ELETRIPTAN METABOLISM BY HUMAN HEPATIC CYP450 ENZYMES AND TRANSPORT BY HUMAN P-GLYCOPROTEIN

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ABSTRACT:

“Reaction phenotyping” studies were performed with eletriptan (ETT) to determine its propensity to interact with coadministered medications. Its ability to serve as a substrate for human P-glycoprotein (P-gp) was also investigated since a central mechanism of action has been proposed for this “triptan” class of drug. In studies with a characterized bank of human liver microsome preparations, a good correlation ($r^2 = 0.932$) was obtained between formation of N-desmethyl eletriptan (DETT) and CYP3A4-catalyzed testosterone $6\beta$-hydroxylation. DETT was selected to be monitored in our studies since it represents a significant ETT metabolite in humans, circulating at concentrations 10 to 20% of those observed for parent drug. ETT was metabolized to DETT by recombinant CYP2D6 (rCYP2D6) and rCYP3A4, and to a lesser extent by rCYP2C9 and rCYP2C19. The metabolism of ETT to DETT in human liver microsomes was markedly inhibited by troleandomycin, erythromycin, miconazole, and an inhibitory antibody to CYP3A4, but not by inhibitors of other major P450 enzymes. ETT had little inhibitory effect on any of the P450 enzymes investigated. ETT was determined to be a good substrate for human P-gp in vitro. In bidirectional transport studies across LLC-MDR1 and LLC-Mdr1a cell monolayers, ETT had a BA/AB transport ratio in the range 9 to 11. This finding had significance in vivo since brain exposure to ETT was reduced 40-fold in Mdr1a$^{-/-}$ relative to Mdr1a$^{+/+}$ mice. ETT metabolism to DETT is therefore catalyzed primarily by CYP3A4, and plasma concentrations are expected to be increased when coadministered with inhibitors of CYP3A4 and P-gp activity.

Eleetran [ETT$^1$; (R)-3-(1-methyl-2-pyrrolidinylmethyl)-5-[2-(phenylsulfonyl)ethyl]-1H-indole] (Fig. 1) is a selective agonist at 5-hydroxytryptamine (serotonin)$_{1B/1D}$ receptors and, like the other triptan class of antimigraine drugs (Goadsby and Hargreaves, 2000). It plays a major role in the oxidative metabolism of both xenobiotics and certain endogenous compounds. Described herein are the first detailed studies to elucidate the P450 forms responsible for ETT metabolism to DETT in human liver. The metabolism of ETT to DETT was chosen to be monitored in our studies since DETT represents a significant metabolite observed to circulate in humans at levels 10 to 20% of those observed for ETT [Product Information for Eletriptan, Pfizer, Sandwich, UK (2001)]. Reaction phenotyping studies (Rodrigues, 1999) were performed by correlation analysis, the use of recombinant human P450 forms, chemical inhibition, and inhibitory antibodies. In addition, to evaluate the potential for drug-drug interactions, the effect of ETT on some selected P450 form enzyme activities was also investigated.

It was of interest also to determine whether ETT could serve as a substrate for P-glycoprotein (P-gp), an efflux transporter expressed on the apical side of gut epithelia and brain endothelia, which can limit oral absorption and brain penetration, respectively. The role of P-gp and P450 in the uptake and metabolism of ETT was investigated because these systems can work in concert to limit systemic exposure following oral dosing (Cummins et al., 2002), and they are also subject to inhibition and/or induction by a variety of xenobiotics (Wandel et al., 1999; Dresser et al., 2003). In addition, the role of P-gp in regulating the uptake of ETT into the brain was of interest because of the intense speculation about a central mechanism of action for the triptan class of antimigraine drugs (Goadsby and Hargreaves, 2000). In this regard, ETT was considered the ideal tool to investigate this...
phenomenon, since it was the first lipophilic agent in this drug class that would be expected to have good intrinsic brain penetration. Our studies describe primarily the role of P-gp and P450 in the disposition of ETT from the perspective of its potential for drug-drug interactions. The role of P-gp in potentially modulating the dose required for antimigraine efficacy is also discussed in the context of a comparison to the other triptan drugs, naratriptan (NARA), rizatriptan (RIZA), sumatriptan (SUMA), and zolmitriptan (ZOLM).

Materials and Methods

Materials. ETT, NARA, RIZA, SUMA, ZOLM (Fig. 1), DETT, and L-844151 [internal standard for liquid chromatography/tandem mass spectrometry (LC/MS-MS)] were synthesized by Merck Sharpe & Dohme (UK) with identity and purity confirmed by NMR, MS, HPLC, and elemental analysis. NADPH, Tris, diethyldithiocarbamate, erythromycin, miconazole, quinidine, sulfaphenazole, trolenadomycin 7-ethoxyresorufin, resorufin, tolbutamide, debrisoquine, chlorozoxzone, testosteron, and 6β-hydroxytestosteron were obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK); and furafylline, S (+)-mephenytoin, methylhydroxytolbutamid, 4′-hydroxymephenytoin, N-desmethyl S-mephenytoin (Nirvanol), 4-hydroxydebrisoquine, and 6-hydroxychlorozoxzone were obtained from Salford Ultrasine Chemicals and Research Ltd. (Manchester, UK). [Guanidine-14C]Debrisoquine, S-[14C]Mephenytoin, and [4-14C]Testosteron (specific activities 53, 56, and 56 mCi/mmol, respectively) were purchased from Amersham Biosciences UK Ltd. (Little Chalfont, Buckinghamshire, UK). A reaction phenotyping kit (Product Number H0500, Version 5) containing 15 individual human liver microsomal preparations, characterized for total P450 content and a range of P450 isoform enzyme activities, was purchased from XenoTech LLC (Kansas City, KS) and stored at −80°C. Microsomes from human B-lymphoblastoid cells containing cDNA-expressed human P450 isoforms (BD Gentest, Woburn, MA) were obtained from Cambridge Bioscience (Cambridge, UK) and stored at −80°C. The samples of human B-lymphoblastoid cell microsomes comprised control cell microsomes (i.e., no transfected human cytochrome P-450 isoform cDNA, but contains native CYP1A1 activity) and cell microsomes containing CYP1A2, CYP2A6 + OR [i.e., CYP2A6 cDNA plus human NADPH-cytochrome P-450 reductase (OR) cDNA], CYP2B6, CYP2C8 + OR, CYP2C9 + OR, CYP2C19, CYP2D6 + OR, CYP2E1 + OR, and CYP3A4 + OR. Neither CYP3A5 nor the effect of cytochrome b5 on P450 activities was investigated in our studies. Inhibitory antibodies against CYP2C8/9, CYP2D6, and CYP3A4 were provided by Merck Research Laboratories (Rahway, NJ) and stored at −80°C. The specificity of these inhibitory antibodies has been demonstrated for CYP3A4 and CYP2D6 (Mei et al., 2002), and for CYP2C8/9, also based on in-house studies (unpublished data).

Human Liver Microsomes. Washed microsomal fractions were prepared in 0.154 M KCl containing 50 mM Tris-HCl, pH 7.4, using standard methods. Two separate batches, designated pools I and II, of pooled human liver microsomes were prepared. Each batch was prepared by pooling liver samples from five subjects. Pool I comprised liver samples from males aged 2.5 and 58 years and females aged 14, 59, and 74 years, whereas pool II comprised liver samples from male subjects aged 44 and 50 years and female subjects aged 11, 31, and 55 years.

Metabolism of ETT by Human Liver Microsomes. The NADPH-dependent metabolism of ETT was studied in incubation mixtures containing 0.05 to 200 μM ETT (added in 0.25 ml of 0.1 M phosphate buffer, pH 7.4), 10 mM MgCl2, 2 mM EDTA, 85 mM phosphate buffer, pH 7.4, and 0.05 to 0.3 mg of microsomal protein in a final volume of 0.5 ml. After a 5-min preincubation at 37°C in a shaking water bath, the reaction was initiated by the addition of 1 mM NADPH. Incubations were performed in duplicate for 5 to 20 min at 37°C and were terminated by the addition of 0.5 ml of ice-cold acetonitrile containing the L-844151 internal standard for LC/MS-MS analysis (see below). Blank incubations contained all components except NADPH.

ETT metabolism to DETT was quantified by LC/MS-MS analysis. An HTS PAL autosampler (Presearch Ltd., Hitchin, Hertfordshire, UK) attached to an HP1100 series high-performance liquid chromatograph (Hewlett Packard Ltd., Bracknell, Berkshire, UK) were used to introduce samples into a Micromass Quattro LC triple quadrupole mass spectrometer (Micromass UK Ltd., Altrinham, Cheshire, UK) using an Electrospray Z-spray source. All incubations were terminated by the addition of an equal volume of ice-cold acetonitrile containing 200 μM L-844151 internal standard. After vortex mixing, the tubes were centrifuged at 10,000g for 10 min at 4°C. Chromatography of 20-μl aliquots of the supernatant was performed with a 150 × 3.0 mm Kromasil C18 5-μm column (Thermo Hypersil-Keystone, Runcorn, Cheshire, UK) and isocratic elution with a mobile phase of 40% (v/v) acetonitrile, 6% (v/v) 250 mM ammonium formate, pH 3.0 and 54% (v/v) ultrapure water, at a flow rate of 0.4 ml/min and a column temperature of 35°C. The eluate was split volumetrically, with 20% passing directly into the triple quadrupole mass spectrometer operating in positive electrospray mode, with probe and source block temperatures of 400 and 100°C, respectively, using nitrogen for desolvation and nebulization, and a capillary voltage of 2.8 kV to initiate ionization. The compounds were selectively detected by multiple reaction monitoring using argon, collision energy 31 V; ETT m/z 383→84, DETT m/z 369→70, and L-844151 internal standard m/z 354→113.

ETT Metabolism Correlation Analysis. Incubation mixtures contained 60 μM ETT (added in 0.25 ml of 0.1 M phosphate buffer, pH 7.4), 10 mM MgCl2, 2 mM EDTA, 85 mM phosphate buffer, pH 7.4, and 0.1 mg of microsomal protein in a final volume of 0.5 ml. After 5 min of preincubation at 37°C in a shaking water bath, the reaction was initiated by the addition of 1 mM (final concentration) NADPH. Incubations were conducted in triplicate with a single blank (no NADPH) for each of the 16 preparations of characterized human liver microsomes and were terminated after 5 min by the addition of 0.5 ml of
ice-cold acetonitrile containing the L-844151 internal standard. Incubations were processed for LC/MS-MS analysis as described above.

**ETT Metabolism by Recombinant P450 Forms.** Incubation mixtures contained 60 μM ETT (added in 0.125 ml of 0.1 M phosphate buffer, pH 7.4) 10 mM MgCl₂, 2 mM EDTA, 1 mM NADPH, and 90 mM phosphate buffer, pH 7.4, in a final volume of 0.25 ml. After a 5-min preincubation at 37°C in a shaking water bath, the reaction was initiated by adding 0.1 mg of B-lymphoblastoid cell microsomal protein with gentle mixing. The incubations were conducted in duplicate with duplicate blank (no microsomes) for each P450 form preparation. Reactions were terminated after either 5 or 30 min with 0.25 ml of ice-cold acetonitrile containing the L-844151 internal standard. Incubations were processed for LC/MS-MS analysis as described above.

**ETT Metabolism Inhibition Studies.** Apart from the studies with the CYP1A2 mechanism-based inhibitor furafylline (Newton et al., 1995), where dimethyl sulfoxide was used, methanol was used as the solvent for these investigations to minimize solvent effects on individual P450 forms.

For the mechanism-based inhibitors requiring preincubation with NADPH, incubation mixtures contained 10 mM MgCl₂, 2 mM EDTA, 1 mM NADPH, 84 mM phosphate buffer, pH 7.4, 0.1 mg of microsomal protein, and either 5 to 50 μM furafylline, 5 to 100 μM diethyldithiocarbamate, 2 to 50 μM troleandomycin, or 2 to 50 μM erythromycin in a volume of 0.25 ml. The inhibitors were added in either methanol (5 μl/tube) or dimethyl sulfoxide (furafylline only, 5 μl/tube), and the concentrations refer to the final concentrations in a 0.5-ml incubation. Following a 30-min preincubation at 37°C in a shaking water bath, 60 μM ETT and 1 mM NADPH (final concentration 2 mM) were added in 0.25 ml of 0.1 M phosphate buffer, pH 7.4, at 37°C. Incubations were performed in duplicate for 5 min at 37°C and were terminated by the addition of 0.5 ml of ice-cold acetonitrile containing the L-844151 internal standard. Incubations were processed for LC/MS-MS analysis as described above.

For compounds not requiring extensive preincubation with NADPH, incubation mixtures contained 10 mM MgCl₂, 2 mM EDTA, 1 mM NADPH, 84 mM phosphate buffer, pH 7.4, 0.1 mg of microsomal protein, and either 2 to 50 μM sulfaphenazole, 2 to 20 μM quinidine, 50 to 500 μM S-mephenytoin, or 2 to 50 μM miconazole, in a volume of 0.25 ml. All the inhibitors were added in methanol (5 μl/tube), and the concentrations refer to the final concentrations in a 0.5-ml incubation. After a 10-min preincubation at 37°C in a shaking water bath, the reaction was initiated by the addition of 60 μM ETT (added in 0.25 ml of 0.1 M phosphate buffer, pH 7.4, at 37°C). Incubations were performed in duplicate for 5 min at 37°C and were terminated by the addition of 0.5 ml of ice-cold acetonitrile containing the L-844151 internal standard. Incubations were processed for LC/MS-MS analysis as described above.

To study the effect of inhibitory antibodies to CYP2C9, CYP2D6, and CYP3A4 forms, incubation mixtures contained 10 mM MgCl₂, 2 mM EDTA, 84 mM phosphate buffer, pH 7.4, 0.1 mg of microsomal protein, and either 0.025, 0.25, or 2.5 μl of the antibody preparation in a final volume of 0.5 ml. After a 10-min preincubation at 37°C in a shaking water bath, the reaction was initiated by the addition of 60 μM ETT and 1 mM NADPH (added in 0.25 ml of 0.1 M phosphate buffer, pH 7.4, at 37°C). Incubations were performed in duplicate for 5 min at 37°C and were terminated by the addition of 0.5 ml of ice-cold acetonitrile containing the L-844151 internal standard. Incubations were processed for LC/MS-MS analysis as described above.

**Effect of ETT on CYP450 Enzyme Activities.** The activities of 7-ethoxyresorufin O-deethylase, tolbutamide methylhydroxylase, debrisoquine 4-hydroxylase, and testosterone 6β-hydroxylase were determined as described previously (Renwick et al., 2000), employing substrate concentrations of 0.2, 180, 150, and 50 μM, respectively. For tolbutamide and debrisoquine metabolism, the tubes were preincubated in duplicate at 37°C in a shaking water bath for 5 min in the presence of either no ETT (controls) or 0.2 to 100 μM ETT and an NADPH-generating system, prior to addition of the P450 substrate. The effect of ETT on 7-ethoxyresorufin metabolism was determined as described for tolbutamide and debrisoquine metabolism, but with an ETT concentration range of 0.1 to 100 μM. For testosterone 6β-hydroxylase, the tubes were preincubated in duplicate at 37°C in a shaking water bath for 5 min in the presence of either no ETT (controls) or ETT (concentration range 0.2–100 μM or 1–100 μM) and 50 μM 4-1C-testosterone (0.5 μCi/tube), prior to the addition of 1 mM NADPH. To evaluate the effect of ETT as a mechanism-based inhibitor of testosterone 6β-hydroxylase activity, the tubes were preincubated at 37°C in a shaking water bath for 30 min in the presence of either no ETT (controls) or ETT (concentration range 0.5–50 μM or 0.2–100 μM) in 1 mM NADPH, prior to the addition of 50 μM 4-1C-testosterone (0.5 μCi/tube) and 1 mM NADPH (final concentration 2 mM). Chlorzoxazone 6-hydroxylase was determined in incubation mixtures containing 45 μM chlorzoxazone, 1 mM NADPH, 0.2 mg of microsomal protein, and 0.1 M phosphate buffer in a final volume of 1 ml. Incubations were terminated after 20 min with 0.2 ml of 30% (v/v) perchloric acid, and the supernatant was analyzed by HPLC. Chromatography was performed as described previously for tolbutamide methylhydroxylase (Renwick et al., 2000), except that the mobile phase consisted of 23% (v/v) acetonitrile and 77% (v/v) 20 mM sodium perchlorate, pH 2.5, and the eluant was monitored at 287 nm. The metabolism of S-mephenytoin to both 4'-hydroxymephenytoin and N-desmethyl S-mephenytoin was determined in incubation mixtures containing 60 μM S-(+)-mephenytoin (0.5 μCi/tube), 1 mM NADPH, 7.5 mM t-isoactic acid, 2 μM isocarboxylase dehydrogenase, 5 mM MgSO₄, 0.5 mg microsomal protein and 0.1 M phosphate buffer pH 7.4 in a final volume of 0.5 ml. Incubations were terminated after 30 min with 0.1 ml of 30% (v/v) perchloric acid and the supernatant analyzed by HPLC. Detection was for appropriate fragmentation transitions as determined above for tolbutamide and debrisoquine. For all P450 form enzyme activities studied, plots were constructed of enzyme activity (percentage of control rate in the absence of added ETT) against ETT concentration (logarithmic scale). Where inhibition of enzyme activity was observed, IC₅₀ values (i.e., concentration of ETT to produce a 50% inhibition of enzyme activity) were calculated by linear regression analysis.

**Brain Penetration of the 5-HT₄R Agonists in Mdr1a⁻/⁻ and Mdr1a⁺/- CF-1 Mice.** The impact of the drug efflux pump, P-gp (Umbenhauer et al., 1997) on the brain entry of the triptan drugs was investigated in vivo using male CF-1 mice. ETT was formulated in 25% polyethylene glycol 300/water at 0.5 mg free base equivalents/ml, and NARA, RIZA, ZOLM, and SUMA were formulated as aqueous solutions at the same concentration. All drugs were dosed at 1 mg free base equivalents/kg as a bolus injection into the tail vein (dose volume 2 ml/kg) to 24 male CF-1 mice (12 Mdr1a⁺/-, 12 Mdr1a⁻/⁻), approximate weight 35 g. Blood (by cardiac puncture into heparinized containers) and brain samples were taken at either 0.5, 2, 4, or 6 h after dosing (n = 3 per time point per genotype). Samples were stored frozen (-80°C) until analysis.

To blood (0.1 ml) was added internal standard (10 μl of a 10 ng/ml solution), 1 M sodium hydroxide (0.05 ml), water (1 ml), and ethyl acetate (4 ml). Samples were vortex mixed and centrifuged. Supernatant was removed and evaporated to dryness (70°C, under nitrogen), and the residue was dissolved in mobile phase (0.1 ml) and transferred to a HPLC vial. Calibration standards covering appropriate ranges were prepared by spiking solutions of analyte at appropriate concentrations into control blood (0.1 ml). To weighed samples of brain cortex were added internal standard (10 μl of a 10 ng/ml solution) and water (2 ml). After homogenization with an ultrasonic probe, 1 M sodium hydroxide (0.2 ml) and ethyl acetate (4 ml) were added. Samples were vortex mixed and centrifuged. Supernatant was removed and evaporated to dryness (70°C, under nitrogen), and the residue was dissolved in mobile phase (0.15 ml) and transferred to a HPLC vial. Calibration standards covering appropriate ranges were prepared by spiking solutions of analyte at appropriate concentration into control brain cortex.

Typically 25-μl injections were made onto a KR100-SC8 HPLC column (150 × 3.2 mm i.d., Hichrom Ltd., Reading, UK) with a mobile phase consisting of acetonitrile (A) and 25 mM ammonium formate, adjusted to pH 3 with formic acid, at a flow rate of 0.4 ml/min, with the following time program: 0 min, 20% A; 2 min, 20% A; 6 min, 50% A; 7 min, 20% A; 10 min, 20% A. Detection was for appropriate fragmentation transitions as determined by infusion of 1 ng/ml solutions at 10 ml/h into the mass spectrometer (Micromass Quattro).

Since three animals per time point were used to construct blood and brain concentration versus time profiles, Bailer’s method for determining area under
the curve (AUC) confidence intervals during sparse sampling was applied
(Bailer, 1988; Nedelman et al., 1995). Standard error of the mean (S.E.M.)
for the brain/blood ratio was determined using the following equation: S.E.M. =
(Brain AUC S.E.M./Blood AUC)^2 + (Brain AUC - Blood AUC S.E.M./
Blood AUC)^2). An unpaired two-tailed test was performed to determine
the significance of the difference between brain/blood AUC 0.5
(Blood AUC 2 ).

Human MDR1 and Mouse Mdr1a P-Glycoprotein Bidirectional Trans-
port Assay. The extent of P-gp-mediated transport of ETT, NARA, RIZA,
ZOLM, and SUMA was also evaluated in LLC-PK1 wild-type, LLC-MDR1
(human), and LLC-Mdr1a (mouse) cell lines (kindly provided by Dr. A. H.
Schinkel, The Netherlands Cancer Institute, Amsterdam, The Netherlands).
Cells were cultured in 199 medium as described. For transport experiments,
cells were cultured in 24-well Transwell plates (Falcon) at a density of 1.5 ×
10^5 cells/well with 0.3 ml of medium in the upper compartment and 35 ml of
medium in the lower nonsubdivided compartment. After culturing for 5 days,
the transport wells were transferred to a 24-well plate. The incubation and
sampling procedure during the transport experiment was performed on a Tecan
Genesis Workstation 150 Robot (Tecan, Zürich, Switzerland). Cells were
washed three times with Hanks’ balanced salt solution containing Hepes (10
mM). Subsequently, anlyte in Hanks’ balanced salt solution containing Hepes
(500 μl) was added either to the apical or basolateral compartment. Analyte
concentrations used were: 10 μM ETT, NARA, RIZA, ZOLM, and SUMA, 10
μM diethyldithiocarbamate (DCC), 5 μM ronavir, and 0.5 μM verapamil. After incubating
for 4 h at 37°C, samples (100 μl) were taken from both the apical and
basolateral compartment and transferred to a 96-well microtiter plate. Samples
were analyzed by positive ion single ion monitoring atmospheric pressure
chemical ionization on a SCIEX API 2000 triple quadrupole mass spectrom-
eter (PerkinElmerSciex, Concord, ON, Canada). The HPLC system used was
a PerkinElmer Series 200 system with 2 Micro pumps (PerkinElmer Instru-
m ents, Shelton, CT) and a Leap HTS PAL autosampler (LEAP Technologies,
Carboro, NC). Samples were chromatographed using an Aquasil C18, 2 × 20
mm, 5-μm HPLC column (Thermo Hypersil, Keystone Scientific Operations,
Belfonte, PA). The mobile phase used was 0.1% acetic acid in water (A) or
in acetonitrile (B) at 2.0 ml/min with a 4:1 split. The HPLC run time was 2
min/sample. Standard curves were prepared by a 2-fold serial dilution in
water/methanol (60:40, v/v) of a standard prepared at 2 times the dosing
solution concentration for an 11-point standard curve. Percentage of transport
was calculated by dividing the concentration of the compound appearing in the
receiver compartment by the sum of the compound concentrations measured in
the receiver and donor compartment (× 100%). The BA/AB ratio was calcu-
lated by dividing the concentration of compound measured in the apical
compartment (resulting from introduction of compound in the basolateral well)
by the concentration of compound measured in the basolateral compartment
(resulting from introduction of the compound in the apical well).

Lipophilicity Measurements. Octanol-pH 7.4 buffer partition coefficients
(logD ) were determined by the shake flask method with HPLC-UV analysis
of the aqueous and organic phases after equilibration (Hansch and Leo, 1979).
Results were derived from the mean of two partition coefficient determinations
performed at overall concentrations of 0.5 and 0.25 mg/ml in 1:1 pH 7.4
buffer/octanol mixtures.

Results
Metabolism of ETT by Human Liver Microsomes. Studies were performed with
two pools, namely pool I and pool II, of human liver microsomes. The total P450
content of the pool I and II microsomal preparations was determined to be 0.72 and
0.51 nmol/mg protein, respectively. The linearity of 50 μM ETT metabolism with respect to
both incubation time and protein concentration was investigated ini-
tially. ETT metabolism to DETT was found to be linear with respect to
incubation time (up to at least 6 min) and protein concentration (up to at least 0.2 mg of protein/incubation).

Kinetics of ETT Metabolism in Human Liver Microsomes. The kinetics of the NADPH-dependent metabolism of 0.05 to 200 μM
ETT to DETT was examined with both the pool I and pool II human

![Graph](https://via.placeholder.com/150)

Fig. 2. Metabolism of ETT to DETT by recombinant human P450 forms.

Incubations were performed with 60 μM ETT and 0.1 mg of B-lymphoblastoid
cell microsomal protein for either 5 or 30 min. Results are presented as the mean of
a duplicate test incubation.

![Graph](https://via.placeholder.com/150)

Fig. 3. Inhibition of ETT metabolism to DETT in human liver microsomes.

The effects of the mechanism-based inhibitors 5 to 50 μM furafylline (●) and 5
to 100 μM diethyldithiocarbamat (○) (A), together with 2 to 50 μM sulfaphen-
zoate (●), 2 to 20 μM quinidine (▲), and 50 to 500 μM S-mephenytoin (△) (B)
were evaluated on the metabolism of 60 μM ETT to DETT. Incubations were
performed with 0.1 mg protein of the pool I human liver microsomal preparation for
5 min. Each point represents the mean of a duplicate test incubation.
liver microsomal preparations. Pooled, rather than individual, liver microsomal preparations were used for these studies since the aim was to identify average $K_m$ (i.e., the concentration of substrate giving half-maximal velocity) values to select a suitable substrate concentration for the reaction phenotyping studies. With both liver microsome pools, DETT formation was detected at all ETT substrate concentrations examined.

The kinetics of ETT metabolism to DETT was examined with Michaelis-Menten and Eadie-Hofstee plots. Kinetic analysis of data for both liver microsome pools revealed mean $K_m$ and $V_{max}$ (i.e., the maximal velocity) values of 27.1 μM (pool I and pool II individual values were 24.3 and 29.9 μM, respectively) and 78 pmol/min/mg protein (pool I and pool II individual values were 105.4 and 50.5 pmol/min/mg protein, respectively). The mean intrinsic clearance ($CL_{int}$; i.e., $V_{max}$ / $K_m$) value was 3.02 l/min/mg protein (pool I and pool II individual values were 4.34 and 1.69 l/min/mg protein).

An ETT substrate concentration of 60 μM was selected for the reaction phenotyping studies described below. This substrate concentration was selected as being approximately 2 times the $K_m$ value for DETT formation.

**ETT Metabolism Correlation Analysis.** The metabolism of 60 μM ETT to DETT was examined with a characterized panel of 16 human liver microsomal preparations. ETT was metabolized to DETT by all 16 human liver microsomal preparations examined. Microsomal ETT metabolism varied from 58 to 635 pmol/min/mg protein, with most of the individual human liver microsomal preparations being more active than the two pooled human liver microsomal preparations.

The rates of ETT metabolism to DETT were correlated with data for total P450 content and a range of P450 form enzyme activities. A good correlation ($r^2 = 0.932$) was observed between ETT metabolism to DETT and CYP3A4-catalyzed testosterone 6β-hydroxylation. Although some correlation ($r^2 = 0.541$) was observed between ETT metabolism to DETT and total P450 content, only low correlations were observed ($r^2 = 0.000–0.361$) with P450 form enzymatic markers for CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP4A9/11. These enzyme activities (with $r^2$ values in parentheses) comprised 7-ethoxyresorufin O-deethylase (0.023, CYP1A2), coumarin 7-hydroxylase (0.022, CYP2A6), S-mephenytoin N-demethylase (0.310, CYP2B6), paclitaxel 6α-hydroxylase (0.361, CYP2C8), diclofenac 4'-hydroxylase (0.104, CYP2C9), S-mephenytoin 4'-hydroxylase (0.145, CYP2C19), dextromethorphan O-demethylase (0.098, CYP2D6), chlorzoxazone 6-hydroxylase (0.004, CYP2E1), and lauric acid 12-hydroxylase (0.000, CYP4A9/11).

**Metabolism of ETT by Recombinant P450 Forms.** The metabolism of 60 μM ETT to DETT was examined with rCYP1A2, rCYP2A6, rCYP2B6, rCYP2C8, rCYP2C9, rCYP2C19, rCYP2D6, rCYP2E1, and rCYP3A4, together with microsomes from control cells (which contain native CYP1A1) was studied. Incubations were performed for 5 and 30 min. Only trace amounts of DETT were observed in incubations with the control cell microsomes (data not shown) and with rCYP2A6, rCYP2B6, and rCYP2E1 preparations (Fig. 2). Low rates of DETT formation were observed with the rCYP1A2 and rCYP2C8 preparations. Pooled, rather than individual, liver microsomal preparations were used for these studies since the aim was to identify average $K_m$ (i.e., the concentration of substrate giving half-maximal velocity) values to select a suitable substrate concentration for the reaction phenotyping studies. With both liver microsome pools, DETT formation was detected at all ETT substrate concentrations examined.
Inhibition of ETT Metabolism. The effect of some human P450 form inhibitors and one P450 form substrate (S-mephenytoin) on the metabolism of ETT to DETT in human liver microsomes was studied. For the mechanism-based inhibitors (Newton et al., 1995) furafylline (CYP1A2), diethyldithiocarbamate (CYP2E1), erythromycin (CYP3A4), and troleandomycin (CYP3A4), the compounds were preincubated for 30 min at 37°C with liver microsomes and NADPH prior to the addition of ETT and another aliquot of NADPH. In the studies with the inhibitors sulfaphenazole (CYP2C9) and quinidine (CYP2D6), and the substrate S-mephenytoin (CYP2C19), the compounds were preincubated with liver microsomes for 10 min at 37°C prior to the addition of NADPH and ETT.

ETT metabolism to DETT was not markedly affected by 5 to 50 μM furafylline, 2 to 50 μM sulfaphenazole, 50 to 500 μM S-mephenytoin, and 2 to 20 μM quinidine (Fig. 3, A and B). Whereas 100 μM diethyldithiocarbamate produced some inhibition of ETT metabolism, little effect was observed with lower concentrations of this inhibitor (Fig. 3A).

The effect of erythromycin, miconazole, and troleandomycin on ETT metabolism to DETT was studied with both pools of human liver microsomes. ETT metabolism to DETT was markedly inhibited to 3 to 13% of control (mean of both liver microsome pools) by 2 to 50 μM miconazole and to 20 to 35% of control by 2 to 50 μM troleandomycin (Fig. 4). Erythromycin also produced a concentration-dependent inhibition of ETT metabolism, with enzyme activity being reduced to 23% of control by 50 μM erythromycin.

The effect of inhibitory antibodies to CYP2C8/9, CYP2D6 and CYP3A4 on ETT metabolism to DETT in human liver microsomes was studied. With both pools of human liver microsomes treatment with 0.05–5 μl/ml incubation of either the CYP2C8/9 or the CYP2D6 antibodies had little effect on ETT metabolism (Fig. 5A and B). In contrast, the CYP3A4 antibody produced a marked inhibition of the metabolism of ETT to DETT in human liver microsomes.

Effect of ETT on P450 Form Enzyme Activities. The effect of addition of ETT at concentrations of up to 100 μM on some P450 form enzyme activities in human liver microsomes was studied. ETT produced a weak inhibition of CYP2D6-dependent debrisoquine 4-hydroxylase activity, with a calculated IC₅₀ value (i.e., concentration of ETT to produce a 50% inhibition of enzyme activity) of 84 μM (Table 1). The addition of ETT had no effect (IC₅₀ values all ≥100 μM) on 7-ethoxyresorufin O-deethylase (CYP1A2), S-mephenytoin N-demethylase (CYP2B6), tolbutamide methylhydroxylase (CYP2C9), S-mephenytoin 4′-hydroxylase (CYP2C19), and chlorozoxazone 6-hydroxylase (CYP2E1) activities. ETT produced a weak inhibition of CYP3A4-dependent testosterone 6β-hydroxylase activity, with a calculated IC₅₀ value (mean of two liver microsome pools) of 95 μM (Table 1). When ETT was evaluated as a mechanism-based inhibitor of testosterone 6β-hydroxylase activity, an observed IC₅₀ value (mean of two microsome pools) of 40 μM was obtained. Using a 30-min preincubation period with NADPH, 20 μM troleandomycin (positive control) inhibited testosterone 6β-hydroxylase activity to 18% of control levels.

Effect of ETT on some P450 enzyme activities in human liver microsomes

<table>
<thead>
<tr>
<th>P450</th>
<th>Enzyme Activity</th>
<th>Control Ratea,b</th>
<th>ETT IC₅₀c/μM</th>
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<tr>
<td>CYP1A2</td>
<td>7-ethoxyresorufin O-deethylase</td>
<td>39</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>S-mephenytoin N-demethylase</td>
<td>14</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Tolbutamide methylhydroxylase</td>
<td>98</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-mephenytoin 4′-hydroxylase</td>
<td>4.1</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Debrisoquine 4-hydroxylase</td>
<td>60</td>
<td>84</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Chlorozoxazone 6-hydroxylase</td>
<td>400</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Testosterone 6β-hydroxylase</td>
<td>819 (836, 802)</td>
<td>95 (87, 100)</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Testosterone 6β-hydroxylase (mechanism-based inhibition)</td>
<td>723 (760, 685)</td>
<td>40 (38, 42)</td>
</tr>
</tbody>
</table>

a Enzyme activity in the absence of added ETT.
b Incubations were performed with the pool II human liver microsomal preparation, except for testosterone 6β-hydroxylase, where both the pool I and II human liver microsomal preparations were used.
c The effect of ETT on the P450 enzyme activities was determined over a concentration range of up to 100 μM.

Human MDRI and Mouse Mdr1a P-Glycoprotein Bidirectional Transport Assay. The transport of NARA, RIZA, SUMA, and ZOLM across LLC-PK1 parent cell monolayers was poor, with 5% of each drug being transported in either the B to A or A to B direction over a 4-h period. This diffusion rate was too low to reliably test whether these compounds were substrates for P-gp. In contrast, the transport of ETT was >10% in this parent cell line, a value consistent with the extent of transport observed for the positive control compounds used: dexamethasone, ritonavir, and verapamil (Table 2). In transport experiments across LLC-MDR1 and LLC-Mdr1a cell monolayers, the BA/AB ratio for ETT was in the range 9 to 11, highlighting it to be a good substrate of human and mouse P-gp in vitro, and similar to the positive control compounds studied.

Discussion

The role of CYP3A and human P-gp in the metabolism and transport of ETT in vitro has been investigated. These studies were performed to understand the potential for ETT to be involved in drug-drug interactions with other drugs that inhibit or induce these
proteins. These are the first detailed studies of this kind to be reported for ETT. In addition, since a central mode of action for RIZA, NARA, and ZOLM has been proposed (Goadsby, 2000; Goadsby and Hargreaves, 2000; Goadsby et al, 2002), factors that regulate the brain penetration of ETT, relative to NARA, RIZA, SUMA, and ZOLM, were investigated also and included measurements of lipophilicity and brain penetration in CF-1 wild-type (Mdr1a+/+) and P-gp-deficient (Mdr1a−/−) mice.

The NADPH-dependent N-demethylation of ETT to DETT was investigated in two preparations of pooled human liver microsomes and in 16 individual liver microsome preparations, and was concluded to be mediated by CYP3A, a good correlation ($r^2 = 0.932$) being observed between DETT formation and testosterone 6β-hydroxylase activity. The effect of erythromycin, miconazole, and tro前列mycin on ETT metabolism to DETT was also studied with both preparations of pooled human liver microsomes. No significant differences were noted between the two human liver microsome pools in the effects of the three compounds on DETT formation. The mechanism-based inhibitors tro前列mycin and erythromycin, together with miconazole, produced a marked concentration-dependent inhibition of ETT metabolism to DETT in human liver microsomes (Fig. 4). Previous studies have demonstrated that tro前列mycin is a specific inhibitor of human hepatic CYP3A4 (Newton et al., 1995). In addition, both erythromycin and miconazole are also known to inhibit CYP3A4-catalyzed reactions in human liver microsomes (Thummel and Wilkinson, 1998).

Prototypical inhibitors of CYP3A2, CYP2C9, CYP2C19, CYP2D6, or CYP2E1 (Newton et al., 1995; Ono et al., 1996; Rodrigues, 1999) had little effect on the metabolism of ETT to DETT in human liver microsomes (Fig. 3). Although DETT formation was inhibited by high concentrations of diethylthiocarbamate, this is unlikely to reflect metabolism by CYP2E1 but, rather, that this compound also inhibits other P450 forms (Ono et al., 1996).

The NADPH-dependent metabolism of 60 μM ETT to DETT by recombinant P450 forms was also studied. The highest rates of DETT formation were observed with rCYP2D6 and rCYP3A4 preparations (Fig. 2). Although ETT was also metabolized to DETT by rCYP2C9 and rCYP2C19 preparations, rates of DETT formation were either much lower or essentially undetectable with the rCYP1A2, rCYP2A6, rCYP2B6, rCYP2C8, and rCYP2E1 preparations.

The observation that ETT is extensively metabolized to DETT by rCYP3A4 is in agreement with the results of the correlation analysis, chemical inhibition, and inhibitory antibody studies. The finding that ETT is also extensively metabolized by rCYP2D6 appears to have little significance when other competing enzymes are present. This finding is borne out by the correlation analysis study, and the lack of effect of quinidine and the CYP2D6-inhibitory antibody on DETT formation in liver microsomes. This result is probably attributable to the relatively higher levels of CYP3A4 compared with those of CYP2D6 in human liver. Levels of CYP3A4/CYP3A4 in human liver microsomes have been reported to range from 44 to 250 pmol/mg protein, whereas those for CYP2D6 have been reported to range from only 5 to 25 pmol/mg protein (Shimada et al., 1994; Rodrigues, 1999).

Many examples of drug-drug interactions in human subjects have been described, and these may be due to either the induction or inhibition of P450-dependent and other xenobiotic metabolizing enzyme activities (Lin and Lu, 1998). The implication of our in vitro data, that of ETT dependence on CYP3A4 for metabolic clearances and its potential for drug interactions when coadministered with CYP3A4 inhibitors or inducers, is that plasma concentrations of ETT in patients would be expected to either increase or decrease, respectively. With regard to coadministration of ETT (Relpax) with CYP3A4 inhibitors, this is borne out in the Product Information for Eletriptan [Pfizer, Sandwich, UK (2001)]. In clinical studies with erythromycin (1000 mg) and ketoconazole (400 mg), which are both strong inhibitors of CYP3A4, significant increases in ETT plasma concentrations were observed (Newton et al., 1995). In addition, both erythromycin and ketoconazole are also known to inhibit CYP3A4-catalyzed reactions in human liver microsomes (Thummel and Wilkinson, 1998).

Prototypical inhibitors of CYP1A2, CYP2C9, CYP2C19, CYP2D6, or CYP2E1 (Newton et al., 1995; Ono et al., 1996; Rodrigues, 1999) had little effect on the metabolism of ETT to DETT in human liver microsomes (Fig. 3). Although DETT formation was inhibited by high concentrations of diethylthiocarbamate, this is unlikely to reflect metabolism by CYP2E1 but, rather, that this compound also inhibits other P450 forms (Ono et al., 1996).

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The release of, for example, the powerful vasodilator calcitonin gene-hibination of meningeal perivascular trigeminal nerves, which reducesstriction of dilated, pain-producing meningeal blood vessels and in-graine attacks. The first two invoke peripheral mechanisms: vasocon-
cance clinically since it was reported recently that administration of
monolayers in the range 9 to 11 (Table 2). This finding has signifi-
tional transport studies across LLC-MDR1 and LLC-Mdr1a cell
interactions in vivo with other drugs that are metabolized by these
data suggest that ETT is unlikely to produce any significant inhibitory
CYP2C19, CYP2D6, CYP2E1, and CYP3A4 studied (Table 1). These
related peptide. The third mechanism, for brain-penetrant compounds,
is antinociceptive activity within the brain stem sensory trigeminal
nuclei; anatomical studies having shown a high concentration of
5-HT1B/1D receptors in the trigeminal ganglion of animals and hu-
mans. It might be that each of these mechanisms operates in a complementary manner to reduce the intense central trigeminal input
that occurs during a migraine headache (Goadsby and Hargreaves, 2001).
If the central site is important for antimigraine efficacy, then
P-gp might be expected to limit the accessibility of ETT to central
sites of action. An analysis of the available pharmacokinetic data for
lead triptans indeed indicates that higher free drug levels of ETT, the
most potent of all the triptans at 5HT1B/1D receptors in vitro, are
required for clinical efficacy when compared with ZOLM or RIZA at
doses that provide equivalent efficacy (Table 3; Goadsby et al., 2002).
These higher plasma levels of ETT may reflect a requirement to provide sufficient free drug levels in the brain for adequate occupancy
of central 5-HT1B/1D receptors. The potential theoretical gains of
increased potency and lipophilicity on improved efficacy and barrier
transport (gut and brain) for ETT may, therefore, have been effec-
tively offset by relatively high plasma protein binding and good
substrate specificity for P-glycoprotein (Fig. 6B, Table 3).

It is recognized that the triptan class of compounds do generally
have poor brain penetration characteristics when compared with typical
CNS marketed drugs (Doan et al., 2002). The extent of brain

### Table 2

<table>
<thead>
<tr>
<th>Analyte</th>
<th>LLC-PK1</th>
<th>MDR1</th>
<th>Mdr1a</th>
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<tbody>
<tr>
<td></td>
<td>BA/AB Ratio</td>
<td>% Transport at t = 4 h</td>
<td>BA/AB Ratio</td>
</tr>
<tr>
<td>Eletroptan</td>
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<td>23</td>
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<td>Naratriptan</td>
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<td>3.8</td>
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<tr>
<td>Rizatriptan</td>
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<td>4.9</td>
<td>4.9</td>
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<tr>
<td>Sumatriptan</td>
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<td>Zolmitriptan</td>
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<tr>
<td>Ritonavir</td>
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<td>Verapamil</td>
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### Table 3

<table>
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<tr>
<th>Compound Dose</th>
<th>mol. wt.</th>
<th>fS</th>
<th>5-HT1B Potency</th>
<th>5-HT1D Potency</th>
<th>Human Pharmacokinetic Parameters</th>
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<td>Doses that provide superior efficacy to SUMA100</td>
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<tr>
<td>ETT80</td>
<td>382</td>
<td>0.24</td>
<td>7.7 ± 0.02</td>
<td>9.2 ± 0.02</td>
<td>226  142  1486  934</td>
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<td>ZOLM5</td>
<td>287</td>
<td>0.75</td>
<td>7.6 ± 0.05</td>
<td>8.9 ± 0.06</td>
<td>7.3–9.1 19–24 52–62 136–162</td>
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<td>RIZA10</td>
<td>269</td>
<td>0.86</td>
<td>7.1 ± 0.04</td>
<td>8.4 ± 0.05</td>
<td>19.8 63 49.6 158</td>
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<tr>
<td>Doses that provide equivalent efficacy to SUMA100</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETT40</td>
<td>295</td>
<td>0.82</td>
<td>7.0 ± 0.03</td>
<td>8.3 ± 0.05</td>
<td>54–78 150–216 419 1164</td>
</tr>
</tbody>
</table>

* Substrate concentrations were 10 μM for ETT, NARA, RIZA, SUMA, and ZOLM. 0.5 μM for verapamil, and 5 μM for ritonavir and dexamethasone.
* Analyses were performed in triplicate and mean data are expressed to two significant figures (percentage CVs were <10% for ETT and <30% for NARA, RIZA, SUMA, and ZOLM).
penetration is, however, a poor guide to central activity, especially with potent agonist drugs such as the triptans, since they, in contrast to most other CNS agents that are antagonists, will require only low fractional receptor occupancy to exert central effects. Further studies are warranted to relate plasma and brain concentrations of the triptans to their occupancy of central antimigraine 5-HT\textsubscript{1B/1D} receptors in vivo to evaluate the potential contribution of central sites to their mechanism of action.

In summary, the results of this study demonstrate that ETT can be metabolized to DETT by human liver microsomal preparations, and this biotransformation is primarily catalyzed by CYP3A4. ETT was shown to be a substrate of human P-glycoprotein. ETT metabolism was markedly reduced in vitro by prototypical CYP3A4 inhibitors. These findings are in agreement with the clinical data inasmuch as plasma levels of ETT are increased in human subjects when ETT is coadministered with inhibitors of CYP3A. ETT was shown to be a substrate of human P-glycoprotein, although the importance of this in the context of a central mechanism of action remains inconclusive.

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References


