with the CYP inhibitors, the applied concentrations were limited by their IC_5 value in Hep G2 cells. This could possibly lead to a lack of effect of some inhibitors due to concentrations below a minimal level needed for complete inhibition.

The metabolites of ET-743 formed by cytochrome P450 in human liver microsomes were found to be less, although not significantly, toxic than ET-743 itself. Together with the decrease in IC_{50} value of ET-743 in combination with specific CYP inhibitors, this indicates that ET-743 is detoxified in the liver.

This study showed that upregulation of CYP3A4, as confirmed with immunoblotting, after induction with rifampicin and dexamethasone did not result in a significant change in the IC_{50} value of ET-743 in the Hep G2 cell line. In-vitro studies by Reid *et al.* [9] and at our laboratory [10] with human liver microsomes showed a high metabolic conversion rate of ET–743, depending

Table 5 Cytotoxicity of ET-743 metabolites (IC_{50} values)

ET-743 incubation	48 h			120 h	
	LDH	WST	SRB	LDH	SRB
No HLM at 37°C	4.28 ± 0.58	2.18 ± 0.77	3.55 ± 1.19	3.22 ± 0.51	1.94 ± 0.17
HLM at 37°C	3.66 ± 0.57	1.71 ± 0.93	3.20 ± 0.71	2.21 ± 0.50	2.05 ± 0.07
HLM at 4°C	4.19 ± 1.08	2.05 ± 0.12	3.42 ± 0.91	2.38 ± 0.41	2.04 ± 0.31

ET-743 (50 μg/ml) was incubated in triplicate with pooled mixed human liver microsomes (HLM) for 3 h at 37°C. ET-743 and its metabolites were retained by ultrafiltration and added to the Hep G2 cells. After 48 or 120 h IC_{50} values (ng/ml) were determined using the SRB, LDH or WST-1 assay and Softmax Pro 3.1 software. Data are the means ± SE obtained from three experiments.

on the CYP3A4 activity levels. Based upon these data, it is expected that the half-life of ET-743 in Hep G2 cells is very short compared to the incubation time in the present experiments, leading to a short exposure compared to its less-toxic metabolites. Shortening this exposure time to the parent compound even further by induction of CYP3A4 may therefore not result in a measurable decrease of the total toxicity during the whole duration of the experiment. However, adding inhibitors of metabolism will most likely increase the exposure time to ET-743 by a significant factor, increasing its contribution to the cytotoxicity. This was confirmed in the CYP inhibition experiments.

In a clinical trial, it was shown that combination therapy of ET-743 with dexamethasone increased the hepatic clearance and reduced the hepatotoxicity [17]. The hepatotoxic potential of ET-743 in female rats was also decreased by pre-treatment with dexamethasone and other modulators of drug metabolism, e.g. indole-3-carbinol [29,30]. However, comparable with our experiments, Donald *et al.* [31] showed that the cytotoxicity of ET-743 in vitro did not significantly decrease in primary rat hepatocytes isolated from animals treated with dexamethasone. This discrepancy of in-vitro and in-vivo results warrants further studies both in-vitro and in-vivo to determine the influence of cytochrome P450 induction on the cytotoxicity and therapeutic efficacy of ET–743 in patients. The mechanism proposed by Donald *et al.* [30] that dexamethasone and indol-3-carbinol interfere with the NF-κB transcriptional activity, and therefore decrease the ET-743 hepatotoxicity, could be valid. However, it does not explain the decrease in ET-743 plasma levels, indicating an increase in clearance. To further investigate the in-vitro effect of CYP induction on the toxicity of ET–743, another experimental set-up should therefore be used, where cytotoxicity is measured after a shorter time period of incubation, with other more sensitive assays and maybe a different in-vitro cell model.

The Hep G2 cell line is the most frequently used and best-characterized human hepatoma cell line [32]. This cell line has a variety of liver-specific metabolic functions. Under standard culturing conditions, the cells show stable, but relatively low, levels of CYP and phase II enzymes, inducible by pre-treatment with inducing agents [32,33]. However, although the relative proportions of CYPs in the Hep G2 cells are comparable to those in normal human livers [10], the overall CYP activity remains low in Hep G2 cells [34]. However, freshly isolated human hepatocytes are difficult to obtain; furthermore, a disadvantage is the high inter-individual variability and a gradual loss of liver-specific functions during cultivation, with special reference to decreased CYP expression [32]. Although the Hep G2 cell line also shows inter-passage variation in the expression of CYP enzymes [35], it is still a good alternative model to study the effect of CYP inhibitors and inducers, and phase II enzyme inhibitors on the cytotoxicity of ET–743. Furthermore, this inter-passage variation in the expression levels of several CYPs and phase II enzymes between different passages of Hep G2 cells could explain the high SDs in ET–743 cytotoxicity between different passages and also the lack of a significant effect of some of the applied inhibitors on the cytotoxicity of ET-743.

Three different cytotoxicity assays were used, each having their own specific endpoint: measurement for the mitochondrial activity (cellular metabolic function) of the cell (WST-1 assay), detection of the release of the cytosolic component LDH into the culture medium (LDH assay) and measurement of cellular protein material attached to the plate (SRB assay) indicative for the number of living cells [18,36,37]. Luber-Narod *et al.* [38] showed that combining different cytotoxicity assays provides the best information about the cytotoxicity of chemotherapeutic drugs as ET-743 and, therefore, all three assays were used. In our experimental set-up the most sensitive and reproducible method was shown to be the SRB assay. Two different time points were used, indicative of short-term (48 h) and long-term (120 h) exposure to ET-743.

Based on the in-vitro cytotoxicity results, there is a potential risk for drug–drug interactions between ET-743 and CYP3A4 inhibitors or inducers, but also with CYP2C9, 2C19, 2D6 and 2E1 substrates [39,40]. They warrant consideration when ET–743 treatment is given in combination with other anti–cancer drugs, e.g. cisplatin, paclitaxel and doxorubicin, which are capable of CYP modulation [17,41,42]. Co–treatment with CYP3A4 inhibitors could lead to reduced metabolism and thus reduced hepatic clearance. In this study, it was shown that metabolites of ET-743 are less toxic than ET-743; therefore, reduced clearance could lead to increased hepatotoxicity. The difficulty with some of the applied compounds is that they are inhibitors (direct effect) and inducers (long-term effect), like paclitaxel. Therefore, in clinical trials with combination therapies, extra attention should be paid to the final effect: hepatic toxicity when plasma levels increase or decreased plasma levels, possibly reducing the efficacy of the therapy. It is important to realize that the changes in hepatotoxicity, as a consequence of changes in the metabolism of ET-743, will also possibly correlate with changes in anti-tumor activity. This aspect deserves attention and should also be studied in patients.

In conclusion, ET-743 showed high cytotoxicity in a human cell line, i.e. Hep G2 cells, which could be increased by CYP2C9, 2C19, 2D6, 2E1 and 3A4 inhibitors, while phase II enzyme inhibitors had no influence on the toxicity. Most likely, CYP metabolism is the rate-limiting step in the detoxification of ET–743. These results show the clinical importance of the

metabolism of ET-743 by CYP enzymes, which could lead to severe drug–drug interactions when ET-743 is combined with other anticancer drugs.

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