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COMPARATIVE ANALYSIS OF CYP3A HETEROACTIVATION BY STEROID HORMONES AND FLAVONOIDS IN DIFFERENT IN VITRO SYSTEMS AND POTENTIAL IN VIVO IMPLICATIONS

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Running title: CYP3A heteroactivation in different in vitro systems

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Abbreviations used: DHEA, dehydroepiandosterone; DHEA-S, dehydroepiandosterone-sulphate; HLMs, human liver microsomes; LC-MS/MS, liquid chromatography-tandem mass spectrometry; AUC, area under plasma concentration time curve; EC_{200}, concentration of effector required to produce 200% control activation; CL_{max}, maximal clearance; fu_{inc}, fraction unbound in the incubation; CYP, cytochrome P450; $fm_{CYP3A4,}$ fraction of a substrate drug metabolized by the heteroactivated pathway via CYP3A4.

ABSTRACT

A systematic analysis of the heteroactivation of CYP3A-mediated carbamazepine 10,11-epoxidation has been investigated in three different in vitro systems, namely recombinant CYP3A4 and CYP3A5, human liver microsomes (HLMs) and cryopreserved human hepatocytes. The effect of ten endogenous steroids and flavonoids was studied over a range of substrate and effector concentrations. A novel heteroactivation model was used to obtain the parameters EC_{200} (concentration of effector required to produce 200% control) and heteroactivation ratio (the ratio of maximum observed reaction velocity to control). The EC_{200} values obtained in HLMs and human hepatocytes were corrected for nonspecific binding. Heteroactivation of CYP3A5 has been demonstrated with mean heteroactivation ratios in CYP3A5, HLMs and hepatocytes on average 2-fold greater than in recombinant CYP3A4 for most of the effectors investigated. In recombinant CYP3A4 heteroactivation was greatest at substrate concentrations below K_m. Heteroactivation increased with effector concentration in a nonlinear manner and differed between effectors (mean heteroactivation ratios varied up to 12-fold). A greater extent of heteroactivation was observed in HLMs than in human hepatocytes for steroid effectors, but the opposite was true for flavonoid effectors. The observed heteroactivation of CYP3A in intact cells supports an in vivo relevance. From the in vitro heteroactivation data, a significant increase in clearance in vivo was predicted for substrates with a high dependence on CYP3A4 to the overall elimination, indicating that heteroactivation of CYP3A may be a potential source of inter-individual variability.

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Cytochromes P450 (CYPs) 3A are the most abundant P450 enzymes in the human liver and small intestine (Paine et al., 2006). In vitro assays of CYP3A mediated metabolism are used routinely in the drug discovery effort to quantitatively predict in vivo pharmacokinetic parameters. However, these estimates may be confounded by atypical (non Michaelis-Menten) kinetics such as autoactivation (homotropic cooperativity, evident as a sigmoidal kinetic profile), heteroactivation (heterotropic cooperativity, the activation of a substrates metabolism by a separate effector compound) or substrate inhibition (inhibition of a substrates own metabolism) (Houston and Galetin, 2005). A number of studies have addressed the issue of atypical kinetics (Davydov et al., 2007, Tang and Stearns, 2001). Heteroactivation is displayed by a number of CYP3A substrates in vitro, including the antiepileptic, carbamazepine (Nakamura et al., 2002, Egnell et al., 2003a). Heteroactivator-enzyme interaction has been rationalized either by the simultaneous binding of substrate and effector molecules (Shou et al., 1994) or assuming the existence of a separate effector-binding site (Houston and Galetin, 2005).

Evidence exists that heteroactivation of CYP3A may occur in vivo. Co-administration of quinidine resulted in an acute increase in diclofenac clearance in monkeys, an effect also observed in monkey liver microsomes and not explained by alteration of protein binding or blood-plasma partitioning (Tang et al, 1999). Lasker et al., (1984) observed a 3-5-fold increase in CYP3A-mediated zoxazolamine 6-hydroxylation in rats when co-administered with a range of flavonoids. Egnell et al., (2003a) demonstrated that heteroactivation of carbamazepine metabolism by felbamate is the likely mechanism of the clinically observed drug-drug interaction. It has been speculated that the CYP3A enzyme exists in a fully activated state in vivo (Houston and Kenworthy, 2000), and that in vitro the enzyme exists in a reduced activity state, which may contribute to the under-prediction of clearance from in vitro data (Ito and Houston, 2005). Since heteroactivation may be a mechanism by which the enzyme is returned to its “natural” state, a detailed understanding of the phenomenon in vitro is necessary to allow incorporation into in vitro-in vivo extrapolation.

The heteroactivation phenomenon is not exclusive for CYP3A4 (Galetin et al., 2003) and has been reported for other CYPs including CYP2C9 (Egnell et al., 2003b, Hummel et al., 2005). However, previous studies reporting heteroactivation (Nakamura et al., 2002, Nakamura et al., 2003, Torimoto et al., 2003) have been limited in design, using either a single high effector concentration (≥100 μM) or a single in vitro system. The lack of studies covering a range of effector concentrations, particularly encompassing physiological values, confounds any conclusions on the in vivo relevance of heteroactivation.

Carbamazepine is metabolized by both CYP3A4 and CYP3A5 to the active metabolite carbamazepine 10,11-epoxide (Huang et al., 2004), with a minor contribution of CYP2C8 (Kerr et al., 1994). This metabolic pathway is estimated to be responsible for up to 85% of carbamazepine clearance at steady state (Eichelbaum et al., 1985, Sumi et al., 1987, Svinarov and Pippenger, 1996). CYP2C8 mediated epoxidation also occurs, but to a much lesser extent. Carbamazepine 10,11-epoxide is subsequently converted to carbamazepine 10,11-trans-diol by epoxide hydrolase and excreted in the urine (Svinarov and Pippenger, 1996). Thus any alteration of the CYP3A mediated metabolic pathway has a significant impact on the clearance of carbamazepine.

Several compounds have been observed to be heteroactivators of CYP3A mediated metabolism. The flavonoid α-naphthoflavone was generally regarded as the prototypical effector of CYP3A4 mediated drug metabolism (Shou et al., 1994), although many other compounds including steroid hormones, flavonoids and drugs have been shown to heteroactivate CYP3A mediated metabolism pathways (Nakamura et al., 2002, Nakamura et al., 2003).

In the current study ten different effectors including endogenous steroids testosterone, progesterone, androstenedione, aldosterone, cortisol, DHEA and DHEA-S, the flavonoids flavone and α-naphthoflavone, and quinidine are employed to investigate the heteroactivation of CYP3A mediated carbamazepine 10,11-epoxidation. The heteroactivation phenomenon is investigated in vitro using three systems with differential complexity, namely recombinant CYP3A4 and CYP3A5, HLMs and cryopreserved human hepatocytes. The effect of a range

of substrate and effector concentrations on the extent of heteroactivation has been assessed. A mechanistic model analogous to that used for the prediction of metabolic inhibition-mediated drug-drug interactions has been derived in order to predict the potential in vivo implications of heteroactivation observed in vitro.

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Materials and Methods

Chemicals. Testosterone, androstenedione, progesterone, aldosterone, DHEA, DHEA-S, cortisol, α-naphthoflavone, flavone, quinidine, carbamazepine, carbamazepine 10,11-epoxide, triazolam, β-NADP, isocitric acid, DL-isocitric dehydrogenase, HEPES, sodium bicarbonate, trypan blue solution 0.4%, hydrochloric acid and sodium hydroxide were purchased from Sigma Chemicals Co. (Poole, Dorset, UK). Methanol and acetonotrile were obtained from BDH laboratory supplies (VWR International Ltd., Leicestershire, UK). Williams E dry powder was obtained from Invitrogen Ltd. (Paisley, UK). All other reagents and solvents were of high analytical grade. Strata™ impact protein precipitation plates were obtained from Phenomenex Inc. (Macclesfield, Cheshire, UK). Multiscreen® polyolefin filter plates with ultracel PPB membranes were obtained from Millipore (UK) Limited (Stonehouse, Gloucestershire, UK). Cryopreserved pooled human liver microsomes (n=60 livers) and cryopreserved pooled human hepatocytes (n=5 livers) were provided by Pfizer Global R&D, Sandwich, Kent, UK.

Microsomal incubation conditions. Effector compounds were selected following a literature search of papers containing examples of in vitro heteroactivation of CYP3A4 mediated metabolism, independent of in vitro system or substrate. Studies were performed in recombinant CYP3A4 and recombinant CYP3A5 expressed in baculovirus infected insect cells (BTI-TN-5B1-4) co-expressed with NADPH P450-oxidoreductase, and in human liver microsomes pooled from 60 individuals, prepared by differential centrifugation of liver homogenate. Incubation time (40 minutes for recombinant enzyme, 20 minutes for HLMs) and protein concentration (40 pmols/ml for recombinant enzyme, 0.82 mg/ml for HLMs) were within the linear range for carbamazepine 10,11-epoxide formation. Microsomes were suspended in phosphate buffer (0.1 M, pH 7.4), effector compound and NADPH regenerating system (1mM β-NADP, 7.5 mM isocitric acid, 10 mM magnesium chloride and 0.2 units isocitric dehydrogenase) and the final incubation volume was 0.25 mL. Controls contained no heteroactivator but the same amount of solvent. Samples were pre-incubated for 5 minutes in a shaking water bath at 37°C and each reaction was initiated with addition of carbamazepine

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substrate (5, 50 or 200μM in recombinant CYP3A4 corresponding to $<K_m$, $\approx K_m$ and $>K_m$ concentrations, or 5 μM in recombinant CYP3A5 and HLMs. The final concentration of the organic solvent (methanol) in the incubation media was 0.25 % v/v. In recombinant CYP3A4 and HLMs, heteroactivation profiles (plot of effector concentration vs % control reaction velocity) were generated for each effector at each substrate concentration over a wide effector concentration range (0.01-100μM). In recombinant CYP3A5, heteroactivation profiles were generated for testosterone, progesterone, androstenedione and flavone, selected as representative effectors. For the remaining effectors, heteroactivation ratio was estimated using the effector concentration producing maximum heteroactivation in recombinant CYP3A4. The reaction was terminated by addition of 0.25 mL ice-cold acetonitrile containing 1μM triazolam (internal standard). Samples were centrifuged at 13,400g for 5 min and the supernatant analyzed by LC-MS/MS.

Cryopreserved human hepatocyte incubation conditions. Studies were performed in cryopreserved human hepatocytes pooled from 5 individuals. Incubation time (120 minutes) and cell concentration (0.5×10^6 cells/ml) were within the linear range for carbamazepine 10,11-epoxide formation. Hepatocytes were rapidly thawed and diluted 1:100 in sterile carbogenated Williams' hepatocyte media (supplemented with L-glutamine, 26mM $NaHCO_3$, 50mM HEPES, carbogenated for 30 min, pH 7.4). Tubes containing the hepatocytes were centrifuged at 90g at 37°C for 5 minutes, and the pellet re-suspended in media. Cell concentration and viability were assessed by trypan blue exclusion. Viability was greater than 70% in all cases. Hepatocytes were suspended in carbogenated Williams' hepatocyte media containing effector compound and the final incubation volume was 0.1 mL. Controls contained no effector compound but the same amount of solvent. Samples were pre-incubated for 5 minutes with shaking in a Haraeus Hera Cell incubator at 37°C, 5% CO_2, and each reaction was initiated with addition of carbamazepine (5μM). The final concentration of the organic solvent (methanol) in the incubation media was 0.25 % v/v. Heteroactivation profiles were generated for each effector over a wide concentration range (0.01-100μM). The reaction

was terminated by addition of 0.1 mL ice-cold acetonitrile containing 1μM triazolam (internal standard). Samples were centrifuged at 8000g for 10 min and the supernatant filtered through protein precipitation plates by vacuum. Samples were evaporated to dryness by nitrogen gas at 40°C and reconstituted in mobile phase before being analyzed by LC-MS/MS.

Microsomal sample analysis. Carbamazepine 10,11-epoxide was quantified by LC-MS/MS using triazolam as internal standard. Separation was achieved on a Luna C18(2) 50x4.6 mm 3μm column (Phenomenex, UK) at 40 °C using a tertiary gradient maintained at 1 ml/min by a Waters Alliance 2795 HT LC system. An initial mobile phase of 90 % 0.001M ammonium acetate/10 % acetonitrile was maintained for 2 minutes before being ramped immediately to 15 % 0.05% formic acid / 10 % acetonitrile, 45% 0.001 M ammonium acetate/10% acetonitrile and 40% 0.001 M ammonium acetate/90% acetonitrile. This was maintained for 1 minute before being ramped immediately to 15 % 0.05% formic acid/ 10% acetonitrile and 85% 0.001 M ammonium acetate/90% acetonitrile and maintained for a further minute. The initial ratio was immediately re-established and maintained to 5 minutes. The retention times were approximately 3.29 (carbamazepine 10,11-epoxide) and 3.76 (triazolam) minutes. The compounds were detected and quantified by atmospheric pressure electrospray ionisation MS/MS using a Micromass Quattro Ultima triple quadrupole mass spectrometer. The LC column eluate was split and ¼ was delivered into the MS where the desolvation gas (nitrogen) flow rate was 600 l/hr, the cone gas (nitrogen) flow rate was 100 l/hr and the source temperature was 125 °C. Using positive ion mode, protonated molecular ions were formed using a capillary energy of 3.5 kV and cone energies of 35 V (carbamazepine 10,11-epoxide) and 80 V (triazolam). Product ions formed in argon at a pressure of 2 $x10^{-3}$ mbar and at collision energies of 20 eV (carbamazepine 10,11-epoxide, m/z 253.05→180.2) and 25 eV (triazolam, m/z 343.0→308.3) were monitored as ion chromatograms which were integrated and quantified by quadratic regression of standard curves using Micromass QuanLynx 3.5 software.

Hepatocyte sample analysis - Carbamazepine 10,11-epoxide was quantified by LC-MS/MS using triazolam as internal standard. Separation was achieved using an onyx monolithic C18 100x4.6mm column (Phenomenex, UK) at 25°C using a tertiary gradient maintained at between 0.5 and 1 ml/min by an Agilent 1100 LC binary pump system. An initial mobile phase of 40 % aqueous (90% H_2O, 10% MeOH)/60% organic (10% H_2O, 90% MeOH) was ramped linearly to 100 % organic at 6.5 minutes and held for 3 minutes, before being ramped linearly back to 40 % aqueous/60% organic at 9.5 minutes and held for 0.5 minutes to re-equilibrate. The retention times were approximately 4.1 (carbamazepine 10,11-epoxide) and 5.7 (triazolam) minutes. The compounds were detected and quantified by atmospheric pressure electrospray ionisation MS/MS using an Applied Biosystems MDS Sciex API 4000 triple quadrupole mass spectrometer. The LC column eluate was split and 20% was delivered into the MS where the source temperature was 650 °C. Using positive ion mode, protonated molecular ions were formed using a capillary energy of 4.5 kV and cone energies of 56 V (carbamazepine 10,11-epoxide) and 35 V (triazolam). Product ions formed in argon at collision energies of 31 eV (carbamazepine 10,11-epoxide, m/z 253.1→180.3) and 25 eV (triazolam, m/z 344.0→309.0) were monitored as ion chromatograms which were integrated and quantified by quadratic regression of standard curves using MDS Sciex Analyst 1.4 software.

Kinetic assays. Kinetic profiles (plot of substrate concentration vs reaction velocity) were obtained for carbamazepine 10,11-epoxidation in all systems. Substrate was incubated at 0.5-1000μM, in the same conditions for each system as previously described (in the absence of effector compound). Samples were analysed as previously described. Carbamazepine kinetic data in different in vitro systems were analysed using the Michaelis-Menten equation with the weighting factor of 1/y using GraFit 5 (Erithacus Software, Horley, Surrey, UK). In the case of sigmoidal kinetics, kinetic parameters V_{max}, K_s (substrate dissociation constant) and α (defining changes in binding affinity - homotropic cooperativity) were estimated from untransformed data using the two-site kinetic model (Houston and Kenworthy, 2000). In the

case of Michaelis-Menten kinetics, intrinsic clearance (CL_{int}) was estimated from the V_{max}/K_m ratio. When the metabolic profile was consistent with homotropic cooperativity, the CL_{max} (the maximum clearance when the enzyme is fully activated) (Houston and Galetin, 2005) was estimated from the fitted curve.

In order to compare clearance values from different in vitro systems, data are scaled to give values in the units µl/min/g of liver. HLM and hepatocyte data were scaled using the scaling factors described by Barter et al. (2007) i.e. 40mg microsomal protein/g of liver and 99×10^6 cells/g of liver. rCYP3A4 and rCYP3A5 data were scaled to µl/min/mg protein as described by Venkatakrishnan et al. (2000), using the CYP3A4 and CYP3A5 abundance data for the pooled HLMs used in this study of 120pmols CYP3A4/mg microsomal protein and 17 pmols CYP3A5/mg microsomal protein.

Protein Binding. Fraction unbound in the incubation (fu_{inc}) was estimated in pooled HLMs and pooled human hepatocytes for all the heteroactivators investigated. Androstenedione, testosterone, aldosterone, α-naphthoflavone, quinidine, cortisol and progesterone were incubated at 1µM with incubation matrix and unbound compound collected by ultracentrifugation at 2000g for 50min at 37ºC through a Multiscreen® filter plate (Millipore, Gloucestershire, UK). Metabolism of compounds was avoided by absence of regenerating system in HLM assays and the use of nonviable hepatocytes. HLM concentration was 0.82 mg protein/ml and hepatocyte concentration was 0.5×10^6 cells/ml. Samples were analysed by LC/MS/MS in the following way: sample protein was reduced using an Opti-Lynx C18 40µM 100Å 2.1×15mm Bumble Bee column. Separation was achieved using an onyx monolithic C18 100x4.6mm column (Phenomenex, UK) at 25°C using a tertiary gradient maintained at 3 ml/min by an Agilent 1100 LC binary pump system. An initial mobile phase of 100 % aqueous (90% H_2O, 10% MeOH)/0% organic (10% H_2O, 90% MeOH) was held for 0.3 minutes before being ramped linearly to 100 % organic at 0.8 minutes. At 2 minutes the mobile phase was ramped linearly back to 100 % aqueous/0% organic at 2.1 minutes and held for 0.4 minutes to re-equilibrate. The retention times were less than 2.5 minutes for all compounds, which were detected and quantified by atmospheric

pressure electrospray ionisation MS/MS using an Applied Biosystems MDS Sciex API 4000 triple quadrupole mass spectrometer. The LC column eluate was split and 20% was delivered into the MS where the source temperature was 650 °C. Using positive ion mode, protonated molecular ions were formed using a capillary energy of 4.5 kV and cone energies of 35 V (testosterone, aldosterone, progesterone, quinidine, α-naphthoflavone and androstenedione) and 91 V (cortisol). Product ions formed in argon at collision energies of 20 eV (testosterone, m/z 287.3→97.2 and androstenedione, m/z 289.1→97.2), 23 eV (cortisol, m/z 363.4→327.2), 25 eV (aldosterone, m/z 361.1→343.2), 39 eV (progesterone, m/z 315.3→109.1), 40 eV (quinidine, m/z 325.2→184.0) and 50 eV (α-naphthoflavone, m/z 273.0→115.2) were monitored as ion chromatograms which were integrated and quantified by quadratic regression of standard curves using MDS Sciex Analyst 1.4 software. Cone energy was 35 V.

Since flavone, DHEA and DHEA-S were not detectable by LC/MS/MS with sufficient sensitivity, microsomal binding was estimated using the prediction equation described by Hallifax and Houston (2006). Experimental log P values of 3.56 and 3.23 for flavone and DHEA were taken from Hansch et al. (1995). A log P value of 2.99 was calculated for DHEA-S using the LogKow online log P calculator (http://www.syrres.com/esc/est_kowdemo.htm). For these three effectors, the fu_{inc} values in the hepatocyte were extrapolated from the microsomal values, assuming a linear relationship between the extent of microsomal and hepatocyte binding at 1mg/mL and 10^6 cells/mL (Austin et al., 2005).

Data Analysis. In the heteroactivation studies, rate of carbamazepine 10,11-epoxide formation data was expressed as % of the control value. Heteroactivation profiles were analyzed in GraFit 5 (Erithacus Software, Horley, Surrey, UK) using the following heteroactivation equation:

$$v = \frac{\text{range}}{1+\left(\frac{EC_{50}}{E}\right)^{S}} + 100 \qquad (1)$$

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where v is the rate of carbamazepine 10,11-epoxide formation in % control, range is the maximum theoretical rate minus control, EC_{50} is the concentration of effector required to produce 50% of maximum heteroactivation, E is the effector concentration and S is the slope. The parameter EC_{200} (concentration of effector required to produce 200% control) was estimated from the fitted curve as an endpoint measure of effector potency. The parameter heteroactivation ratio (the ratio of maximum observed reaction velocity to control) was calculated from the data, and values greater than 2 were considered to represent significant heteroactivation. The values of EC_{200} obtained in HLMs and human hepatocytes were corrected for microsomal protein binding, whereas it was assumed to be negligible in recombinant enzymes.

Predicted impact on in vivo kinetics. In order to assess the impact of heteroactivation of CYP3A mediated carbamazepine metabolism in vivo a mechanistic model (Appendix) shown in equation 2 was used.

$$\frac{CL_{int,act}}{CL_{int,cont}} = \frac{AUC_{cont}}{AUC_{act}} = fm_{CYP3A4}(1+[E]/EC_{200}) + (1-fm_{CYP3A4}) \quad (2)$$

Where CL_{act} and CL_{cont} represent clearance in the presence and absence of heteroactivator, respectively; AUC_{act} and AUC_{cont} represent area under plasma concentration-time profile in the presence and absence of heteroactivator, respectively, [E] is the effector concentration, fm_{CYP3A4} represents the fraction of a substrate drug metabolised by the heteroactivated pathway via a particular P450 enzyme, $(1-fm_{CYP3A4})$ represents clearance via other P450 enzymes and/or renal clearance. The EC_{200} was used as a pragmatic indicator of heteroactivator potency. An experimental parameter reflecting affinity of the heteroactivators (analogous to inhibition constant K_i) would be preferable for the in vitro-in vivo extrapolation of the heteroactivation data; however, lack of detailed mechanistic knowledge does not allow derivation of such parameter. This equation assumes that the conditions of the well-stirred liver model exist, that the effector does not affect either the intestinal absorption or plasma protein binding of the substrate, that the substrate is eliminated by a single metabolic pathway that is subject to heteroactivation, and that the AUC ratio of orally administered substrate in

the presence and absence of effector reflects the ratio of clearances, as used previously for the prediction of inhibition drug-drug interactions (Ito et al., 2005). Since many of the effectors used in this study are endogenous steroids and unbound plasma concentrations are not available, total plasma concentrations were used for the purposes of predicting in vivo heteroactivation of carbamazepine epoxidation. While concentrations of effector far greater than the EC_{50} would result in saturation of heteroactivation, it is assumed that such concentrations are unlikely to be reached in vivo.

Results

Kinetic properties of carbamazepine metabolism. Carbamazepine 10,11-epoxidation displayed Michaelis-Menten kinetics in recombinant CYP3A4, whereas sigmoidal kinetics were observed in recombinant CYP3A5, HLMs and human hepatocytes (Table 1). The extent of sigmoidicity (assessed by the value of α) was system-dependent, with the most pronounced cooperativity in HLM and the least in recombinant CYP3A5. The sigmoidal kinetics observed was consistent with previous reports in HLMs (Nakamura et al., 2002, Egnel et al., 2003b), but contrasts with a previous report in human hepatocytes (Pelkonen et al., 2001). The Michaelis-Menten kinetics observed in recombinant CYP3A4 was in contrast to the autoactivation reported by Egnel et al. (2003a). CYP3A4 and CYP3A5 showed comparable activity for carbamazepine 10,11-epoxidation, in agreement with previous findings by Huang et al. (2004). When the data were scaled using CYP3A4/3A5 microsomal abundance to express carbamazepine 10,11-epoxidation in pmols/min/g liver, there was a good agreement between recombinant CYP3A and HLMs, but CL_{max} in human hepatocytes was approximately a quarter of those values (Table 1).

Heteroactivation of CYP3A-mediated carbamazepine metabolism in recombinant CYP3A4 and CYP3A5. Initial studies were conducted in recombinant CYP3A4 enzyme. An effector concentration-dependent effect was observed (Fig. 1), as the increasing effector concentrations resulted in increasing extent of heteroactivation approaching the plateau (used to calculate the heteroactivation ratio). No significant heteroactivation was observed at effector concentrations <0.1μM, and greatest heteroactivation was generally observed within the 50-100μM effector concentration range (Fig. 2). Heteroactivation ratio and EC_{200} were estimated using the heteroactivation model for all the effectors and in all in vitro systems investigated, as shown in Table 2. A substrate concentration-dependent heteroactivation effect was noted, with the greatest heteroactivation ratio at the lowest concentration of carbamazepine of 5μM ($<K_m$) with all the effectors investigated (Fig. 2); hence, this substrate concentration was employed for all further heteroactivation studies.

The degree of heteroactivation of carbamazepine 10,11-epoxidation varied between effectors (Fig. 2). There was no heteroactivation of carbamazepine metabolism by either DHEA-S or quinidine, contrary to the in vitro heteroactivation of midazolam 1'-hydroxylation observed by Galetin et al (2002). Greatest heteroactivation ratios were observed for aldosterone and DHEA (6.3 and 6.0, respectively). DHEA and α-naphthoflavone displayed greatest heteroactivator potency, i.e., the lowest EC_{200} (Table 2).

Heteroactivation studies in recombinant CYP3A5 were conducted using the concentration of effector producing maximum heteroactivation in recombinant CYP3A4 (100μM in all cases except androstenedione (50μM) and α-naphthoflavone (5μM)) and at 5μM concentration of carbamazepine. Heteroactivation ratios >2 were observed for all effectors except quinidine and α-naphthoflavone. In contrast to the results in recombinant CYP3A4, heteroactivation was observed in CYP3A5 in the presence of DHEA-S (heteroactivation ratio 3.6) suggesting that this effector is selective for the CYP3A5 enzyme (Fig. 3). Heteroactivation in recombinant CYP3A5 was on average 2-fold greater than in CYP3A4. Greatest heteroactivation ratios were observed for testosterone and DHEA (11.9 and 15.7, respectively), and the rank order of effectors according to heteroactivation ratio was different compared to CYP3A4 (Table 2).

Heteroactivation in pooled HLM. Heteroactivation ratios >2 were observed for 8 effectors except DHEA-S and quinidine, consistent with the heteroactivation trends observed in the recombinant CYP3A4. The finding was not surprising as the CYP3A content of the pooled HLMs (137 pmols/mg protein) consisted of 87% CYP3A4; therefore, the impact of the CYP3A5 in the HLMs will probably be less pronounced. However, heteroactivation was on average 2-fold greater in HLMs than in recombinant CYP3A4, similar to that observed in recombinant CYP3A5. Effector potency, as measured by EC_{200}, was also generally greater in HLMs compared to recombinant CYP3A4 (mean EC_{200} values of 3.4 and 11.0, respectively). Greatest heteroactivation ratios were observed for androstenedione and DHEA (18.5 and 14.1, respectively), and the rank order of effectors based on the heteroactivation ratio differed from that seen with recombinant enzymes (Table 2, Fig.3).

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Heteroactivation in pooled human hepatocytes. Heteroactivation ratios >2 were observed with 9 effectors except quinidine. Heteroactivation was on average 1.7-fold greater in hepatocytes than in recombinant CYP3A4; the mean ratio was comparable to the values observed in HLM and recombinant CYP3A5. Greatest heteroactivation ratios were observed for flavone and DHEA (19.5 and 11.0, respectively), and the rank order of effectors based on either the heteroactivation ratio or the EC_{200} differed to the previously investigated in vitro systems (Table 2). Mean effector potency (EC_{200}) was 5.8μM, representing a greater potency than in recombinant CYP3A4, and a comparable potency to HLMs.

Nonspecific Binding. Fraction unbound in the incubation (fu_{inc}) was estimated for all the effectors investigated in HLM and hepatocytes, as shown in Table 2. For some highly protein-bound compounds (e.g., α-naphthoflavone, flavone, progesterone), the correction for microsomal binding resulted in a large increase in apparent activation potency (decrease in EC_{200}) and good agreement between values obtained in HLMs and hepatocytes for the 8 effectors investigated.

Prediction of heteroactivation in vivo. The possible impact of heteroactivation on in vivo carbamazepine clearance was estimated from the in vitro data generated in the current study (Fig. 4). The fm_{CYP3A4} value of carbamazepine 10,11-epoxidation by CYP3A in vivo was estimated to be 0.4-0.8 based on the range of literature reported values (Eichelbaum et al., 1985, Sumi et al., 1987, Svinarov and Pippenger, 1996). Since carbamazepine is a potent inducer of CYP3A4 expression and usually chronically dosed, higher values of fm_{CYP3A4} (associated with chronic dosing) may be of greater relevance (Eichelbaum et al., 1975, Eichelbaum et al., 1985). In vivo plasma concentrations of the effectors used in the current study are generally sub-micromolar, but with notable exceptions: progesterone is highly elevated during pregnancy, typically above 0.5μM (Elenkov et al., 2001)). Flavonoids have been detected at concentrations up to 15μM (Hollman and Katan, 1997). Predicted increase in carbamazepine clearance ratio at 0.5μM progesterone was below 2 (1.8 and 1.2-fold using data from HLMs and human hepatocytes, respectively), even under the assumption of 80% contribution of CYP3A4 to the elimination of carbamazepine. This minimal predicted in vivo

effect can be explained by low (<1) ratio of effector concentration and potency (E/ EC_{200}) seen regardless of the source of in vitro heteroactivation data. However, a 3.2 and 6.7-fold increase in carbamazepine clearance was predicted by flavone assuming an in vivo concentration of 5μM using data from HLMs and human hepatocytes, respectively (Table 3). The substrate-related parameter (fm_{CYP3A4}) and in vivo effector concentration/effector potency ([E]/ EC_{200}) both had a significant impact on the predicted changes in carbamazepine clearance and AUC. Simulation in Figure 4 indicates that effectors with [E]/ EC_{200} <1 are expected to have a minor impact on the in vivo kinetics (i.e., < 2-fold change in clearance of the victim drug). However, an increase in clearance of up to 11-fold is predicted for potent effectors and substrates where CYP3A contribute >80% to the overall elimination.

Discussion

The current study provides a comprehensive, systematic study of heteroactivation of the CYP3A enzymes. The study utilises 10 effectors over a wide concentration range, at several carbamazepine concentrations and in four different in vitro systems, namely recombinant CYP3A4, CYP3A5, human liver microsomes and cryopreserved hepatocytes.

Substrate-concentration dependent heteroactivation. In recombinant CYP3A4, the greatest heteroactivation was observed at the lowest concentration of carbamazepine, equivalent to a substrate concentration below the K_m value, reducing at larger substrate concentrations (Fig. 1). Some previous studies have investigated in vitro heteroactivation phenomenon using only a single arbitrarily selected high substrate concentration (Nakamura et al 2002, Torimoto et al 2003), which might not provide a true estimation of the extent of heteroactivation. Moreover, it should be noted that concentrations of drugs in both in vitro drug metabolic stability studies and in vivo in plasma are usually in the low μM region. Therefore, a low substrate concentration when performing heteroactivation studies should hold greater in vivo relevance than a high substrate concentration.

In vitro system-dependent heteroactivation. Heteroactivation of CYP3A4 mediated metabolism has been previously reported in recombinant enzymes (Galetin et al., 2002, Nakamura et al., 2003) and HLMs (Nakamura et al., 2002) but little evidence previously existed for heteroactivation in intact cells or for CYP3A5 mediated metabolism. In the current study, heteroactivation of carbamazepine 10, 11-epoxidation was observed in all in vitro systems. Although carbamazepine is known to be a potent inducer of CYP3A4 and can induce its own metabolism in man (Eichelbaum et al., 1975), the increase in clearance observed in hepatocytes in the present study is easily distinguished by the acute nature of the effect; induction of CYP3A4 by carbamazepine requires synthesis of new enzyme and it is only detectable over a time course of days, whereas incubations in the current study occurred over a maximum time course of 120 minutes. It is unlikely that the observed increase in carbamazepine epoxide production in hepatocytes is due to the inhibition of epoxide hydrolase mediated carbamazepine 10,11-trans-diol formation since the magnitude of the

observed increase in carbamazepine epoxide was large (heteroactivation ratio up to 19.5) and no evidence exists that the compounds used in this study are capable of potent inhibition of epoxide hydrolase. Indeed, potentiation of mammalian epoxide hydrolase activity in the presence of both flavone and α-naphthoflavone has been previously reported (Alworth et al., 1980).

The current study has shown significant differences between CYP3A4 and CYP3A5 heteroactivation potential. This is not unexpected since inhibitors of CYP3A typically have a differential potency for inhibition of CYP3A4 and CYP3A5 in terms of both reversible and irreversible inhibition (Gibbs et al., 1999, McConn et al., 2004). This finding suggests that CYP3A5 may play a more important role in in vivo interactions involving heteroactivation than involving inhibition. While most Caucasians have low CYP3A5 expression relative to CYP3A4, higher expression levels are found in African American populations, with CYP3A5 comprising of about half of all CYP3A expression in many individuals (Xie et al., 2004) indicating an increased potential for clinically relevant interactions involving heteroactivation in this population.

Generally, there was a good agreement in the type of carbamazepine kinetics and heteroactivation between HLMs and human hepatocytes. Both systems displayed autoactivation kinetics explained by the existence of two separate substrate binding sites (Houston and Galetin, 2005, Tang and Stearns, 2001) and had comparable effector potency values once they had been corrected for nonspecific binding. Some differences were evident as the extent of heteroactivation by the steroid effectors was greater in HLMs than in hepatocytes, the reverse being true for the flavonoid effectors (Table 2), and the specific rank order of effector EC_{200}, and heteroactivation ratio differed between systems. Although inter-donor differences may also contribute to this effect, it is likely that the differences result from differences at the cellular level.

Recombinant P450 enzymes represent a cleaner system with fewer experimental complexities and easier interpretation of the data. However, the choice of recombinant system, the concentration of accessory proteins (e.g., NADPH P450-oxidoreductase and cytochrome b_5) and their relative ratio to the P450 protein may also influence the degree to which

heteroactivation occurs, as suggested recently by Jushchyshyn et al. (2005). Use of recombinant CYP3A4 and CYP3A5 with co-expressed b5 (not present in the current study) may have increased the comparability between this system and HLM in the extent of heteroactivation. Addition of DHEA-S to the hepatocyte incubation resulted in heteroactivation of carbamazepine metabolism similar to the effect observed in the recombinant CYP3A5. This result was different to that in the HLM, but since the relative contribution of CYP3A5 to total CYP3A activity in the hepatocytes was unknown, it is possible that a large proportion of total CYP3A was CYP3A5. Alternatively, a separate mechanism specific to the intact cells may facilitate apparent DHEA-S mediated heteroactivation of CYP3A4.

Effector-dependent heteroactivation. The magnitude of heteroactivation varied between all 10 effectors in all in vitro systems (Fig. 3), consistent with previously observed differential inhibition in the case of CYP3A (Galetin et al., 2003). Some similarities between groups of effectors were evident, such as the greater heteroactivation in HLMs by steroids versus greater heteroactivation in hepatocytes by flavonoids.

Substrate-dependent heteroactivation. Quinidine did not show activation of carbamazepine metabolism in any in vitro system, despite the evidence that exists in the literature for quinidine induced heteroactivation of CYP3A mediated metabolism of other substrates including diclofenac, warfarin and felodipine (Tang et al, 1999, Galetin et al 2002). The specific interaction between substrate and effector within the active site that leads to heteroactivation of substrate metabolism is therefore likely to be governed not only by the structure of the effector compound, but by that of the substrate.

Prediction of impact on in vivo kinetic parameters. The current study has shown conclusively that heteroactivation is not simply a microsomal phenomenon, and occurs in intact human liver cells. Many of the effectors used in this study are either endogenous (steroids) or similar to many compounds ingested in a normal diet (flavonoids), indicating a potential in vivo relevance of heteroactivation. While steroid concentrations in the human liver are yet to be determined accurately, serum concentrations over 0.5µM in the case of progesterone during pregnancy have been observed (Di Renzo et al., 2005). While the

concentrations quoted in this study are total plasma concentration and likely to overestimate the unbound concentrations in vivo, it is possible that many of these steroids may have an additive effect, resulting in a combined concentration required to produce a significant increase in clearance of some substrates. This hypothesis coincides with a clinically observed increase in carbamazepine clearance during pregnancy (Lander and Eadie, 1991).

The predicted 6.7-fold increase in carbamazepine clearance in the presence of 5μM flavone (assuming an fm_{CYP3A4} value of 0.8 for carbamazepine in vivo) suggests the possibility of a significant increase in drug clearance in the presence of a high concentration of a similar flavonoid, and the loss of substrate resulting in sub-therapeutic concentrations. Since plasma concentrations of some flavonoids in individuals taking herbal supplements can exceed 5μM (Hollman and Katan, 1997), a clinically relevant interaction may be possible. Indeed, drug interactions involving other flavonoids are well established – St John's Wort and grapefruit juice are both known to alter the pharmacokinetics of CYP3A substrates in vivo. Since the unregulated use of herbal supplements is currently increasing worldwide, the potential for further drug interactions between the pharmacologically active components of these supplements and prescribed CYP3A substrates is increasing. In addition, a significant proportion of CYP3A mediated metabolism may also occur in the gut (Paine et al., 2006, Galetin and Houston, 2006). Since flavonoids are commonly ingested in many foods such as nuts and wine as well as in supplement form, it is likely that gut concentrations of these compounds during absorption phase may be high in many individuals, with the potential for heteroactivation of CYP3A metabolism at this site and reduced substrate bioavailability.

In summary, the systematic analysis of in vitro heteroactivation of CYP3A mediated drug metabolism performed in this study suggests that a number of factors influence the degree of heteroactivation observed, and that careful consideration of the experimental conditions should be made before in vitro heteroactivation data can be interpreted. Heteroactivation of CYP3A5 mediated metabolism has been comprehensively studied for the first time, and the extent of heteroactivation was greater than that in recombinant CYP3A4. HLMs and human hepatocytes contain both isoforms of the P450 enzymes, and represent an environment closer to in vivo than a

recombinant CYP3A system. This suggests that these systems represent a better choice for studying heteroactivation compared to recombinant P450. This study has demonstrated heteroactivation in intact human hepatocytes by a range of endogenously expressed compounds, supporting the hypothesis that heteroactivation may occur in vivo. Increase in drug clearance >100% have been predicted based on the in vitro heteroactivation data reported in this study. Even greater magnitude of in vivo heteroactivation is predicted for a substrate with a predominant contribution of CYP3A4 (e.g. triazolam, felodipine, fm_{CYP3A4} >0.8), where heteroactivation may have a large impact on the inter-individual variability in the drug clearance.

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FOOTNOTES

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Figure Legends

Fig. 1. Concentration-effect profiles for progesterone mediated heteroactivation of carbamazepine metabolism by rCYP3A4. Carbamazepine concentration in the study was 5μM (○), 50μM (□) and 200μM (△). Data represent the mean of duplicates. Progesterone is shown as representative of all effectors.

Fig. 2. Concentration-effect profiles for A) testosterone, B) androstenedione, C) progesterone and D) flavone mediated heteroactivation of carbamazepine metabolism by rCYP3A4 (○), rCYP3A5 (●), HLMs (△) and human hepatocytes (□). Data represent the mean of duplicates. Carbamazepine concentration of 5μM was employed for all heteroactivation studies.

Fig. 3. Summary of heteroactivation ratios for 10 effectors in rCYP3A4, rCYP3A5, HLMs and human hepatocytes. Effectors investigated are aldosterone (A), testosterone (B), progesterone (C), androstenedione (D), DHEA (E), DHEA-S (F), cortisol (G), flavone (H), α-naphthoflavone (I) and quinidine (J). Carbamazepine concentration in all the heteroactivation studies was 5μM.

Fig. 4. Predicted impact of an effector on in vivo carbamazepine clearance. Hypothetical values of $[E]/EC_{200}$ (0.1-10) were used to predict CL ratio (CL_{act}/CL_{cont}) over a range of physiologically relevant fm_{CYP3A4} values (0.4-1.0). $fm_{CYP3A4,}$ represents fraction of a substrate drug metabolized by the heteroactivated pathway via CYP3A4.

TABLE 1

Summary of kinetic parameters for carbamazepine 10,11-epoxidation in different in vitro systems

In Vitro System	Type of Kinetics	V_{max} (pmols/min/pmol CYP or mg protein or 10^6 cells)	K_m or Ks (μM)	α	αKs (μM)	CL_{int} or CL_{max} (μl/min/pmol CYP or mg protein or 10^6 cells)	CL_{int} or CL_{max} (μl/min/g liver)
rCYP3A4	Michaelis-Menten	5.3	630	n/a	n/a	0.0083[1]	36 (rCYP3A4 + rCYP3A5)
rCYP3A5	Sigmoidal[2]	10	2300	0.092	210	0.0077	
HLMs	Sigmoidal[2]	440	1900	0.012	23	1.1	34
Human Hepatocytes	Sigmoidal[2]	27	840	0.045	38	0.076	7.5

[1] The value represents CL_{int}.[2] The kinetic parameters were determined using the two-site kinetic model assuming the equivalence of two active sites (Houston and Kenworthy, 2000)

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TABLE 2

Extent of heteroactivation and nonspecific binding in the incubation (fu_{inc}) for all 10 effectors in all *in vitro* systems investigated. Carbamazepine concentration was 5µM in all cases.

Effector	Parameter	rCYP3A4	rCYP3A5	HLMs	Human Hepatocytes
Androstenedione	Heteroactivation Ratio	4.9	7.6	18.5	7.3
	Unbound EC_{200} (µM)	2.5	-	0.9	0.5
	fu_{inc}	-	-	0.54	0.70
Testosterone	Heteroactivation Ratio	4.0	11.9	8.8	6.1
	Unbound EC_{200} (µM)	3.2	-	0.8	1.2
	fu_{inc}	-	-	0.54	0.62
Progesterone	Heteroactivation Ratio	4.7	10.8	6.7	4.3
	Unbound EC_{200} (µM)	3.1	-	0.5	2.2
	fu_{inc}	-	-	0.17	0.43
Aldosterone	Heteroactivation Ratio	6.3	8.4	10.5	5.5
	Unbound EC_{200} (µM)	24.1	-	7.1	31.5
	fu_{inc}	-	-	0.65	0.70
α-Naphthoflavone	Heteroactivation Ratio	2.5	-	2.9	3.7
	Unbound EC_{200} (µM)	1.2	-	0.2	0.1
	fu_{inc}	-	-	0.02	0.05
Flavone	Heteroactivation Ratio	5.8	3.8	7.2	19.5
	Unbound EC_{200} (µM)	5.9	-	1.8	0.7
	fu_{inc}	-	-	0.15	0.15
DHEA	Heteroactivation Ratio	6.0	15.7	14.1	11.0
	Unbound EC_{200} (µM)	2.2	-	1.2	0.8
	fu_{inc}	-	-	0.49	0.49
DHEA-S	Heteroactivation Ratio	-	3.6	-	2.6
	Unbound EC_{200} (µM)	-	-	-	9.3
	fu_{inc}	-	-	0.33	0.33
Cortisol	Heteroactivation Ratio	2.3	2.4	2.4	3.6
	Unbound EC_{200} (µM)	46	-	14.4	5.5
	fu_{inc}	-	-	0.25	0.35
Quinidine	Heteroactivation Ratio	-	-	-	-
	Unbound EC_{200} (µM)	-	-	-	-
	fu_{inc}	-	-	0.45	0.72

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TABLE 3

Predicted impact of progesterone and flavone on in vivo carbamazepine clearance via heteroactivation of CYP3A. Predictions of clearance ratio were made from EC_{200} data obtained in HLMs and human hepatocytes.

Effector	**In vivo [E] (μM)**	**In vitro system**	**E/EC_{200}**	**Clearance ratio (predicted fold increase)**
Progesterone	0.5	HLMs	1	1.8
		Hepatocytes	0.2	1.2
Flavone	5	HLMs	2.8	3.2
		Hepatocytes	7.1	6.7

Figure 1

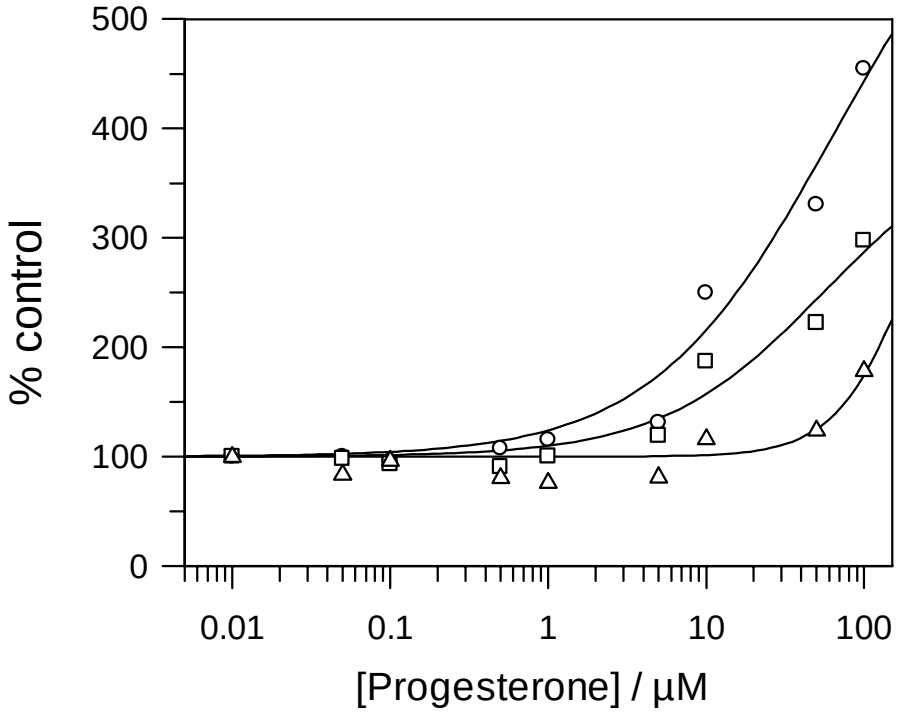

Figure 2

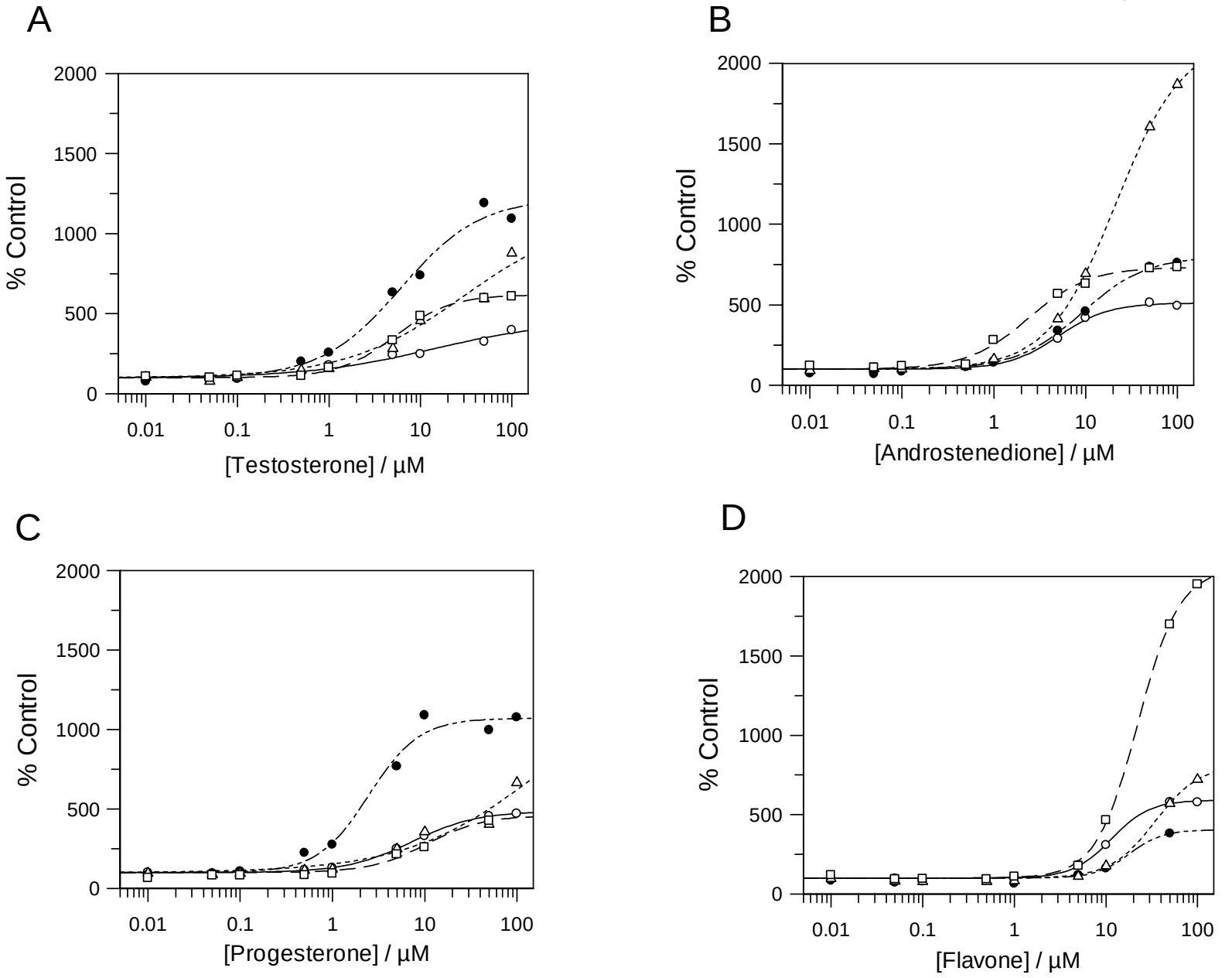

Figure 3

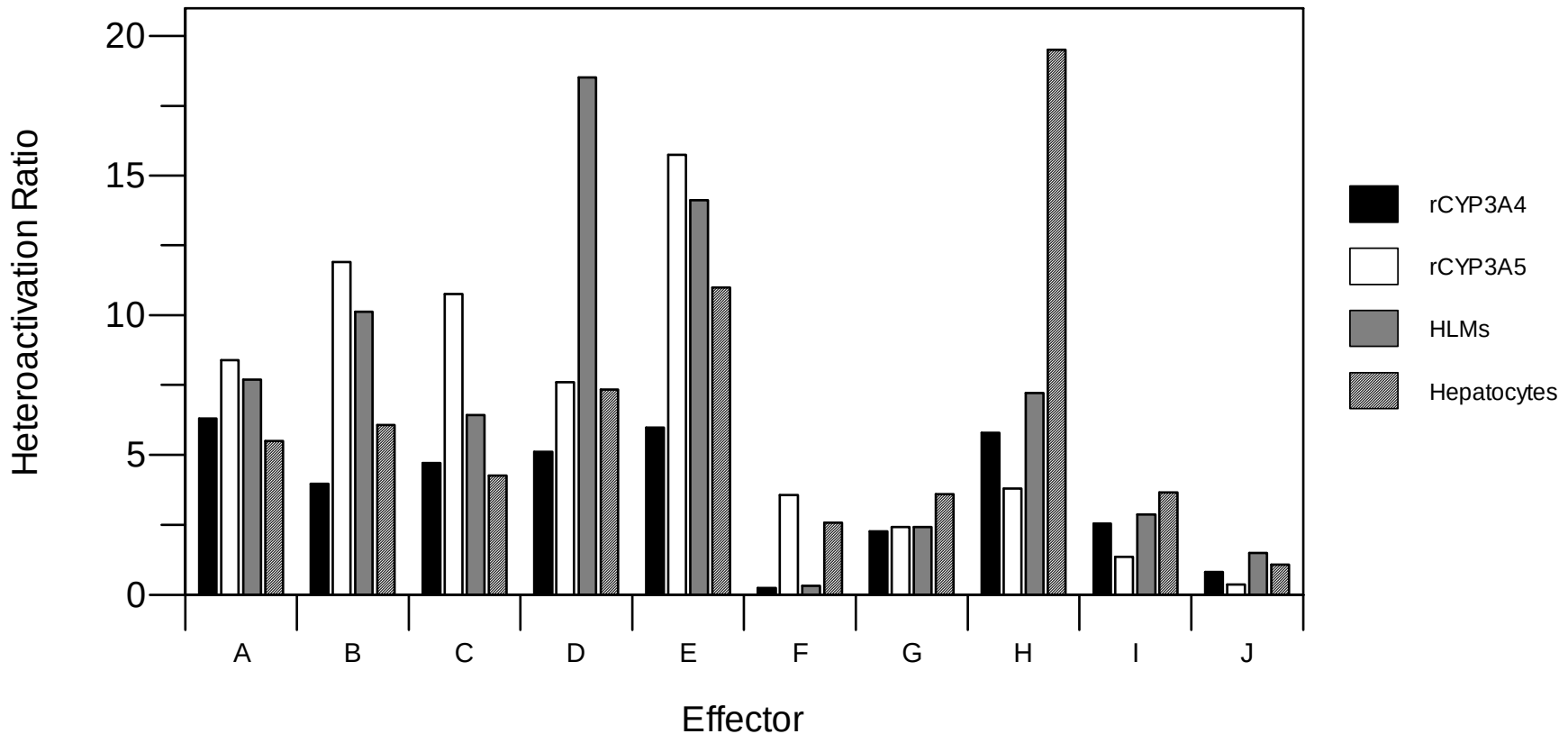

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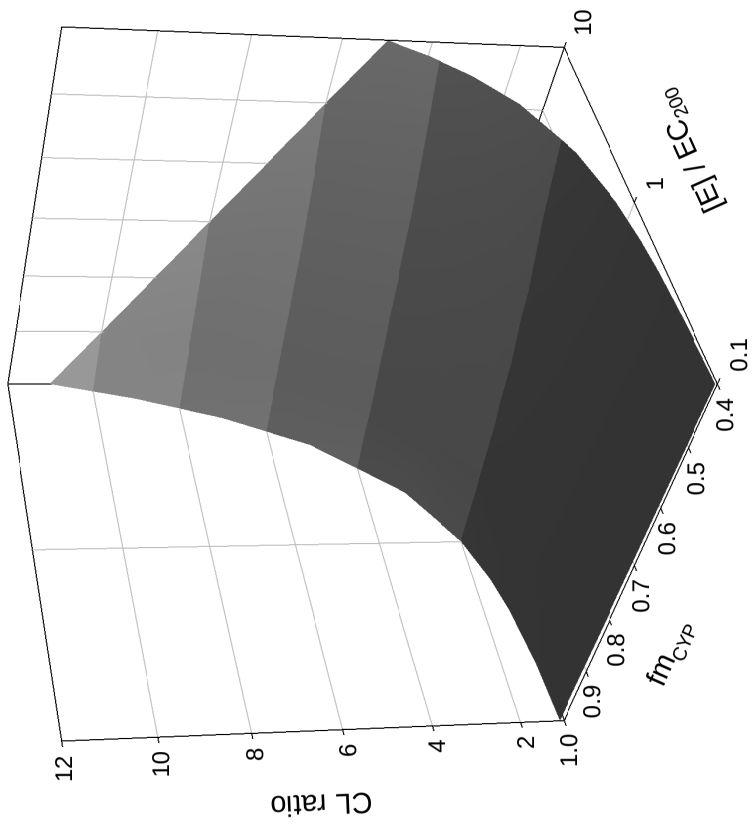
Figure 4



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Appendix

Prediction of heteroactivation in vivo from in vitro data. Hepatic intrinsic clearance, assuming linear metabolism kinetics (substrate concentration much smaller than the K_m), can be defined as the sum of the ratios of V_{max} and K_m for the individual pathways/enzymes. In the case of substrate clearance by one CYP pathway and a separate, undefined second pathway:

$$CL_{int} = \frac{V_{max1}}{K_{m1}} + \frac{V_{max2}}{K_{m2}} = fm_{CYP} CL_{int} + (1 - fm_{CYP}) CL_{int} \quad (3)$$

where 1 refers to a particular CYP pathway (i.e. CYP3A-mediated carbamazepine 10,11-epoxidation) and 2 refers to other metabolic pathways (Ito et al., 2005). In the case of heteroactivation by an effector compound, either the effector increases the rate of product formation or binding affinity, as shown in the equations 4 and 5, respectively.

$$V_{max,act} = V_{max}(1 + [E]/EC_{200}) \quad (4)$$

$$K_{m,act} = \frac{K_m}{1 + [E]/EC_{200}} \quad (5)$$

Assuming that the effector does not affect both metabolic pathways, the intrinsic clearance in the presence of the effector ($CL_{int,\ act}$) can be expressed as follows, again assuming linear metabolism kinetics, since the substrate concentration does not approach the K_m:

$$CL_{int,act} = \frac{V_{max1}(1 + [E]/EC_{200})}{K_{m1}} + \frac{V_{max2}}{K_{m2}} \quad (6)$$

Therefore:

$$CL_{int,act} = fm_{CYP3A4} CL_{int}(1 + [E]/EC_{200}) + (1 - fm_{CYP3A4}) CL_{int} \quad (7)$$

The ratio of the CL_{int} and AUC in the presence and absence of the effector can then be expressed by the following equation:

$$\frac{CL_{int,act}}{CL_{int,cont}} = \frac{AUC_{cont}}{AUC_{act}} = fm_{CYP3A4}(1 + [E]/EC_{200}) + (1 - fm_{CYP3A4}) \quad (8)$$