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ORIGINAL ARTICLE

Delineation of the interactions between the chemotherapeutic agent eribulin mesylate (E7389) and human CYP3A4

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Abstract
Purpose Eribulin mesylate (E7389), a structurally simplified, synthetic analog of the marine natural product halichondrin B, acts by inhibiting microtubule dynamics via mechanisms distinct from those of other tubulin-targeted agents. Eribulin is currently in Phase III clinical trials for the treatment of metastatic breast cancer. Since drug-induced modulation of cytochrome P450 enzymes, particularly CYP3A4, is a frequent cause of drug–drug interactions, we examined the effects of eribulin on the activity and expression of hepatic and recombinant CYP3A4 (rCYP3A4) in vitro.
Methods Identification of the enzyme(s) responsible for eribulin metabolism was based on compound depletion and metabolite formation in reaction mixtures containing subcellular liver fractions or primary human hepatocytes, plus recombinant Phases I and II metabolic enzymes. The role of the enzyme(s) identified was confirmed using enzyme-selective inhibitors and the correlation with prototypic enzyme activity. The effect of eribulin on enzymatic activity was characterized using both microsomal preparations and recombinant enzymes, while the possible modulation of protein expression was evaluated in primary cultures of human hepatocytes.
Results Eribulin was primarily metabolized by CYP3A4, resulting in the formation of at least four monooxygenated metabolites. In human liver microsomal preparations, eribulin suppressed the activities of CYP3A4-mediated testosterone and midazolam hydroxylation with an apparent K_i of approximately 20 μM. Eribulin competitively inhibited the testosterone 6β-hydroxylation, nifedipine dehydration, and *R*-warfarin 10-hydroxylation activities of rCYP3A4, with an average apparent K_i of approximately 10 μM. These inhibitions were reversible, with no apparent mechanism-based inactivation. Eribulin did not induce the expression or activities of CYP1A and CYP3A enzymes in human primary hepatocytes, and clinically relevant concentrations of eribulin did not inhibit CYP3A4-mediated metabolism of various therapeutic agents, including carbamazepine, diazepam, paclitaxel, midazolam, tamoxifen, or terfenadine.
Conclusions Eribulin was predominantly metabolized by CYP3A4. Although eribulin competitively inhibited the testosterone 6β-hydroxylation, nifedipine dehydration, and *R*-warfarin 10-hydroxylation activities of rCYP3A4, it did not induce or inhibit hepatic CYP3A4 activity at clinically relevant concentrations. As eribulin does not appear to affect the metabolism of other therapeutic agents by CYP3A4, our data suggest that eribulin would not be expected to inhibit the metabolism of concurrently administered drugs that are metabolized by CYP3A4, suggesting a minimal risk of drug–drug interactions in the clinical setting.

Keywords Cancer therapy · Cytochrome p450 enzymes · Drug–drug interactions · Eribulin

Some data from this study have previously been reported in poster format at the 2003 North American International Society for the Study of Xenobiotics meeting in Providence, RI, USA.

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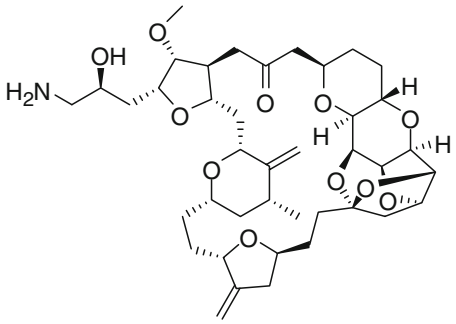
Introduction

Eribulin mesylate (E7389) is a structurally simplified, synthetic analog of the marine natural product halichondrin B [1]. Like its parent compound, eribulin (Fig. 1)



eribulin

Fig. 1 Chemical structure of eribulin

exerts its chemotherapeutic effect through binding to tubulin and inhibiting microtubule dynamics. This prevents mitotic spindle formation and results in irreversible mitotic block which, in turn, triggers cell-cycle arrest at the G_2/M phase and apoptosis [2–5]. Compared with other tubulin-targeted agents, eribulin has a novel effect on microtubule dynamics, as it suppresses microtubule polymerization, exhibits no effect on depolymerization, and sequesters tubulin into nonfunctional aggregates [3]. In preclinical studies, eribulin appeared to be pharmacologically potent with a wide therapeutic window against a number of cancer-derived cell lines and human tumor xenografts [5]. Eribulin is the first analog of halichondrin B to reach clinical testing. In clinical trials, preliminary data indicate that eribulin has promising activity and a manageable tolerability profile in the treatment of metastatic breast cancer in patients who have previously received a median of four chemotherapy regimens, including an anthracycline and a taxane [6]. Furthermore, preclinical data and early clinical results indicate that eribulin has a broad spectrum of activity beyond the treatment of breast cancer [5, 7–10], and it is currently in development for the treatment of non-small-cell lung cancer, prostate cancer, soft-tissue sarcoma, and ovarian cancer.

Many natural product-derived chemotherapeutic agents interact with, and are metabolized by, cytochrome P450 enzymes, especially CYP3A4 [11]. CYP3A4 is abundantly expressed in human liver and intestinal epithelia, and is primarily responsible for the metabolism of nearly half of currently available therapeutic agents [12]. For example, paclitaxel is a widely used chemotherapeutic agent and a high affinity substrate for CYP2C8 and CYP3A4 that inhibits several metabolic CYP enzymes [13] and upregulates the CYP3A4 gene via activation of the human pregnane X receptor (hPXR) [11]. Moreover, several chemotherapeutic agents and associated metabolites (e.g., tamoxifen and its metabolite *N*-desmethyltamoxifen) are converted to reactive intermediates and then bind covalently to the very enzymes responsible for their metabolism. This mode of enzyme inactivation following substrate bioactivation is known as mechanism-based inactivation, which has been recognized as a key contributor to drug–drug interactions (DDIs) in the clinical setting [14].

The DDIs are frequently reported in cancer therapy because cancer patients often take a plethora of drugs, either as part of combination therapy or for the treatment of concurrent diseases and treatment-derived complications [15]. However, modulation of drug activities between concomitantly administered medications can sometimes be favorable. For example, a drug may inhibit the inactivation and clearance of concurrently administered agents, thus prolonging their therapeutic presence and enhancing their efficacy [16]. Nevertheless, understanding the metabolism of eribulin is essential to its clinical use, either for the prevention of potentially negative DDIs or for planning its use in combination therapy. Consequently, we investigated the interactions of the chemotherapeutic agent eribulin with the metabolic enzyme, CYP3A4, in both human liver microsomes (HLMs) and primary hepatocytes. Due to the wide substrate specificity and induction by a vast array of structurally diverse compounds, we have investigated multiple aspects of potential interactions, including modulation of CYP3A4 enzyme expression.

Methods

Materials

Chemicals

Eribulin was synthesized at Eisai Research Institute (Andover, MA, USA). Testosterone and 6β-hydroxytestosterone were purchased from Steraloids (Newport, RI, USA). Desmethyltamoxifen was obtained from Toronto Research Chemical (Toronto, Canada). (±)-Bufuralol, (±)-1′-hydroxybufuralol, 6-hydroxychlorozoxazone, *S*-mephenytoin, 4′-hydroxy-*S*-mephenytoin, nifedipine, dehydronifedipine, and monohydroxylated warfarin metabolites (6-, 7- and 10-hydroxywarfarin), and 3α-hydroxypaclitaxel were purchased from Discovery Labware, BD Biosciences (Bedford, MA, USA). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from the National Cancer Institute Chemical Carcinogen Reference Standard Repositories (MRI, Kansas City, MO, USA). 6′,7′-dihydroxy-bergamottin (DHB) was obtained from Ultrafine Chemical Corp. (Manchester, UK). Optically pure *S*- and *R*-warfarin (>98%) were prepared from racemic mixture as described [17]. All other chemicals were purchased from Sigma-Aldrich Corp. (St Louis, MO, USA).

Enzymes and hepatocytes

Pooled or individual HLM, S9 preparations, and recombinant cytochrome P450 enzymes (rCYPs) were purchased from Discovery Labware, BD Biosciences, Xenotech Corp. (Kansas City, KS, USA), or Invitrogen (Madison, WI, USA). Primary cultures of human hepatocytes were obtained from In Vitro Technologies, CellzDirect (Pittsboro, NC, USA), or Discovery Labware, BD Biosciences. None of the hepatocyte donors had liver disease or damage (Table 1).

Antibodies

Polyclonal goat anti-human CYP1A1/2 and CYP3A4 antibodies were obtained from Discovery Labware, BD Biosciences. Polyclonal rabbit and monoclonal mouse anti-human CYP1A1 antibodies were purchased from Chemicon International Corp. (Temecula, CA, USA), and monoclonal anti-human CYP1A2 from Invitrogen. Secondary antibodies were from Sigma-Aldrich Corp.

Eribulin biotransformation assays

Eribulin [at 1, 5, or 80 μM in either Tris or phosphate-buffered saline (PBS) buffers] was incubated in reaction mixtures containing 0.5 mg HLM proteins or 25–50 pmol of recombinant enzymes. After incubating at 37°C for 1 min, reactions were initiated by adding 25 μl NADPH (20 mg/ml) or the co-substrates (4 mM UDPGA, 0.4 mM AAPS, 100 mM GSH, or 5 mM acetyl CoA), and incubated for 30–120 min. Reactions were terminated by the addition of methanol or 0.1% formic acid in methanol.

Incubations containing liver microsomes from individual donors were used to study the correlation between eribulin metabolism and CYP3A4 activity (testosterone 6β-hydroxylation). Substrate and chemical inhibitors, terfenadine, ketoconazole, and DHB were used to confirm CYP3A4 involvement in eribulin metabolism. These were prepared in methanol, and 5 μl was added 5 min prior to eribulin.

CYP inhibition profiling and characterization

Incubation mixtures (250 μl) contained 0.5 mg HLM protein or 25–50 pmol of recombinant human CYPs, eribulin (1 or 5 μM), a probe substrate (*R*-, *S*-warfarin, chlorzoxazone, *S*-mephenytoin, bufuralol, testosterone, or nifedipine), and 0.5 mg of NADPH in 50 mM Tris buffer. Eribulin was added 5 min prior to the addition of the probe substrate, then incubated for 1 min at 37°C. The reaction was initiated with 25 μl NADPH solution (20 mg/ml), and incubated for 8–60 min.

The CYP3A4 substrates midazolam, nifedipine, testosterone, and *R*-warfarin, and eribulin (0–40 μM), were used to determine the inhibition constant (K_i). Apparent K_i values were estimated by Dixon plots and simultaneous nonlinear regression analyses (SNLR).

Time- and NADPH-dependent CYP3A4 inhibition was performed in comparison with the prototypic controls (terfenadine and DHB) [18]. Briefly, 5 μl eribulin stock solution (10 mM) was pre-incubated with rCYP3A4 for up to 60 min at 37°C. CYP3A4 activities were examined by incubating with nifedipine for 15 min. Samples were analyzed by LC/MS/MS.

Table 1 Demographic information of hepatocyte donors

Batch	Demographic information						
	Age	Race	Gender	Alcohol	Tobacco	Drug	Medications
Donor 1[a]	48	Caucasian	F	No	Yes	No	Unknown
Donor 2[a]	6	Caucasian	F	No	No	No	Unknown
Donor 3[a]	52	Caucasian	M	No	Used to	No	Unknown
Donor 4	65	Caucasian	F	No	No	No	Unknown
Donor 5	69	Caucasian	F	Previous[b]	Yes	No	Unknown
Donor 6	45	Caucasian	F	No	Yes	No	No
Donor 7	52	Caucasian	M	Yes	Previous[c]	Previous[c]	No
Donor 8	0	African American	F	No	No	No	No
Donor 9	64	Caucasian	M	Yes	Yes	No	Unknown
Donor 10	31	Hispanic	F	No	No	No	Unknown
Donor 11	48	Caucasian	F	No	No	No	Antidepressants
Donor 12	5	African American	M	No	No	No	No
Donor 13	60	Caucasian	M	Yes	Yes	No	Unknown
Donor 14	55	Caucasian	F	Yes	No	No	Unknown
Lot 098[d]	44	Caucasian	F	No	No	No	Unknown
Lot VTA[d]	52	Caucasian	M	Yes	Yes	No	Unknown

[a] Hepatocytes were studied for CYP induction

[b] Abused alcohol for 15 years, quit for 30 years

[c] Used for 10 years, then quit

[d] Cryopreserved cells

Drug–drug interactions

CYP3A4 inhibition by eribulin was compared with that of ketoconazole. The CYP3A4 substrates paclitaxel [13], carbamazepine [19], diazepam [20], tamoxifen [21], midazolam [13], and terfenadine [22], were chosen to study DDIs. Each substrate (at $\sim$1–3 $\times$ K_m) was co-incubated with eribulin at concentrations up to 10 μM in human hepatocyte suspensions. The positive control was ketoconazole, whereas the negative control contained only methanol. The CYP3A-mediated activities were measured by LC/MS/MS.

The protein binding capacity of 100–1,000 ng/ml eribulin was determined in human plasma by equilibrium dialysis.

CYP induction assays

Freshly isolated human hepatocytes were plated in six-well dishes and maintained in culture media containing streptomycin, gentamicin, and penicillin at 5% CO_2 and 37°C. Cells were treated with vehicle, CYP1A inducer TCDD (20 nM), CYP3A inducer rifampicin (50 μM) [23], and eribulin (1 and 5 μM) for 72 h, and CYP isozyme-specific activities and protein expression were measured by LC/MS/MS and Western blot, respectively.

Electrophoretically resolved proteins were probed with anti-human CYP1A and CYP3A antibodies, followed by horseradish peroxidase-labeled secondary antibodies. After exposure to peroxidase substrate (enhanced chemiluminescence reagents), the fluorescence detected from labeled proteins was recorded on X-ray film.

Instrumentation

Qualitative analyses for metabolite profiling

Either a SCIEX API2000 (Applied Biosystems, Foster City, CA, USA) or a triple quadruple-ion trap mass spectrometer (SCIEX API4000 Q-TRAP; Applied Biosystems) was used for quantitation. Metabolites were separated on an RP8 column. The mobile phase was a mixture of 10 mM ammonium acetate (pH 5.0) and acetonitrile or methanol. MS was operated at positive electrospray ionization (ESI). Transition ions of multiple reaction monitoring (MRM) for eribulin and metabolite detection were 730.4→712.4 and 746.4→728.4, respectively.

Quantitative analyses of CYP activities

CYP1A (*R*-warfarin 6- and 8-hydroxylation), CYP2C9 (*S*-warfarin 7-hydroxylation) and CYP3A4 (*R*-warfarin 10-hydroxylation) activities were quantified as reported [24], as were the quantitative LC/MS/MS methods for CYP2C19-mediated *S*-mephenytoin 4′-hydroxylation and CYP2D6-mediated bufuralol 1′-hydroxylation [25].

The activities of CYP3A4-mediated testosterone 6*β*-hydroxylation and nifedipine dehydration were determined using LC/MS/MS, with either C18 or RP8 columns. The mobile phase was a mixture of methanol, acetonitrile, and 0.1% formic acid, pumped at 300 μl/min. MS/MS was operated at positive ESI with a 5 kV ionization potential and ion source temperature of 350°C. MRM transition ions for 6*β*-hydroxytestosterone, dexamethasone, nitrendipine, dehydronifedipine, 6-hydroxychlorzoxazone, and niflumic acid were 305→269, 393→237, 361→315, 345→284, 184→120, and 281→237, respectively. The method for CYP2E1-mediated chlorzoxazone 6-hydroxylation was previously published [26].

In DDI studies, LC/MS/MS was used for carbamazepine epoxidation, diazepam hydroxylation (C8 columns), and terfenadine oxidation (RP18 columns). Mobile phases comprised of 10 mM ammonium acetate, and acetonitrile, pumped at 250 μl/ml. The MS/MS was operated at positive ESI with a 5 kV ionization potential and a 400°C ion source temperature. The MRM transition ions for carbamazepine 10,11-epoxide, temazepam, and the alcohol-hydroxylated terfenadine were 253→236, 301→255, and 488→452, respectively. Mobile phases for tamoxifen comprised of 10 mM ammonium acetate, pH 5.0 and acetonitrile; those for paclitaxel comprised of 0.1% formic acid and methanol, pumped at 250 or 300 μl/ml, respectively. The MS/MS was operated at positive ESI with a 5 kV ionization potential and a 450°C ion source temperature. MRM transition ions for detecting demethylated tamoxifen and 3′-hydroxypaclitaxel were 358→129 and 870.5→569.4, respectively. The IS transition ions were 393→237.

Data analysis

The concentrations of metabolites were quantified using the calibration curves ($y = a\,x + b$, $y = ax^2 + bx + c$ or $y = ax^b + c$) of the peak area ratios of metabolites to the respective IS. Statistical analyses were performed with SigmaStat (Version 2.03, Systat, Inc., Chicago, IL, USA). Apparent inhibition constants were estimated using Dixon plots constructed based on the linear regression, and then determined based on SNLR analyses [27]. The derived equations for the turnover rates were as follows: competitive inhibition $V = V_{max}/(1 + K_m/S/(1 + I/K_i)$; uncompetitive inhibition: $V = V_{max}/(1 + I/K_i + K_m/S)$; noncompetitive inhibition: $V = V_{max}/(1 + K_s/S)/(1 + I/K_i)$; mixed inhibition: $V = V_{max}/((1 + I/K_i') + (1 + K_s/S)(1 + I/K_i)$. Here, S represents the substrate concentration; I, inhibitor concentration; V_{max}, maximum rate of turnover; K_m, substrate concentration at which the turnover rate is half of the maximum; K_i, competitive inhibition constant; K_i', uncompetitive

inhibition constant; and K_s, dissociation constant of the enzyme-substrate complex.

Results

CYP-mediated metabolism

The metabolism of eribulin was determined by monitoring the disappearance of eribulin or the appearance of eribulin metabolites. Eribulin was slowly metabolized in mixtures containing HLMs, but not in S9 or cytosolic fractions, suggesting the primary involvement of CYP- or FMO-mediated metabolism. Moreover, rCYP3A4 converted 39% of 1 μM eribulin and 20% of 5 μM, with estimated turnover rates of 130 and 330 pmol/min/nmol, respectively. A minor cofactor-dependent compound depletion (<15%) was observed for CYP2D6 and Phase II metabolizing enzymes, such as the hepatic UGT members (data not shown).

Several monooxygenated metabolites were detected using LC/MS/MS in mixtures containing either HLMs or rCYP3A4 (Fig. 2). The sites of monooxygenation were not identified due to nonspecific MS/MS fragmentations that formed following the neutral losses of 18 amu (water) or 32 amu (presumably methanol) under normal collision energy (20–50 eV). Interestingly, the addition of oxygen to eribulin appeared somewhat resistant to MS/MS collision-associated dissociation. Thus, product ions from eribulin exhibited *m/z* at 712.4 (MH^+), 698.4, 694.4, 680.4, and 662.4, whereas the product ions from the metabolites were consistently 16 amu higher, exhibiting *m/z* at 728.5 (MH^+), 714.4, 710.4, 696.4, and 678.5.

The chemical inhibitors DHB, ketoconazole, and terfenadine were used to characterize the role of CYP3A4 in eribulin metabolism in HLMs from 16 individuals. The rates of metabolism in pooled HLMs were significantly suppressed by terfenadine, ketoconazole, and DHB (Fig. 3). The inhibitory effects were consistent at both concentrations of eribulin evaluated (1 and 5 μM). CYP3A4 activity appeared highly heterogeneous, exhibiting a 17-fold variation among these individual HLM preparations. Eribulin metabolic turnover was significantly correlated with testosterone hydroxylation in the HLMs, with correlation coefficients (r^2) of 0.814 ($P < 0.01$) at 1 μM and 0.663 ($P < 0.01$) at 5 μM eribulin. Thus, CYP3A4 appeared to be the major CYP in eribulin metabolism based on both the statistically significant correlations with CYP3A4 activities and the effective suppression of eribulin metabolism by the CYP3A4-selective chemical inhibitors.

Characterization of CYP inhibition

Eribulin-mediated inhibition of the major drug-metabolizing CYP members, including 1A2, 2C9, 2C19, 2D6, 2E1, and 3A4, was determined in HLMs (Fig. 4). Eribulin (5 μM) inhibited CYP3A4-mediated testosterone 6β-hydroxylation and *R*-warfarin 10-hydroxylation ($P < 0.01$) in a concentration-dependent fashion, and the inhibitory effects appeared to be negligible at 1 μM ($P > 0.05$). The activities of CYP3A4-mediated testosterone 6β-hydroxylation and midazolam 1′-hydroxylation in HLMs were reduced in the presence of eribulin, with apparent K_i values of 25–30 and 10–15 μM, respectively. In contrast, the effect of eribulin on the activities of the remaining CYP members examined was negligible.

CYP3A4 suppression was further characterized using recombinant enzymes and three prototypic CYP3A4 substrates: testosterone, nifedipine, and *R*-warfarin. Both the potential mechanism of the inhibition as well as the inhibition constants were examined using Dixon plots. The apparent K_i values were determined by SNLR [28]. All reversible inhibitory models, including competitive, uncompetitive,

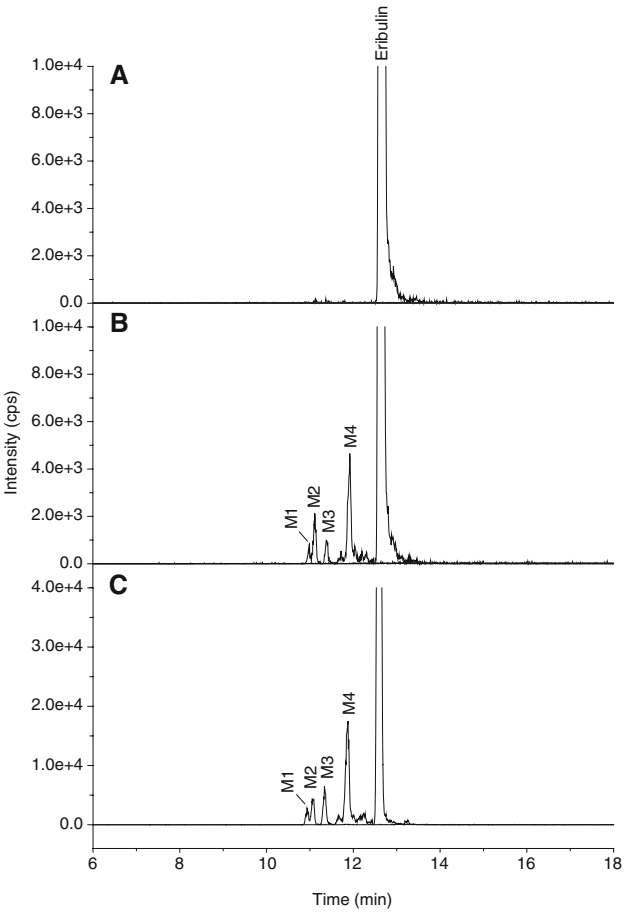


Fig. 2 Profiles of eribulin metabolites generated by pooled human liver microsomes and rCYP3A4. Liquid chromatography and tandem mass spectrometry analyses of reaction mixtures containing eribulin (100 μM) and human liver microsomes (2 mg/ml) prior to (**a**) and after (**b**) a 2-h incubation, or containing rCYP3A4 (~25 nM) after a 2-h incubation (**c**)

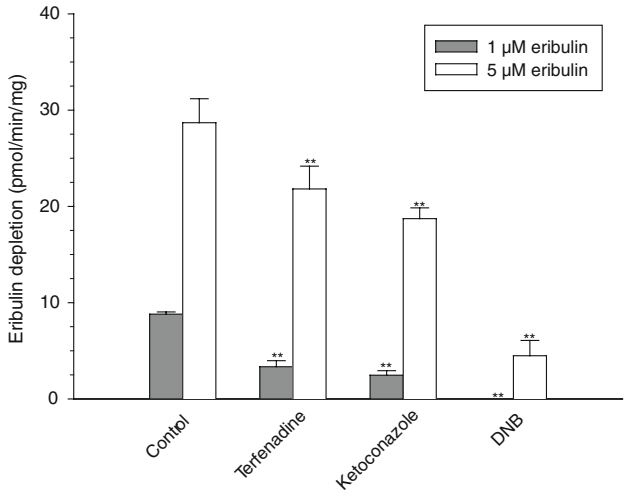


Fig. 3 Effect of chemical inhibitors on eribulin metabolism. Inhibitors were the CYP3A4 prototypic substrate (terfenadine), the competitive inhibitor (ketoconazole), and the mechanism-based inhibitor 6′,7′-dihydroxybergamottin) ($n = 3$)

noncompetitive, and mixed inhibition, were applied to determine the best fit. The direct active site competition model was found to fit the data best ($r^2 > 0.94$) (Table 2). The apparent K_i values were approximately 10 μM, which agree with the initial estimates suggested by the Dixon plots.

The mechanism(s) by which eribulin inhibits CYP3A4 was further examined by assessing the characteristic time- and NADPH-dependent mechanism-based inhibition of CYP3A4. Terfenadine (a substrate competitor) and DHB (a mechanism-based inhibitor) [18, 22], were included as controls. The pre-incubation of terfenadine or eribulin at 37°C did not alter CYP3A4-mediated nifedipine dehydration, either in the presence or absence of NADPH. In contrast, the pre-incubation of DHB in the presence of NADPH markedly enhanced DHB's inhibitory effect on CYP3A4-mediated nifedipine dehydration in a time-dependent manner. The results suggest that eribulin acts as a CYP3A4 substrate competitor and not as a mechanism-based inhibitor, in a manner similar to terfenadine.

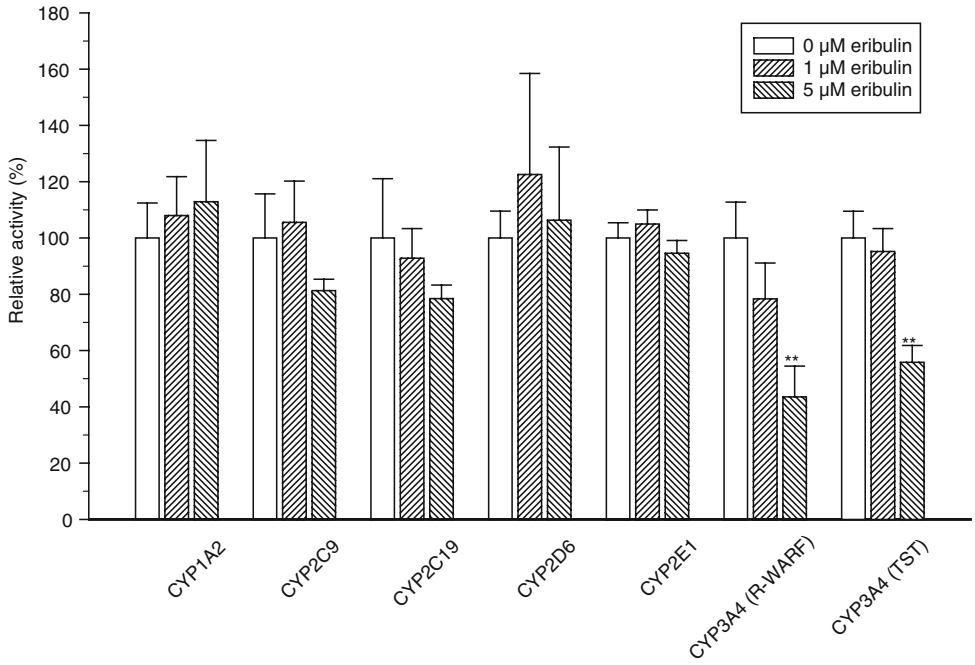


Fig. 4 Effect of eribulin on drug-metabolizing CYP enzymes. Enzyme activities were determined from triplicate (Mean ± SD). $^{**}P < 0.01$ versus vehicle controls

Table 2 Kinetics of eribulin inhibition of CYP3A4-mediated nifedipine dehydration, testosterone 6β-hydroxylation, and *R*-warfarin hydroxylation determined using simultaneous nonlinear regression analyses

	Probe	Competitive	Uncompetitive	Noncompetitive	Mixed
Equation		$V_{max}/((1 + K_m/S/(1 + I/K_i))$	$V_{max}/(1 + I/K_i + K_m/S)$	$V_{max}/(1 + K_s/S)/(1 + I/K_i)$	$V_{max}/((1 + I/K_i') + (1 + K_s/S)(1 + I/K_i))$
K_i	Nifedipine	11.2 μM ($r^2 = 0.953$)	8.2 μM ($r^2 = 0.911$)	23.7 μM ($r^2 = 0.940$)	4.1 μM or ∞ ($r^2 = 0.911$)
	Testosterone	16.7 μM ($r^2 = 0.947$)	ND	21.5 μM ($r^2 = 0.941$)	ND
	R-warfarin	3.2 μM ($r^2 = 0.985$)	7.2 μM ($r^2 = 0.854$)	12.0 μM ($r^2 = 0.935$)	3.6 μM or ∞ ($r^2 = 0.854$)

ND not determined because of the weak correlation ($r^2 < 0.7$)

DDI in vitro and in vivo prediction

In vitro CYP3A4 inhibition by eribulin was compared with inhibition by ketoconazole, a known inhibitor of CYP3A4 in the clinical setting [29]. As shown in Table 3, a number of CYP3A4-mediated activities were markedly inhibited by ketoconazole, with an attenuated suppression on tamoxifen *N*-demethylation. In contrast, only 10 μM eribulin, the highest concentration evaluated, elicited minor inhibitory effects on a few CYP3A4-mediated biotransformation activities, consistent with the modest K_i values.

The maximal plasma concentration (C_{max}) of eribulin has been projected to range from 0.0484 to 0.642 μM (corresponding to doses of 0.25–4.0 mg/m^2), based on data obtained from clinical Phase I studies [30]. The unbound fractions (f_u) of eribulin in plasma were 35% at 0.121 μM, 51% at 0.605 μM, and 50% at 1.21 μM. Thus, the unbound fraction of maximal plasma concentration ($C_{max,u}$ or $f_u C_{max}$) was expected to fall between 0.0219 and 0.291 μM. Finally, $C_{max,u}/K_i$, a predictive indicator for DDI risk potential in the clinical setting [31], was calculated to lie in the range 0.00211–0.0280. This value is considerably lower than 0.1, the threshold value for remote DDI risk [31], indicating that DDIs mediated by CYP3A4 inhibition are unlikely with eribulin.

CYP induction

The CYP induction assays were conducted to establish whether eribulin can affect drug clearance rates by altering metabolic enzyme expression via transcriptional activation of the encoding genes. Primary hepatocytes from three human donors were used in these experiments. These cells showed a few treatment-induced morphologic changes, most of which involved variations in initial cell confluence. The polyclonal CYP3A antibodies potentially recognized several CYP3A members (i.e., CYP3A4 and 3A5), whereas antibodies against CYP1A1 and 1A2 were more form-specific (Fig. 5). As expected, TCDD induced CYP1A2 expression [32] and rifampicin induced the expression of CYP3A [23] (Fig. 5). The activities of *R*-warfarin 6-hydroxylation and testosterone 6β-hydroxylation were markedly elevated at least 19- and 7-fold following exposure to TCDD and rifampicin, respectively. In contrast, eribulin administration did not alter CYP1A or 3A protein levels (Fig. 5) or activities. The results indicate that eribulin does not cause enhanced drug clearance via altered expression of CYP1A and 3A enzymes.

Discussion

Eribulin, is a non-taxane microtubule dynamics inhibitor that is a structurally simplified, synthetic analog of the marine natural product halichondrin B. Eribulin is the first synthetic analog of halichondrin B to enter clinical testing and is currently in Phase III clinical trials for the treatment of metastatic breast cancer. Like halichondrin B, eribulin is believed to exert its chemotherapeutic activity via a novel mechanism of action distinct from those of other known classes of tubulin-targeted agents, including paclitaxel [3]. CYP3A4 has a broad substrate spectrum and poor ligand specificity [33], so it is not surprising that eribulin functions as a substrate of this enzyme. Indeed, many natural products, including erythromycin, cyclosporine, and paclitaxel, are metabolized by CYP3A4 and bind to the active site(s) [34, 35]. In this study, eribulin was primarily metabolized by hepatic CYP3A4 in HLMs, resulting in the formation of a series of monooxygenated metabolites (M1–4). The MS/MS product ion spectra of eribulin and its metabolites were not structurally informative, specifically because the losses of water (−18 amu) and methanol (−32 amu) made identification of metabolites difficult. However, the high tendency for retention of metabolically incorporated oxygen, as evidenced by the MS/MS spectra, is reminiscent of cyclic core monooxygenations. CYP3A4-mediated eribulin biotransformation, based on slow compound depletion and metabolite formation, appeared to be of low capacity, resulting in a desirably slow hepatic clearance, as seen in clinical trials [30].

Table 3 Effect of eribulin on CYP3A4 activities in the suspensions of the freshly isolated human hepatocytes

Reaction	Activity (%)[a]			
	Ketoconazole	Eribulin		
	5 μM	1 μM	5 μM	10 μM
Midazolam 1′-OH	<10.3[b]	103.3 ± 21.3	101.6 ± 16.3	90.1 ± 23.7
Carbamazepine EPO	<20.0[b]	91.6 ± 8.1	81.0 ± 10.4	71.9 ± 11.6
Diazepam 3′-OH	6.0 ± 3.6	93.5 ± 8.0	90.8 ± 6.1	82.8 ± 9.80
Terfenadine OXID	25.7 ± 9.0	100.0 ± 11.8	100.4 ± 6.70	94.7 ± 8.50
Tamoxifen DEME	50.1 ± 6.2	81.7 ± 1.5	88.1 ± 3.7	82.3 ± 4.90
Paclitaxel 3′-OH	<47.2[b]	96.1 ± 10.8	84.2 ± 6.8	71.8 ± 6.20

DEME demethylation; *EPO* epoxidation; *OXID* oxidation

[a] Relative to the activities without the inhibitor or eribulin (three batches of cells per activity)

[b] Below the quantifiable level

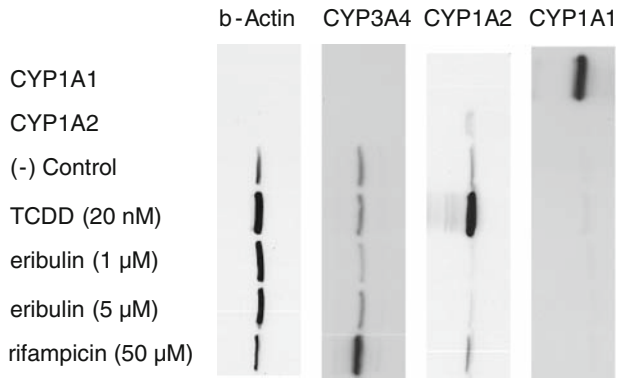


Fig. 5 Effect of CYP inducers and eribulin on CYP protein expression in the primary culture of human hepatocytes

In contrast, the affinity of CYP3A4 for eribulin metabolism may be high, as demonstrated by experiments with a selection of CYP3A4 inhibitors or prototypic substrates. In pooled HLM reaction mixtures, the suppression of eribulin metabolism by the chemical inhibitors was consistent with their respective inhibitory actions. For example, DHB, a CYP3A4 inhibitor that acts as both a reversible and as an irreversible (or mechanism-based) inhibitor [18], significantly suppressed eribulin metabolism. Conversely, the competitive inhibitors ketoconazole and terfenadine attenuated microsome-mediated biotransformations weakly [22]. Moreover, in the reaction mixtures containing HLMs prepared from 16 individual donors, the turnover of 1 or 5 μM eribulin correlated significantly with prototypic CYP3A4 substrate activity. Thus, it is likely that CYP3A4 converts eribulin with high affinity.

Eribulin, as a high affinity substrate, would be anticipated to compete with other ligands for the CYP3A4 active site. Interestingly, both the competitive and noncompetitive mechanisms were suggested by the close fit of the regression curves to each of these models (Table 2). However, based on the inhibitory mechanism depicted by the Dixon plots, the competitive model appears to be the best fit. This implies that SNLR alone may not be sufficient for delineating the inhibitory mechanism, and other biochemical computations (e.g., Dixon plots) should be employed for the analysis of data obtained from initial studies [36]. Collectively, the inhibitory results suggest a typical substrate competition by eribulin, with no indications of the atypical kinetic behavior frequently detected in CYP3A4 interactions [37]. Such atypical kinetic behavior of CYP3A4 might evolve from simultaneous binding of two (or more) small ligands, as suggested by the active site topography [35]. However, the complexities of CYP3A4 actions and the interactions between enzyme and ligands are not fully understood, even in the light of available crystal structures [35, 38]. The sizes of erythromycin (M_r 734) and eribulin (M_r 729 for the free-base portion) are comparable and erythromycin has been shown to reside in the binding pocket as a single molecule, in contrast to the potential double occupancy for small molecules such as ketoconazole [35]. Thus, it is reasonable to speculate that eribulin, like erythromycin, interacts with the enzyme within the cavity of active site(s) as a single molecule [35].

Based on the substrate-dependent binding and other potential intricacies of enzyme actions [38–41], the inhibitory effect of eribulin on CYP3A4 activities was further evaluated using alkaline nifedipine, neutral testosterone, and acidic *R*-warfarin as CYP3A4 substrates [22, 40]. In addition to the structures of these substrates, hydrophobicity and hydrogen-binding were two additional factors considered in selecting them [39]. The results of eribulin-mediated CYP3A4 suppression in the presence of these substrates were consistent with modest competitive inhibition. Among the substrates evaluated, only the metabolism of *R*-warfarin appeared to be inhibited by eribulin. However, *R*-warfarin is pharmacologically inactive compared with the active enantiomer *S*-warfarin, which is not a CYP3A4 substrate [42].

The purpose of the in vitro DDI study was to determine whether the inhibition of CYP3A4 by eribulin was clinically significant. Ketoconazole, a potent CYP3A4 inhibitor, induces DDI in the clinical setting [29] and markedly suppressed all CYP3A4-mediated activities with the exception of tamoxifen *N*-demethylation, which was slightly attenuated. This reduced effect on tamoxifen metabolism may be attributed to the involvement of other enzymes, such as CYP1A2, 2C9, and 2D6 [21]. Nevertheless, in contrast to ketoconazole, eribulin demonstrated little effect on the CYP3A4-mediated metabolism of any of the drugs evaluated in freshly isolated human hepatocytes from many subjects, including tamoxifen, paclitaxel, carbamazepine, diazepam, terfenadine, and midazolam. Moreover, based on the exposure level determined in clinical trials, the values of $C_{max,u}/K_i$ were much smaller than 0.1, the threshold value for remote DDI risk [31]. Finally, NADPH- and time-dependent inhibition of CYP3A4-catalyzed nifedipine dehydration, characteristic of a mechanism-based inhibition, was not observed for eribulin. Thus, eribulin appears to act as a CYP3A4 substrate competitor, similar to terfenadine, rather than a mechanism-based inhibitor. Taken together, these observations suggest that although eribulin is a substrate competitor for CYP3A4, inhibition of CYP3A4 is reversible and may not be clinically significant at the concentrations used in patients. However, it is important to note that the intracellular concentrations of some drugs may be higher than those achieved and examined in plasma; if so, then DDI could not be ruled out without knowing eribulin levels achieved in hepatocytes.

CYP3A4 substrates and inhibitors, including the tubulin-targeted chemotherapeutic agents, taxanes, may potentially

induce metabolic or inhibitory enzymes via transcriptional activation of the encoding genes [43]. Such induction of enzyme expression was partially attributed to the alleviation of therapeutic efficacies and the escalation of tumor resistance. However, as a substrate, eribulin did not induce the expression or activities of either CYP3A or CYP1A in primary cultures of human hepatocytes derived from several donors, ruling out the possible attenuation of efficacy as a result of enhanced metabolic clearance. Given a low capacity of biotransformation mediated by CYP3A4, DDI due to the potential alteration of eribulin metabolism by CYP3A4 activator or inhibitor would be unanticipated, although such analyses have yet to be carried out.

Eribulin has been shown to be a substrate of P-glycoprotein (Pgp) in cell-based studies [44]. Further support for this comes from the observation that oral bioavailability of eribulin in CF-1 Pgp deficient mice was much higher (53%) compared with that observed in CF-1 wild-type mice (7%) (unpublished data). Although not specifically investigated in the current study, it remains possible that eribulin may affect CYP3A4-mediated metabolism via mechanisms potentially involving Pgp [45].

In summary, eribulin was primarily metabolized by CYP3A4, did not induce CYP3A or CYP1A expression or activity, and potentially inhibited CYP3A4-mediated metabolism as a substrate competitor. However, this reversible inhibition was determined to be not clinically relevant, based on the enzyme-kinetic characterization and the lack of NADPH- and time-dependent enzyme inhibition. Moreover, the predicted value of $C_{max,u}/K_i$ was less than 0.1, suggesting that administration of eribulin poses a minimal risk for DDI in the clinical setting. Although it is possible that the intracellular concentrations of some drugs may be higher than those in plasma, and clinically significant DDIs could occur if eribulin is administered with a CYP3A4 activator or inhibitor, the results to date suggest that eribulin would not be expected to inhibit the metabolism of concurrently administered drugs that are metabolized by CYP3A4 suggesting a minimal risk of drug–drug interactions in the clinical setting.

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