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Biotransformation of Fluticasone: *In Vitro* Characterization

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Abbreviations: Fluticasone, 6α, 9α-difluoro-11β-hydroxy-17α-(propionyloxy)-16α-methyl-3-oxoandrosta-1, 4-diene-17β-carbothioate; fluticasone 17β-carboxylic acid (M1), 6α, 9α-difluoro-11β-hydroxy-17α-(propionyloxy)-16α-methyl-3-oxoandrosta-1, 4-diene-17β-carboxylic acid; CYP, cytochrome P450; ketoconazole, cis-1-acetyl-4-[4[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine; α-naphthoflavone, 7,8-benzoflavone; mifepristone, 11β-(4-dimethylamino)phenyl-17β-hydroxy-17-(1-propynyl)estra-4,9-dien-3-one; MS, mass spectrometry; omeprazole, 5-methoxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulfinyl]-1*H*-benzimadazole; quinidine, dextro-6´-methoxycinchonan-9-ol; sulfaphenazole, 4-amino-*N*-(1-phenyl-1H-pyrazol-5-yl)benzenesulfonamide; ticlopidine, 5-(*o*-chlorobenzyl)-4,5,6,7-tetrahydrothieno(3,2-c)pyridine.

Abstract

Fluticasone propionate (FTP) is a synthetic, trifluorinated glucocorticoid with potent anti-inflammatory action that is commonly used in patients with asthma. After oral or intranasal administration, FTP undergoes rapid hepatic biotransformation; the principal metabolite formed is a 17β-carboxylic acid derivative (M1). M1 formation has been attributed largely to cytochrome P450 (CYP) 3A4, however, there are no published data that confirm this assertion. Hence, *in vitro* studies were conducted to determine the role that human CYPs play in the metabolism of FTP. Consistent with *in vivo* data, human liver microsomes catalyzed the formation of a single metabolite (M1) at substrate concentrations ≤10 μM (mean plasma C_{max} = 1 nM). Under these conditions, the kinetics of M1 formation in human liver microsomes were consistent with those of a single enzyme ($K_m \cong 5$ μM). Formation of M1 correlated significantly (r >0.95) with CYP3A4/5 activities in a panel of human liver microsomes (n=14) and was markedly impaired by the CYP3A inhibitor, ketoconazole, (>94%), but not by inhibitors of other P450 enzymes (≤10%). Studies with a panel of cDNA-expressed enzymes revealed that M1 formation was catalyzed primarily by CYP3A enzymes at FTP concentrations ≤1 μM. M1 formation was catalyzed by CYPs 3A4, 3A5 and 3A7; in vitro intrinsic clearance values (V_{max} / K_m) were comparable for all three CYP3A enzymes. These results suggest that at pharmacologically relevant concentrations, biotransformation of FTP to M1 is mediated predominantly by CYP3A enzymes in the liver.

Controlling underlying inflammation is a central component of prevention and clinical management of asthma in adults and children. Current National Heart Lung and Blood Institute guidelines emphasize the importance of early intervention with inhaled corticosteroids as first-line anti-inflammatory therapy (National Asthma Education and Prevention Program, 1997). Comparison of the agents within this drug class that are currently available for clinical use reveals that fluticasone propionate (FTP), a synthetic trifluorinated glucocorticoid (Figure 1), has the greatest relative potency (Mager et al., 2003).

Studies using oral dosing of labeled and unlabeled FTP have demonstrated that oral systemic bioavailability is negligible (<1%), primarily due to incomplete absorption and extensive first-pass metabolism in the gut and liver (FLOVENT© product information; Harding, 1990). In the original report by Harding, no unchanged FTP was detected in the plasma of volunteers up to 6 h following a 16 mg oral dose of FTP. In contrast, the majority of the FTP dose administered via inhalation is not subjected to pre-systemic clearance but rather, is absorbed across the pulmonary vascular bed, resulting in an extent of systemic availability ranging from 14 to 31% (mean value, 21.2%) in healthy adults and 8.5 to 20.9% (mean value 13.3%) in adults with chronic obstructive pulmonary disease (Singh et al., 2003).

In the original studies describing the pharmacokinetics of FTP in humans, Harding (1990) reported that up to 40% of a ^{3}H-FTP dose was converted to one principal metabolite, a 17β-carboxylic acid derivative (M1; Figure 1) found to have negligible pharmacological activity; the remainder of the dose was converted to several unidentified, minor metabolites and glucuronide conjugates. FTP biotransformation to M1 has been attributed largely (by inference) to CYP3A4, there are no published data that confirm this assertion and/or examine the relative ability of other CYP3A isoforms (e.g., CYP3A5 and CYP3A7) to catalyze M1 formation. Consequently, the specific metabolic fate of FTP in man remains uncharacterized despite its widespread clinical use in infants, children and adolescents. Characterization of the role that CYPs play in the biotransformation of FTP would provide not only a more complete understanding of the clinical pharmacology of this drug but also provide insight into how developmental changes and/or inter-individual variability in biotransformation capacity might contribute to the production of adverse events (*e.g.*, those seen with dose escalation) (Randell et al., 2003) and

possibly, the reported phenomenon of corticosteroid resistance (Adcock and Ito, 2004). To address this knowledge gap, a series of *in vitro* studies were undertaken to determine the role that human cytochrome P450 enzymes (CYPs) play in the biotransformation of FTP to M1.

Materials and Methods

Chemicals

Ketoconazole, α-naphthoflavone, omeprazole, quinidine, sulphafenazole, 4-methylpyrazole, ticlopidine, mifepristone, glucose-6-phospate, glucose-6-phosphate dehydrogenase, and NADP were purchased from Sigma-Aldrich Co. (St. Louis, MO). FTP and M1 were synthesized by Coral Drugs (New Delhi, India) and by Chemsyn Laboratories (Lenexa, KS), respectively. All other reagents were of analytical grade.

Biological reagents

Microsomal preparations from individual human liver donors and from baculovirus-infected insect cells (SUPERSOMES®) expressing human P450 enzymes (1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, and 3A7) or control vector were purchased from BD GENTEST Corp. (Woburn, MA). All recombinant enzymes were co-expressed with human NADPH-cytochrome P450 reductase; some enzymes (CYP2B6, CYP2C19, CYP2E1, CYP3A4 and CYP3A7) were also co-expressed with human cytochrome b_5. Pooled human liver and pulmonary microsomes, an inhibitory, polyclonal antibody against CYP3A4 and control IgG (both raised in rabbits) were purchased from XenoTech, L.L.C. (Lenexa, KS). The manufacturers provided protein concentration, P450 specific content and enzyme activities for each microsomal preparation. A pool of fetal human liver microsomes was prepared from 10 individual microsomal preparations that were homozygous for CYP3A5 *3*3 and contained no CYP3A5 protein as determined by Western immunoblot (as described in Leeder et al., 2005). Vials of microsomes were stored at -70ºC until use. Microsomes were rapidly thawed in room temperature water and placed on ice prior to use.

Analytical method

FTP and M1 were resolved on a reversed-phase Agilent Technologies (Palo Alto, CA) Zorbax XDB C-8 column (4.6 mm x 75 mm, 3.5 μm particle size) using a Hewlett Packard HP1100 HPLC system (HP1100 de-gasser, binary pump, auto-sampler, column heater, and diode array detector)

equipped with a HP1100 mass spectral detector (Hewlett Packard Instruments, Santa Clara, CA). The mobile phase consisted of 0.25% aqueous NH_3OH (solvent A) and methanol (solvent B) and was delivered at a constant flow of 0.8 ml/min. The solvent program was as follows: 0-1 min, 50%B; 1-1.9 min, a linear gradient from 50 to 70% B; 1.9-6.5 min, 70% B; 6.5-6.51 min a linear gradient from 70 to 50% B; 6.51-10 min, 50% B. The column temperature was maintained at 30ºC. Under these conditions, M1 and FTP eluted at ~1.7 and 7.2 min, respectively. The column effluent was monitored by UV detection (254 nm) and by atmospheric pressure electrospray detection with a mass spectrometer operating in a selective negative ion-monitoring mode. Ion detection was optimized for M1 detection. The drying gas temperature and flow were maintained at 300ºC and 10.0 L/min, respectively, and the nebulizer pressure was set at 30 psig. The vaporizer temperature was maintained at 400ºC. The capillary voltage was set at 2.7 kV. Under these conditions, M1 yielded [M^-] ions at *m/z* 451 whereas FTP was monitored as a molecular ion [M^-] at *m/z* 499. Data were collected and integrated with Hewlett Packard Chemstation V A.0401 software. M1 and FTP were quantified by comparison of their peak areas (determined by mass spectral analysis) with those of analytical standards. The lower limit of quantification for M1 detection was 0.055 pmol (~1 ng/ml). The analytical method was linear ($r^2 > 0.99$) over a standard concentration range of 0.055 to 110 pmol M1 (~1-1000 ng/ml). For low, midpoint and high concentrations of M1 throughout the linear range, intra-day standard CVs ranged from 1-12%, whereas inter-day standard CVs ranged from 4-11%.

In vitro incubation conditions

In vitro enzyme assays were performed in 96-well microtiter plates. Each 100-µl incubation reaction contained human liver or pulmonary microsomes (5-30 µg of liver and 10-100 µg of pulmonary microsomal protein) or insect cell microsomes containing bacculovirus-expressed cytochrome P450 enzymes (2.5 pmol) co-expressed with P450 reductase, potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), and FTP (10 nM to 30 µM). Reactions were initiated by the addition of an NADPH-generating system, consisting of NADP (1 mM), glucose-6-phosphate (1 U/ml), glucose-6-

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phosphate dehydrogenase (5 mM), placed in a shaking incubator at 37 ± 0.1°C, and terminated after 10 to 15 minutes for human liver microsomes or 30 minutes for recombinant P450s by the addition of 100 µl of ice-cold methanol. Protein was precipitated by centrifugation at 10,000 g_{max} for 10 minutes. An aliquot (50-100 µl) of the supernatant was analyzed by HPLC/MS via direct injection.

Initial experiments indicated that under these conditions, metabolite formation was proportional to incubation time and protein concentration. Metabolism of the parent compound was ≤ 10%. The concentration of methanol present in the incubations was ≤ 1% to minimize inhibition of product formation. Three replicate experiments were performed with two replicate samples per condition for correlation and competitive inhibition experiments (n=6 determinations). All other experiments were performed in duplicate or triplicate.

Mechanism-based inhibition experiments

Human liver microsomes (10 µg microsomal protein) were pre-incubated with mechanism-based inhibitors (100 µM) at 37±0.1°C for 20 minutes in the presence of an NADPH-generating system. The pre-incubation mixture was then diluted 10-fold in an incubation mixture containing potassium phosphate (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), and FTP (100 nM). Reactions were initiated by the addition of a second aliquot of NADPH-generating system, placed in a shaking incubator at 37 ± 0.1°C, and terminated with ice-cold methanol after 15 min. Protein was sedimented by centrifugation and an aliquot of the supernatant was analyzed by reversed-phase HPLC/MS via direct injection.

In related experiments, human liver microsomes were pre-incubated with an inhibitory, polyclonal antibody against CYP3A enzymes or control IgG. After the pre-incubation period, potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), and FTP (100 nM) were added to the incubation mixtures. Reactions were initiated by the addition of a second aliquot of NADPH-generating system and conducted as described above.

Data analysis

Coefficients of determination (r^2) between the rates of M1 formation and the activities of cytochrome P450 enzymes were determined using least-squares linear regression analysis. Significance was determined by Pearson's regression analysis from two-tailed t tables (α=0.05). Enzyme kinetic parameters were estimated by non-linear regression without weighting (GraFit 5; Erithacus Software Ltd, Surrey, UK).

Results

Metabolism of fluticasone by cDNA-expressed human P450s

To determine which P450 enzymes were capable of catalyzing M1 formation, a relatively high concentration of FTP (1 μM as compared to average peak plasma concentrations during therapeutic administration of ≈ 1 nM) was incubated with control vector or cDNA-expressed CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, and 3A7. A supra-physiological concentration of FTP was chosen for an initial screen of the recombinant enzymes in order to identify candidates for subsequent kinetic analyses. Of the recombinant enzymes examined, only CYPs 2C19, 3A4, 3A5 and 3A7 produced M1 at rates greater than the control vector, and these results are depicted in Figure 2. Furthermore, CYP3A isoforms catalyzed M1 formation at rates that were at least 25 times those of CYP2C19.

Determination of kinetic parameters

The kinetics of M1 formation were investigated in pooled adult and fetal human liver microsomes, in three individual preparations of human liver microsomes, and in microsomes containing recombinant CYP3A4, CYP3A5 or CYP3A7. Although there was an indication of substrate activation at the lowest concentrations of fluticasone studied (as suggested by the hooked portion of the Eadie-Hofstee plot depicted in Figure 3), the majority of the data points obtained with human liver microsomal preparations were consistent with Michaelis-Menten kinetics for a single enzyme. This suggested that M1 formation is catalyzed predominantly by a single P450 enzyme or possibly, by multiple enzymes with similar kinetic properties. A representative Eadie-Hofstee plot is shown in Figure 3, and the mean kinetic parameters (K_m, V_{max} and V_{max} / K_m) from the aforementioned experiments are contained in Table 1.

Comparable to the results obtained with human liver microsomes, formation of M1 by recombinant CYP3A4, CYP3A5 and CYP3A7 appeared to conform to typical Michaelis-Menten kinetics, based upon evaluation of Eadie-Hofstee plots for each isoform (plots not shown). Kinetic parameters for

each CYP3A isoform are also contained in Table 1. Examination of the V_{max} / K_m ratio, a parameter reflecting in vitro intrinsic clearance (Cl_{int}), revealed comparable values for all three CYP3A enzymes.

Correlation experiments

Human liver microsomes prepared from 14 donors were examined for their ability to metabolize FTP at two substrate concentrations (20 and 100 nM). All of the microsomal samples examined converted FTP to M1 to some extent; formation of other metabolites of FTP was not observed. The rates of M1 formation varied ~16.6- and ~26.4-fold among the microsomal samples at substrate concentrations of 20 and 100 nM, respectively {range (rates ± S.D.): 20 nM, 0.14 ± 0.06 to 2.33 ± 0.42; 100 nM, 0.52 ± 0.16 to 13.7 ± 0.4 pmol/mg protein/min}. As illustrated in Table 2, significant correlations were observed between the rates of M1 formation and the activity of CYP3A4/5 ($r^2 \geq 0.920$) and to a lesser extent, with CYP2B6 ($r^2 \geq 0.457$) and CYP2C19 ($r^2 \geq 0.299$) activities. M1 formation was not significantly associated with any other cytochrome P450 activities. It should be noted that CYP3A4/5 activity was significantly correlated with CYP2B6 activity ($r^2 = 0.67$) which suggests that the relationship between M1 formation and CYP2B6 activity may largely be fortuitous, particularly since recombinant CYP2B6 failed to catalyze formation of M1 (data not shown).

Competitive inhibition experiments

The effects of various concentrations of selective P450 inhibitors on the conversion of FTP (100 nM) to M1 by pooled human liver microsomes are illustrated in Figure 4. Ketoconazole, a potent CYP3A4/5 inhibitor, markedly inhibited the formation of M1 (~50%) at the lowest concentration examined (0.1 μM) and virtually eliminated M1 formation at the higher concentrations examined (1 and 10 μM). It should be noted, however, that the effect of 10 μM ketoconazole on M1 formation may not be attributed solely to inhibition of CYP3A4/5, because at this concentration, ketoconazole is no longer a selective inhibitor for CYP3A4/5 (Newton et al., 1995). In contrast to the effects observed with ketoconazole, the P450 inhibitors coumarin (CYP2A6), sulfaphenazole (CYP2C9), omeprazole (CYP2C19), quinidine (CYP2D6) and 4-methylpyrazole (CYP2E1) caused little or no inhibition of M1

formation (< 20%). The CYP1A1/2 inhibitor, α-naphthoflavone, caused a slight decrease (~21%) in the formation of M1 at a concentration of 1 μM, however bracketing concentrations of α-naphthoflavone (0.1 and 10 μM) failed to cause a significant decrease in M1 formation (Figure 4). This finding suggested that CYP1A enzymes are likely not involved in catalyzing M1 formation and that the data observed with the 1 μM concentration was likely an experimental artifact.

Mechanism-based inhibition experiments

Pooled human liver microsomes were pre-incubated with mechanism-based inhibitors of the P450 isoforms that were demonstrated to have the capacity to catalyze M1 formation. Ticlopidine, a CYP2C19 mechanism-based inhibitor, caused little or no inhibition in the rate of M1 formation compared to the positive control sample that contained an equal amount of methanol but no inhibitor. In contrast, mifepristone, a mechanism-based inhibitor of CYPs 3A4 and 3A7 but not of CYP3A5, virtually eliminated (i.e., > 92%) formation of M1 (Figure 5). In related experiments, pooled human liver microsomes were pre-incubated with an inhibitory, polyclonal antibody against CYP3A enzymes or control IgG. The antibody against CYP3A4/5 markedly (> 90%) inhibited the conversion of FTP to M1 whereas IgG had no discernable effect (Figure 5). Collectively, these results suggest that the CYP3A enzymes, most notably CYP3A4, account for most, if not all, of the formation of M1 from FTP.

Biotransformation by human pulmonary microsomes

Two pools of human pulmonary microsomes, prepared from surgical specimens of lung tissue from non-smoking (n=10) and smoking (n=10) donors, were examined for their ability to convert FTP (100 or 1000 nM) to M1. Under the most favorable experimental conditions (eg., high concentrations of fluticasone and microsomal protein; 1000 nM and 1 mg/ml, respectively), no M1 appeared to be formed by either of the pools of pulmonary microsomes. However, it should be noted that these microsomal preparations also failed to demonstrate any detectable CYP3A activity, as measured by testosterone 6β-hydroxylation (data not shown).

Discussion

Our experimental data demonstrate that conversion of FTP to its carboxylic acid metabolite, M1 (Figure 1), appears to be catalyzed primarily by CYP3A isoforms. Although M1 formation was significantly correlated with only CYP3A4/5, CYP2B6 and CYP2C19 activities in our studies using human liver microsomes, cDNA-expressed CYP3A enzymes catalyzed M1 formation at rates >20 times those of the next most active P450 enzyme, namely CYP2C19, whereas cDNA-expressed CYP2B6 did not appear to catalyze M1 formation. As well, M1 formation was markedly inhibited by the potent CYP3A inhibitor, ketoconazole, in a concentration dependent manner, but not by selective, competitive inhibitors of other P450 enzymes. An inhibitory antibody against CYP3A4/5 eliminated >90% of M1 formation. Interestingly, mifepristone (RU-486), an inhibitor of CYP3A4 but not of CYP3A5, inhibited >95% of M1 formation in human liver microsomes. These results suggest that at pharmacologically relevant concentrations of FTP, formation of M1 is mediated predominantly by CYP3A4 and/or CYP3A7, although in certain individuals capable of expressing CYP3A5, this enzyme may also play a role in M1 formation.

As previously reviewed by de Wildt, et al. (1999), CYP3A isoforms are located in the liver and several extrahepatic organs/tissues that include the small intestine, the kidney and the lung. CYP3A5 is the predominant CYP3A isoform expressed in human lung, whereas CYP3A4 appears to be expressed in only about 20% of Caucasian lungs (Antilla et al., 1997). In general, CYP3A5 is capable of generating the same metabolites as CYP3A4 qualitatively, but is typically much less active (Williams et al., 2002). Given that FTP is predominantly administered to patients via inhalation and the potential importance of the lung as regards its local and systemic metabolism, we elected to evaluate its biotransformation using human pulmonary microsomes. In these experiments, no discernible M1 appeared to be formed from FTP independent of smoking history associated with the lung specimens. It is not surprising that M1 formation was lacking in the microsomes prepared from donors that smoked as CYP3A5 expression has been shown to be suppressed in cigarette smokers (Hukkanen, 2003).

The potential clinical relevance of identifying CYP3A4 as the predominant enzyme capable of catalyzing the biotransformation of FTP resides with its potential to produce systemic effects when given via inhalation. As recently shown by Singh et al. (2003), the mean (range) systemic availability of a single, 1000 μg inhaled dose of FTP was 21.2 % (14.3-31.4%) and 13.3% (8.5-20.9%) in healthy controls and adults with chronic obstructive pulmonary disease, respectively. Thus, when a therapeutic dose of FTP is delivered to the airway under controlled circumstances (*e.g.*, supervised, proper administration techniques), greater then negligible systemic exposure may result. The potential adverse consequences of long-term, high-level systemic exposure to FTP is illustrated by a recent review of more than 50 case reports of adrenal crises associated with high-dose FTP administration (Randell et al., 2003). It has also been shown that long-term, high-dose FTP administration can impair recovery of the hypothalamic-pituitary-adrenal axis following discontinuation of oral corticosteroids used to treat asthma (Kennedy et al., 2002).

Conditions other than high-dose administration of FTP may serve to increase the extent of systemic drug exposure over that predicted from its innate pharmacokinetic characteristics (Singh et al., 2003). Using the CYP3A4/5 substrate alfentanil, Klees, et al. (2005) demonstrated that ketoconazole (a commonly used azole antifungal agent) was an order of magnitude more potent than troleandomycin as an inhibitor of CYP3A4. The pharmacokinetic consequences of CYP3A4/5 inhibition by ketoconazole are illustrated by a recent study of the anticancer drug Gleevec (imatinib mesylate; a substrate for CYP3A4/5) conducted in adults where a single 400 mg dose of ketoconazole increased the Gleevec area under the curve (a parameter reflecting the extent of systemic exposure associated with a given drug dose) by 40% (Dutreix et al., 2004). As we have demonstrated in our *in vitro* study, ketoconazole produced approximately 50% and 100% inhibition in M1 formation at concentrations of 0.1 and 1.0 μM, respectively (Figure 4). Thus, it would be predicted that administration of a potent CYP3A4/5 inhibitor such as ketoconazole would have the potential to markedly increase the systemic availability of FTP via inhibition of its biotransformation in both the small intestine (for the fraction of an inhaled dose swallowed upon administration) and liver and thus, increase its adverse event potential.

In addition to the reduction in CYP3A4/5 activity produced by the co-administration of agents capable of inhibiting these enzymes, the impact of development must also be considered as it pertains to the biotransformation of FTP. As recently reviewed by de Wildt, et al. (1999) and Kearns, et al. (2003), the ontogeny of CYP3A4/5, as reflected by pharmacokinetic studies of drugs known to be substrates for these isoforms, indicates that during the neonatal period the activity of these enzymes is substantially reduced. Hepatic CYP3A4 expression is virtually non-existent prior to birth, but begins to increase dramatically at about 1 week of age, reaching 30% of adult levels by 1 month of age (LaCroix et al., 1997). As CYP3A4 expression increases, a simultaneous decline typically occurs in the expression of CYP3A7, resulting in total CYP3A protein expression that remains relatively constant over the entire neonatal period (Lacroix et al., 1997). A recent publication (Sim et al., 2005) reported that relatively high levels of CYP3A7 protein expression could be detected in a subset of adult human livers, which may have clinical implications regarding xenobiotic biotransformation in adults and children. In view of the fact that FTP and other inhaled corticosteroids are being increasingly used in neonates and young infants with acute (Wong et al., 2002) and chronic lung disease (Lister et al., 2003), developmentally-associated reductions in CYP3A activity have the potential to significantly increase the systemic availability of FTP by the inhaled route and by inference, the potential for adverse effects if adjustments in dosing to compensate for reduced enzyme activity are not made.

In conclusion, the *in vitro* studies presented here suggest that CYP3A isoforms are the predominant enzymes associated with the biotransformation of FTP in humans. Establishing the importance of these enzymes in the metabolism of this commonly used drug enables clinical consideration of concomitant events (eg., drug-enzyme and drug-drug interactions) and conditions (eg., developmental and/or disease-associated reductions in enzyme activity) that may serve to influence the dose-concentration-effect relationship for FTP *in vivo*.

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Footnote to Title

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Figure legends

Figure 1. Metabolic scheme for the conversion of fluticasone propionate (FTP) to fluticasone 17β-carboxylic acid (M1).

Figure 2. M1 formation by human recombinant cytochrome P450 enzymes. To evaluate which P450 enzymes were capable of catalyzing M1 formation, FTP (1 μM) was incubated with heterologously expressed human P450 enzymes (CYPs 1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, and 3A7) or vector as described under "Materials and Methods". Only those enzymes capable of catalyzing M1 formation at rates greater than the vector are depicted. Each bar represents the mean ± S.D. of three determinations.

Figure 3. Representative Eadie-Hofstee plot of M1 formation in pooled human liver microsomes (prepared from 50 individuals). FTP (10 nM to 30 μM) was incubated with pooled human liver microsomes (0.1 mg microsomal protein/ml) in 100-μl reaction mixtures at 37±0.1°C, and terminated after 10 min by the addition of methanol (100 μl). Following precipation of microsomal protein, an aliquot of the supernatant was analyzed by HPLC/MS via direct injection, respectively, as described under Materials and Methods. Similar plots were obtained for individual human liver microsomal preparations (H042, H056, and H064) and for recombinant CYP3A4, CYP3A5 and CYP3A7.

Figure 4. Effects of various P450 isoform-selective inhibitors on the formation of M1 by human liver microsomes. Pooled human liver microsomes (prepared from 50 individuals) were incubated with FTP (100 nM) in the presence or absence of various chemical inhibitors, as described under "Materials and Methods". Inhibitable P450 enzymes are indicated in

the brackets. Each bar represents the mean ± S.D. of six determinations. The uninhibited rate of M1 formation was 6.93 ± 0.26 pmol/mg/min.

Figure 5. Effects of mechanism-based chemical inhibitors or of inhibitory CYP3A4/5 antibodies on M1 formation by pooled human liver microsomes. Pooled human liver microsomes (prepared from 50 individuals) were pre-incubated with mechanism-based inhibitors (100 μM) at 37±0.1°C for 20 min in the presence of an NADPH-generating system. The pre-incubation mixture was then diluted 10-fold, and a second incubation was conducted with FTP (100 nM) as substrate, as described under "Materials and Methods". Alternatively, human liver microsomes were pre-incubated for 20-min at room temperature with an inhibitory, polyclonal antibody against CYP3A enzymes or control IgG, followed by incubation with FTP (100 nM), as described under "Materials and Methods". Each bar represents the mean ± S.D. Mechanism-based chemical inhibition experiments were performed in triplicate, whereas antibody-inhibition experiments were performed in duplicate.

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Table 1. Apparent kinetic parameters for the formation of M1 in human liver microsomes and cDNA-expressed CYP3A enzymes.

Kinetic Parameters	Human Liver Microsomes					cDNA-Expressed Enzymes		
	H42	H56	H64	Adult Pool	Fetal Pool	CYP3A4	CYP3A5	CYP3A7
K_m [a]	6.3	3.4	5.5	2.6	4.8	5.3	3.8	5.2
V_{max} [b]	132	68	21	83	32	206	145	182
V_{max}/K_m [c]	21	20	4	32	7	39	38	35

Units are [a] μM, [b] pmol/mg protein/min for human liver microsomes and pmol/nmol P450/min for cDNA-expressed enzymes and [c] μl/min/mg protein for human liver microsomes and μl/min/nmol P450 for cDNA-expressed enzymes.

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Table 2. Regression analysis (r^2) of the relationship between the rates of fluticasone conversion to fluticasone 17β-carboxylic acid (M1) with the sample-to-sample variation in cytochrome P450 activity in human liver microsomes.

Enzymatic Reaction (Enzyme)[a]	*20 nM Fluticasone* [b]		*100 nM Fluticasone* [b]	
	r^2	*P*	r^2	*P*
Phenacetin O-deethylation (CYP1A2)	0.001	0.931	0.000	0.997
Coumarin 7-hydroxylation (CYP2A6)	0.000	0.951	0.000	0.968
S-Mephenytoin *N*-hydroxylation (CYP2B6)	**0.457**	0.008	**0.474**	0.007
Paclitaxel 6α-hydroxylation (CYP2C8)	0.113	0.239	0.093	0.289
Diclofenac 4′-hydroxylation (CYP2C9)	0.120	0.227	0.099	0.272
S-Mephenytoin 4′-hydroxylation (CYP2C19)	**0.299**	0.043	**0.305**	0.040
Bufurolol 1′-hydroxylation (CYP2D6)	0.138	0.194	0.129	0.207
Chlorzoxazone 6-hydroxylation (CYP2E1)	0.225	0.085	0.257	0.064
Testosterone 6β-hydroxylation (CYP3A4/5)	**0.927**	<0.0001	**0.920**	<0.0001
Lauric acid 12-hydroxylation (CYP4A9/11)	0.054	0.423	0.059	0.403

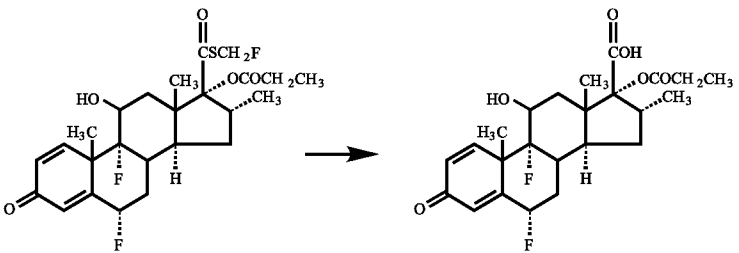
Values in bold indicate significant coefficients of determination between P450 activity and rate of M1 formation at $P<0.05$.

r^2, Coefficient of determination.

[a]Panel consisted of microsomal preparations from 14 different human livers.

[b]Substrate concentration.

Figure 1



Fluticasone Propionate

Fluticasone 17β-carboxylic Acid (M1)

Figure 2

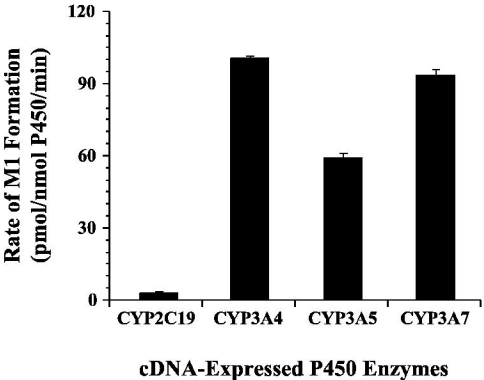

120
90
60
30
0
Rate of M1 Formation
(pmol/nmol P450/min)
CYP2C19
CYP3A4
CYP3A5
CYP3A7
cDNA-Expressed P450 Enzymes

Figure 3

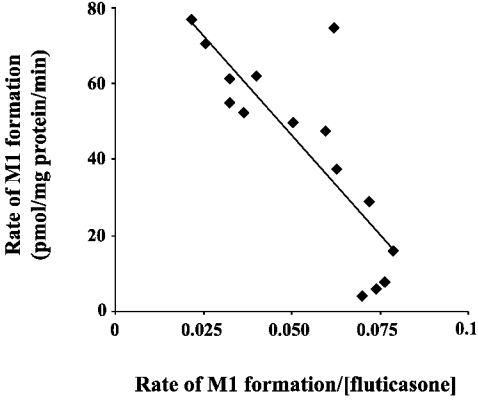

Rate of M1 formation (pmol/mg protein/min)
80
60
40
20
0
0
0.025
0.050
0.075
0.1
Rate of M1 formation/[fluticasone]

Figure 4

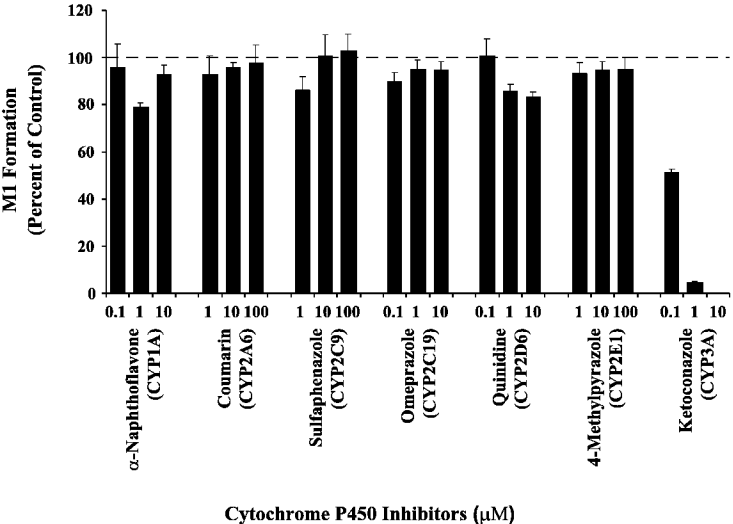
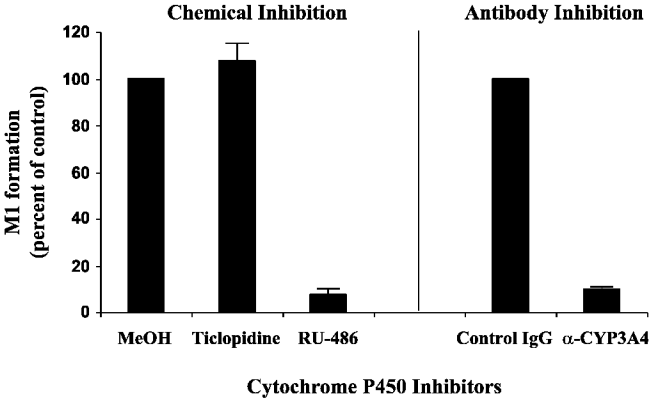

M1 Formation
(Percent of Control)
120
100
80
60
40
20
0
0.1 1 10
1 10 100
1 10 100
0.1 1 10
0.1 1 10
1 10 100
0.1 1 10
α-Naphthoflavone (CYP1A)
Coumarin (CYP2A6)
Sulfaphenazole (CYP2C9)
Omeprazole (CYP2C19)
Quinidine (CYP2D6)
4-Methylpyrazole (CYP2E1)
Ketoconazole (CYP3A)
Cytochrome P450 Inhibitors (μM)

Figure 5


Chemical Inhibition
Antibody Inhibition
M1 formation
(percent of control)
120
100
80
60
40
20
0
MeOH
Ticlopidine
RU-486
Control IgG
α-CYP3A4
Cytochrome P450 Inhibitors