Characterization of kinetics of human cytochrome P450s involved in bioactivation of flucloxacillin: inhibition of CYP3A-catalyzed hydroxylation by sulfaphenazole

Authors: Stefan J. Dekker, Floor Dohmen, Nico P.E. Vermeulen and Jan N.M. Commandeur*

Affiliation:
Division of Molecular Toxicology, Amsterdam Institute for Molecules Medicine and Systems (AIMMS), Vrije Universiteit, De Boelelaan 1108, 1081HZ, Amsterdam, the Netherlands.

Running head: Bioactivation of flucloxacillin by human CYPs.

Correspondence: Dr. Jan N.M. Commandeur, Division of Molecular Toxicology, Amsterdam Institute for Molecules Medicine and Systems (AIMMS), Vrije Universiteit, De Boelelaan 1108, 1081HZ, Amsterdam, the Netherlands. Phone: (31)205987595. E-mail: j.n.m.commandeur@vu.nl

Number of figures: 8

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bph.14548

BACKGROUND AND PURPOSE

The aim of this study was to characterize the human cytochrome P450s (CYPs) involved in oxidative bioactivation of flucloxacillin to 5-hydroxymethylflucloxacillin, a metabolite with high cytotoxicity toward biliary epithelial cells.

EXPERIMENTAL APPROACH

The CYPs involved in hydroxylation of flucloxacillin were characterized using recombinant human CYPs, pooled liver microsomes in presences of CYP-specific inhibitors, and by correlation analysis using a panel of liver microsomes from 16 donors.

KEY RESULTS

Recombinant CYPs showing the highest specific activity were CYP3A4, CYP3A7 and to lower extent CYP2C9 and CYP2C8. Michaelis Menten enzyme kinetics were determined for pooled HLM, recombinant CYP3A4, CYP3A7 and CYP2C9. Remarkably, sulfaphenazole appeared a potent inhibitor of 5'-hydroxylation of FLX by both recombinant CYP3A4 and CYP3A7.

CONCLUSIONS AND IMPLICATIONS

The combined results show that the 5'-hydroxylation of FLX is primarily catalysed by CYP3A4, CYP3A7 and CYP2C9. The large variability of the hepatic expression of these enzymes could affect the formation of 5'-hydroxymethylfucloxacillin, which may determine the differences in susceptibility to flucloxacillin-induced liver injury. Additionally, the strong inhibition in CYP3A-catalyzed flucloxacillin metabolism by sulfaphenazole suggest that unanticipated drug-drug interactions could occur with coadministered drugs.

Abbreviations:

BEC, biliary epithelial cells; CYP, cytochrome P450; DILI, drug-induced liver injury; FLX, flucloxacillin; GWAS, genome-wide association studies; HLM, human liver microsomes; 5'-HM-FLX, 5'-hydroxymethyl flucloxacillin; NRS, NADPH-regenerating system.

What is known about this subject

- There are large inter-individual differences in plasma concentrations of flucloxacillin and 5'-hydroxymethyl flucloxacillin.
- 5'-hydroxymethyl flucloxacillin is much more toxic to biliary epithelial cells than flucloxacillin
- A previous study on four human CYPs showed highest activity of CYP3A4 in flucloxacillin hydroxylation, however, only a single substrate concentration was used, without quantification of specific activity due to lack of reference metabolite.

What this story adds

- Quantification of specific activities and enzyme kinetics of FLX-hydroxylation by HLM and fourteen recombinant human CYPs.
- Next to CYP3A4, CYP3A7 and CYP2C9 may contribute significantly to flucloxacillin hydroxylation.
- Sulfaphenazole, considered as a selective CYP2C9-inhibitor, strongly inhibits CYP3A-mediated FLX-hydroxylation.

CYP3A4 - http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1337

CYP3A7 - http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=263#1339

CYP2C9 - http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1326

CYP2C8 - http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1325

cyclosporine - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=1024

quinidine - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=2342

warfarine - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=6853

NADP - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=3045

bufuralol - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=9834

chlorzoxazone - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=2322

fluvoxamine - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=7189

ketoconazole - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=2568

mephenytoin - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=7223

miconazole - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=2449

quercetin - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=5346

quinidine - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=2342

testosterone - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=2858

ticlopidine - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=7307

Midazolam - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=3342

CYP1A2 - http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1319

CYP2B6 - http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1324

CYP2C8 - http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1325

CYP2C19 - http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=262#1328

CYP2D6 - http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1329

CYP2E1 - http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1330

phenacetin - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=7402

CYP2A6 - http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1321

bupropion - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=7135

amodiaquine - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=10018

diclofenac - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=2714

bufuralol - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=9834

CYP2J2 - http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=262#1332

CYP3A5 - http://www.guidetopharmacology.org/GRAC/FamilyDisplayForward?familyId=263#1338

CYP1A1 - http://www.guidetopharmacology.org/GRAC/ObjectDisplayForward?objectId=1318

hydroxylapatinib - http://www.guidetopharmacology.org/GRAC/LigandDisplayForward?ligandId=5692

Introduction

The isoxazolyl-penicillin flucloxacillin (FLX) is a narrow spectrum β-lactam antibiotic which is active against gram-positive bacteria, such as methicillin-sensitive *Staphylococcus aureus* (Sutherland *et al*., 1970; Leder *et al*., 1999). Although the antibiotic is generally well tolerated at daily doses as high as 12 grams, it causes drug-induced liver injury (DILI) of a cholesteric nature in susceptible patients (Fairley *et al*., 1993; Derby *et al*., 1993; Eckstein *et al.*, 1993; Koek *et al*., 1994; Russmann *et al*., 2005; De Abajo *et al*., 2004; Andrews *et al*., 2009). Although the risk for FLX-induced DILI is in the region of 9 in 100.000 patients (Russmann *et al*., 2005), the fact that it is one of the most prescribed β-lactam antibiotics makes FLX one of the drugs most often involved in DILI (De Abajo *et al*., 2004). The mechanisms underlying FLX-induced cholestasis are still incompletely understood. Association studies have identified several risk factors, such as prolonged treatment with high daily doses and an age over 55 years (Fairley *et al*., 1993; Russmann *et al*., 2005). Furthermore, a genome-wide association study (GWAS) identified the HLA-B*57:01 allele as a strong risk factor for FLX-induced DILI, with an odds ratio of 80.6, indicative for an important role of the immune system in the mechanism of liver toxicity (Daly *et al*., 2009). Many forms of idiosyncratic DILI are believed to have an immunological basis, in which covalent binding of drugs and/or metabolites to proteins may play an important role by generation of a hapten and by causing cellular stress, leading to the release of so-called danger signals which can stimulate the immune response (Uetrecht et al., 2007; Tailor et al., 2015). Like many other penicillin-like antibiotics, the reactive β-lactam-rings of FLX and its active metabolite 5'-hydroxymethyl FLX (5'-HM-FLX, Figure 1) have been shown to react with lysine-residues of proteins thereby forming potential haptens (Jenkins *et al*., 2009). Interestingly, cytotoxic (CD8+) T-cells were only activated by FLX-exposed antigen-presenting cells in cells obtained from patients and volunteers with the HLA B*57:01 genotype, supporting the role of this genotype as risk factor for FLX-induced DILI (Monshi

et al., 2013; Wuillemin et al., 2013; Wuillemin et al., 2014). However, although the association with HLA B*57:01 is one of the strongest ever reported, still only 1 in 1000 patients with the high-risk allele eventually develops FLX-induced DILI. Therefore, non-genetic risk factors, which escape detection by GWAS-studies, may also determine the sensitivity for FLX-induced cholestasis. Because 5'-HM-FLX was shown to be highly cytotoxic to isolated biliary epithelial cells, whereas FLX was without effect, a high internal exposure to 5'-HM-FLX may be considered as a potentially important risk factor (Lakehal *et al*., 2001).

Many studies have investigated the pharmacokinetics of FLX in groups of healthy volunteers or patients (Thijssen *et al*., 1982; Adam *et al*., 1983; Gath *et al*., 1995; Røder *et al*., 1995; Landersdorfer *et al*., 2007; Dijkmans *et al*., 2012; Maier-Salamon *et al*., 2017). Dependent of the dose regimen and route of administration (by i.v. infusion and/or orally) the maximal plasma concentration (C_{max}) of FLX can range 50 to 790 µM. Systemic clearance of FLX appears to be mainly by renal clearance (65-71%) (Landersdorfer *et al*., 2007; Maier-Salamon *et al*., 2017). Non-renal clearance of FLX occurs predominantly by cytochrome P450-mediated hydroxylation of FLX to 5'-HM-FLX and partly by hydrolysis to the corresponding penicilloic acid, Figure 1. In healthy volunteers plasma-concentrations of 5'-HM-FLX are on average approximately 10% of those of FLX, whereas their half-lives are comparable (Thijssen *et al*., 1982). Because of the high contribution of renal excretion of FLX and 5'-HM-FLX, renal impairment was shown to lead to a significant increase in the areas under the curve which may increase the risk for FLX-induced DILI (Gath *et al*., 1995).

The different pharmacokinetics studies have demonstrated that metabolism of FLX shows significant interindividually variability, which may be a reflection of the large interindividual variability of the enzymes involved in hydroxylation of FLX and/or de mammalian dipeptidase involved in hydrolysis of FLX and 5'-HM-FLX to their corresponding penicilloic acids. In the study of Lakehal et al., it was shown that CYP3A4 was able to catalyze the 5'-hydroxylation of FLX (Lakehal *et al*., 2001). However, the involvement of only four CYP-isoforms were studied and FLX-hydroxylation was measured at only one concentration. Another study showed that FLX induces CYP3A4 in cell-lines exposed to 200-400 µM FLX (Huwyler *et al*., 2006). This may explain the decrease of plasma concentrations of cyclosporine A, quinidine, voriconazole and warfarine when coadministered together with FLX (Cynke et al., 1999; Comuth et al., 2012; Muilwijk et al., 2017; Chaudhuri and Wade, 2018). Whether this induction resulted in increased formation of 5'-HM-FLX was not studied, however. Because a recent meta-analysis of Achour et al.

(Achour *et al*., 2014) showed that the hepatic expression levels of all drug metabolizing human CYPs vary considerably, due to genetic polymorphism, enzyme induction and epigenetic factors, it is critically important to fully characterize the CYPs involved in drug metabolism in order to predict the interindividual variability in pharmacokinetics and the risk to be victim of drug-drug interaction. Furthermore, a major involvement of CYP3A4 in drug metabolism has been shown to complicate prediction of pharmacokinetics based on enzyme kinetics because of the occurrence of atypical enzyme kinetics, such as substrate activation (Houston et al., 2000; Ekins et al., 2003; Atkins et al., 2005; Houston et al., 2005). Furthermore, inhibition of CYP3A4 by drugs appears substrate-dependent complicating prediction of drug-drug interactions (Stresser et al., 2000).

The aim of the present study was therefore to characterize the bioactivation of FLX to 5'-HM-FLX in more detail by using a larger set of recombinant CYPs, by inhibition studies using pooled human liver microsomes (HLM) and by correlation analysis using a panel of HLM from 16 donors. Furthermore, for HLM and the most active CYPs the enzyme kinetics of FLX-hydroxylation was determined.

Materials & methods

Chemicals

FLX (CAS: 1847-24-1) was a kind gift of Dr. Bayliss from the University of Liverpool. β-Nicotinamide adenine dinucleotide phosphate disodium salt (NADP; CAS: 24292-60-2), glucose-6-phosphate (G-6-P; CAS: 54010-71-8), G-6-P dehydrogenase from baker's yeast (*Saccharomyces cerevisiae*; CAS: 9001-40-5), (+)-N-3-benzyl-nirvanol (CAS: 790676-40-3), bufuralol (CAS: 60398-91-6), chlorzoxazone (CAS: 95-25-0), diethyldithiocarbamate (CAS: 20624-25-3), fluvoxamine maleate (CAS: 22916-47-8), ketoconazole (CAS: 65277-42-1), (S)-mephenytoin (CAS: 70989-04-7), miconazole (CAS: 22916-47-8), α-naphtoflavone (CAS: 604-59-1), quercetin (CAS: 6151-25-3), quinidine (CAS: 56-54-2), sulfaphenazole (CAS: 526-08-9), testosterone (CAS: 58-22-0), ticlopidine (CAS: 53885-35-1), and troleandomycin (CAS: 2751-09-9) were purchased from Sigma-Aldrich (St. Louis, MO). Midazolam (CAS: 59467-96-8) was from Duchefa Farma (Haarlem, the Netherlands). All other chemicals were of analytical grade purity and obtained from standard suppliers

Gentest Supersomes containing recombinant human CYPs were obtained from Corning BV life sciences (Amsterdam, the Netherlands). The enzymes used were CYP1A1

(Lot No. 456 211), CYP1A2 (Lot No. 456 203), CYP2A6 (Lot No. 456 254), CYP2B6 (Lot No. 456 255), CYP2C8 (Lot No. 456 252), CYP2C9*1(Arg144) (Lot No. 456 258), CYP2C18 (Lot No. 456 222), CYP2C19 (Lot No. 456 259), CYP2D6*1 (Lot No. 456 217), CYP2E1 (Lot No. 456 206), CYP2J2 (Lot No. 456 264), CYP3A4 (Lot No. 456 207), CYP3A5 (Lot No. 456 256) and CYP3A7 (Lot No. 456 237).

Pooled HLM (50 donors, 20 mg/ml, lot No. 0710619) were obtained from Xenotech (Lenexa, USA). A panel of HLM from single donors were prepared from livers kindly provided by Kalycell (Plobsheim, France) (donors: S1399T, S1449T, S1352T, S1342T, B1327T, S1405T, S1336T, S1446T, S1329T, S1339T, S1343T, S1442T, S1344T, S1441T, S1334T and R1341T).

5'-HM-FLX was obtained biosynthetically by hydroxylation of FLX using a drug metabolizing mutant of CYP102A1 from *Bacillus Megaterium*, M11 L437E, which contains mutations R47L, E64G, F81I, F87V, E143G, L188Q, E267V, G415S and L437E compared to wild-type CYP102A1. This mutant appeared to selectively convert FLX to 5'-HM-FLX (Luirink et al., 2018). One mL of a solution of 100 mM FLX in DMSO was added to 49 mL 100 mM potassium phosphate buffer pH 7.4 and incubated in presence of 1 µM of M11 L437E and NADPH-regenerating system (NRS) for 20 hours at room temperature with constant stirring. The NRS consisted of 100 µM NADPH, 10 mM glucose-6-phosphate, and 0.5 units/mL glucose-6-phosphate dehydrogenase (final concentrations). The reaction was terminated by the addition of an equal volume ice-cold methanol containing 2% acetic acid. The mixture was centrifuged at 4000 rpm for 2 hours at 4°C after which the supernatant was filtered through a 0.2 µm Phenex RC membrane filter from Phenomenex (Utrecht, the Netherlands). 5'-HM-FLX was purified by preparative HPLC using a Xbridge Prep C18-MS (5 µm; 10 x 55 mm) column. Samples of 1.5 mL were injected and eluted with a flow rate of 3 mL/min. A gradient was constructed using eluent **A** (99% water/0.9% acetonitril/0.1% formic acid) and eluent **B** (99% acetonitril/0.9% water/0.1% formic acid) and programmed from 25% **B** to 50% **B** in 11 min after which it returned to 25% **B** in half a minute. After re-equilibrating for 4.5 min the next sample was injected. A fraction collector was triggered to collect peaks appearing at UV-detection at 272 nm. The fractions containing 5'-HM FLX were combined and evaporated to dryness and stored at -80°C under N_2. The identity of 5'-HM FLX was confirmed by ^{1}H NMR and ^{19}F NMR using a Bruker Avance 250 (Fallanden, Switzerland) equiped with a cryoprobe operating at 250,1 MHz. The metabolite had a purity of 99% based on HPLC analysis.

Oxidative metabolism of FLX by HLM and recombinant human CYPs

FLX was initially incubated at concentrations of 10 and 100 µM in presence of 100 nM of fourteen Gentest Supersomes in order to identify the most active human CYPs. Reactions were initiated in the presence of NRS (see above). Incubations were performed in a 100 mM potassium phosphate buffer pH 7.4 supplemented with 5 mM $MgCl_2$ and 2 mM EDTA, and at a total volume of 50 µL. The incubations were started by the addition of NRS and incubated for 10 min at 37 °C, during which metabolite production was linear (data not shown). The reactions were terminated as described above. The samples were subsequently centrifuged at 14,000 rpm (20,800g) for 20 min using a table top centrifuge. The supernatant was filtered through 0.2 µm Phenex RC membrane filters from Phenomenex (Utrecht, the Netherlands) and subsequently analysed by LC-MS, as described below. Since these incubations were only performed to identify the most active enzymes duplicate experiments were deemed sufficient since reproducibility of incubations with recombinant enzymes from the same source was always within 5%,

For the most active Gentest Supersomes and pooled HLM, the enzyme kinetics of the formation of 5'-HM-FLX were determined by incubating with eleven concentrations of FLX, ranging from 0 to 500 µM. Each FLX concentration was incubated in duplicate for 10 min with 0.5 mg/mL pooled HLM, 100 nM CYP3A4, CYP3A7 and CYP2C9 Supersomes in 100 mM potassium phosphate buffer, pH 7.4 supplemented with 5 mM $MgCl_2$ and 2 mM EDTA. The reactions were started, stopped and analysed as described above.

Effect of specific inhibitors of CYPs on oxidative metabolism of FLX by pooled HLM.

To study the involvement of individual human CYPs in the hydroxylation of FLX in incubations with pooled HLM from 50 individuals, the effect of selective P450 inhibitors was investigated. FLX was incubated at substrate concentrations of 10 and 100 µM with 0.5 mg/mL HLM, as described above, and in absence or presence of the following inhibitors: 10 µM α-naphtoflavone (αNF; CYP1A2), 3 µM ticlopidine (TIC; CYP2B6), 15 µM quercetin (QCT; CYP2C8), 10 µM sulfaphenazole (SPZ; CYP2C9), 10 µM fluvoxamine (FVX; CYP2C9/CYP1A2), 1 µM (+)-N-3-benzyl-nirvanol (BNV; CYP2C19), 2 µM quinidine (QND; CYP2D6), 20 µM diethylthiocarbamate (DDC; CYP2E1), 3 µM troleandomycin (TAO; CYP3A4); 2 µM miconazole (MCZ; CYP3A4/CYP2C9) and 0.1 µM (CYP3A4) and 1 µM (CYP3A) ketoconazole (KTZ) respectively. At these concentrations the inhibitors are considered to be selective for the indicated P450 (Khojasteh et al., 2011; Kumar et al., 2006; Niwa et al., 2009). The mechanism-based inhibitors TIC and TAO were pre-incubated with

HLM and NRS for 10 minutes before the addition of FLX. The inhibition experiment was performed three times, with each condition incubated in duplicate.

Because the CYP2C9-specific inhibitor sulfaphenazole appeared to almost completely block formation of 5'-HM-FLX by pooled HLM, it was also added at different concentrations to incubations of CYP3A4 and CYP3A7 Supersomes with FLX (10 µM) and testosterone (50 µM).

Variability of oxidative metabolism of FLX by individual HLM and correlation analysis

To determine the variability of 5'-hydroxylation of FLX, FLX was incubated at 10 and 100 µM with a panel of individual HLM-fractions from 16 donors (0.5 mg/mL) which were phenotyped with respect to activities of CYP1A2 (phenacetin O-deethylation), CYP2A6 (coumarine 7-hydroxylation), CYP2B6 (bupropion hydroxylation), CYP2C8 (amodiaquine N-deethylation), CYP2C9 (diclofenac 4'-hydroxylation), CYP2C19 (mephenytoin 4-hydroxylation), CYP2D6 (bufuralol 1-hydroxylation), CYP2E1 (chlorzoxazone 6-hydroxylation) and CYP3A4 (midazolam 1'-hydroxylation and testosterone 6ß-hydroxylation) using the concentrations described previously (den Braver-Sewradj et al., 2017).

Analytical methods

FLX and its metabolites were measured using an Agilent TOF 6230 mass spectrometer with electrospray ionization source coupled with an Agilent 1200 rapid resolution LC system. The mass spectrometer was operated under the following conditions: a capillary voltage of 3500V, 10 L/min nitrogen drying gas and 50 psig nitrogen nebulizing gas at 350°C and operating in positive ion mode. Chromatographic separation of FLX and its metabolites was achieved by reversed-phase liquid chromatography using a Phenomenex Luna C18-column (5 µm; 4.6 x 150 mm). A gradient was constructed using 1% acetonitrile in 0.1% formic acid (**A**) and 99% acetonitrile in 0.1% formic acid (**B**) as eluents. The gradient was programmed from 40% **B** to 99% B in 23.5 min after which the percentage went back to 40% **B** in 0.5 minute. Next the column was re-equilibrated in 40% **B** for 11 min. The flow rate was 0.4 mL/min.

The chromatograms were analysed using Agilent Masshunter Qualitative Analysis software (RRID:SCR_016657). Quantification was performed using a standard curve of 5'-

HM-FLX ranging from 1 nM to 6 µM. The lower limit of quantification of 5'-HM-FLX was 8 nM.

Data analysis

Enzyme kinetic parameters were determined using GraphPad Prism 5.00 for Windows, GraphPad Software (San Diego, USA; RRID:SCR_002798) and fitted to the Michaelis Menten equation. IC_{50}'s were calculated using the log(inhibitor) vs. response function of Graphpad Prism 5.00 for Windows, Graphpad Software (San Diego, USA). The Pearson correlation coefficient was calculated for the specific probe substrates and flucloxacillin 5'-hydroxylation using GraphPad Prism 5.00 for Windows, GraphPad Software (San Diego, USA). A two-tailed p-value was used with a confidence interval of 95%. The data and statistical analysis in this manuscript comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al, 2015). However, randomization of samples was deemed unneccessary since no systematic bias was observed related to the sample position or in the order of sample analysis by LC-MS. Blinding was not undertaken since prior knowledge of sample content was not expected to introduce bias of the measurements. Also data acquisition and data analysis were performed by different individuals.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).

Results

Oxidative metabolism of FLX by HLM and recombinant CYPs.

As shown in Figure 2, initial incubations of 10 µM FLX for 60 min with pooled HLM, in the presence or absence of NRS, resulted in the formation of only one NRS-dependent metabolite eluting at 11.6 min and with m/z ($[M+H]^+$) of 470.052, which corresponded to the retention time and m/z-value of biosynthetical 5'-HM-FLX. The product eluting at 9.3 min has a m/z of 472.078 and was also formed in absence of NRS. The mass increase of 18 relative to FLX (m/z 454.059) indicates that this product most likely corresponds to the penicilloic acid formed by hydrolysis of the beta-lactam ring of FLX

which is not dependent of CYP-activity. The penicilloic acid of 5'-HM-FLX , which was identified in urine (Maier-Salamon et al., 2017), was not yet detectable under the incubation conditions used. After checking the linearity of product formation with time, the enzyme kinetics of 5'-HM-FLX formation by pooled HLM was studied in 10 minute incubations by varying the concentration of FLX from 10 to 500 µM. As shown in Figure 3A, a hyperbolic curve was obtained consistent with Michaelis-Menten kinetics. Enzyme kinetic parameters obtained by non-linear regression were: K_m 284 ± 38 µM and V_{max} 30 ± 2 pmol/min/mg protein.

To characterize which human CYP isoenzymes catalyze the formation of 5'-HM-FLX, eleven different recombinant CYP Supersomes were incubated with 10 and 100 µM FLX. These concentrations were chosen to discriminate between active isoforms at the average C_{max} in patients taking 1000 mg orally (100 µM) and high affinity enzymes active at lower substrate concentration (10 µM). At a FLX-concentration of 10 µM, recombinant CYP3A4, CYP3A7 and to a lesser extent CYP2C9 showed the highest activity in formation of 5'-HM-FLX, Figure 4A. At 100 µM FLX, CYP3A7 was the most active CYP followed by CYP3A4. Low activities of 5'-HM-FLX formation were observed, in decreasing order, in incubations with CYP2C9, CYP2J2, CYP2C8, CYP2E1, CYP3A5, CYP1A1, CYP2D6 and CYP1A2, Figure 4B. Since CYP3A4, CYP3A7 and CYP2C9 Supersomes showed highest activity at both concentrations, the enzyme kinetics of 5'-HM-FLX formation was also determined for these recombinant CYPs (Figures 3B, 3C and 3D). Similar to the incubations with pooled HLM, Figure 3A, Michaelis-Menten kinetics was observed. Using the Michaelis Menten model the following enzyme kinetic parameters were determined: CYP3A4, K_m 124 ± 15 µM, V_{max} 197 ± 9 pmol/min/nmol P450; CYP3A7: K_m 65 ± 8 µM, V_{max} 193 ± 7 pmol/min/nmol P450; and CYP2C9: K_m of 508 ± 82 µM, V_{max} 67 ± 7 pmol/min/nmol P450.

Effect of isoenzyme-selective CYP-inhibitors on FLX metabolism by HLM.

Figure 5 shows the effects of isoenzyme-selective CYP-inhibitors on the oxidative metabolism of FLX by pooled HLM. The three inhibitors of CYP3A-isoforms, KCZ, MCZ and TAO, showed a relatively modest and differential inhibitory effect on 5'-HM-FLX formation. Even at the highest concentration of KTZ, which inhibits the CYP3A-family, still only 50% inhibition was observed at both concentrations of FLX. The other CYP3A4-inhibitors showed only 20 to 40% inhibition at 10 µM FLX-concentration. Although recombinant CYP2C9 showed much lower activity when compared to CYP3A4, Figures 3 and 4, an almost 90% inhibition of 5'-HM-FLX formation was observed in presence of

sulfaphenazole, which is considered to be a selective CYP2C9-inhibitor. However, fluvoxamine, the other CYP2C9-inhibitor used did not show significant inhibition of 5'-HM-FLX formation.

To study whether sulfaphenazole has the ability to inhibit CYP3A-mediated hydroxylation of FLX, sulfaphenazole was added at different concentrations to incubations of CYP3A4 and CYP3A7 with FLX and testosterone as substrates. As shown in Figure 6A and 6B, SPZ appeared to be a potent inhibitor of FLX hydroxylation by recombinant CYP3A4 and CYP3A7 with an IC_{50} of 4.1 ± 0.4 µM and 6.1 ± 0.5 µM respectively. At these SPZ-concentrations, no inhibition of CYP3A4-catalyzed 6β-hydroxylation of testosterone was observed. In HLM SPZ could inhibit both diclofenac 4'-hydroxylation and FLX hydroxylation, but not testosterone hydroxylation, with an IC_{50} of 2.2 ± 1.1 µM for diclofenac and an IC_{50} of 13 ± 1.3 µM for FLX, Figure 6C.

Variability of oxidative metabolism of FLX by individual HLM-fractions and correlation analysis.

Figure 7 shows the variability of oxidative FLX metabolism which was observed in incubations of FLX with HLM-fractions from sixteen donors. When incubating with 10 µM FLX, a 6.5-fold difference was found between the lowest (S1342) and highest (S1441) metabolising donor. When using 100 µM FLX as substrate concentration, the difference between the lowest (S1342) and highest (S1343) metabolising donor was 3.3-fold. As shown in Figure 8 and Table 1, the activities of 5'-HM-FLX formation showed very poor correlations with activities of most CYP-specific reactions. At both concentrations of FLX, the highest correlation coefficients were found with CYP2C9-catalyzed diclofenac 4'-hydroxylation, with Pearson correlation coefficients of 0.78 and 0.58, respectively, and CYP3A-catalyzed 6ß-hydroxylation, with correlation coefficients of 0.69 and 0.58, respectively. Visual inspection of the data points of the correlation between CYP2C9 acitivity and FLX hydroxylation shows a trend that the line does not cross the origin but at 280 pmol/min/mg protein at the x-axis. The fact that none of the correlation coefficiens approaches unity may support the fact that multiple CYPs contribute to FLX hydroxylation.

Discussion

Previously it has been shown that 5'-HM-FLX appeared to be highly toxic to biliary epithelial cells variability in the internal exposure to 5'-HM-FLX should be considered as an important factor determining susceptibility to liver injury (Lakehal *et al*., 2001). Therefore,

the aim of present study was to further characterize the CYPs involved in hydroxylation of FLX by using recombinant CYPs, specific inhibitors and correlation analysis. Furthermore, the enzyme kinetic parameters of FLX hydroxylation were quantified using biosynthetical 5'-HM-FLX as reference.

The results of the present study confirm that, upon incubation of FLX with HLM or recombinant CYPs, 5'-HM-FLX is the only oxidative metabolite formed, Figure 2, consistent with previous studies (Lakehal *et al*., 2001). By using recombinant CYPs, at both substrate concentrations used CYP3A4 and CYP3A7 appeared to be the most active enzymes. At 10 µM FLX, CYP2C9 showed a 8-10 fold lower activity, Figure 4A. At 100 µM FLX, low activities were also observed with CYP1A1, CYP1A2, CYP2C8, CYP2E1, CYP2J2 and CYP3A5. The fact that Lakehal et al. (Lakehal *et al*., 2001) did not detect 5'-HM-FLX in incubations with recombinant CYP1A2, CYP2C9 and CYP3A5 might be attributed to the less sensitive analytical method used (HPLC-UV detection vs LC-MS) and/or the use of yeast-expressed CYPs with lower specific activity than the Supersomes used in the present study. Also, compared to the present study, incubations with recombinant CYPs were performed at lower temperature (28 ^{o}C vs. 37 ^{o}C) and lower enzyme concentration (50 nM vs 100 nM CYP).

The present study confirms the previous study of Lakehal et al. (Lakehal *et al*., 2001). that CYP3A4 is likely to play a major role in the formation of 5'-HM-FLX, since it is on average the most abundant CYP in human liver (mean 93 pmol/mg protein) (Achour *et al*., 2014). However, the meta-analysis of Achour et al. demonstrated that in the 713 human livers analyzed CYP3A4 levels varies dramatically, ranging from 0 to 601 pmol/mg protein(Achour *et al*., 2014). This large variability can contribute to the interindividual variability in plasma-concentrations of 5'-HM-FLX (Maier-Salamon *et al*., 2017). As mentioned, next to CYP3A4 also CYP3A7 showed high activity in FLX-hydroxylation. CYP3A7 was originally considered a CYP only expressed in fetal tissues (Schuetz *et al*., 1994). However, more recent studies have indicated that in approximately 10% of adult livers, CYP3A7 is expressed to significant extents, up to 90 pmol/mg protein, which equals the mean level of CYP3A4 (Sim *et al*., 2005; Ohtsuki *et al*., 2012). This strongly increased CYP3A7 expression appears to be strongly associated with the CYP3A7*1C allele which has arisen through the conversion of 60 bp of the promotor of CYP3A4 into the corresponding region of CYP3A7 (Burk *et al*., 2002). To what extent the variability in CYP3A7-expression contributes to the variability of FLX-hydroxylation remains to be established, and is complicated by the present lack of specific inhibitors for the CYP3A-isoforms. The relatively

poor correlation between the rate of FLX-hydroxylation with the CYP-specific reactions, Figure 8 and Table 1, may be explained by involvement of more than one enzymes.

Involvement of CYP3A-isoforms in drug metabolism is well known to result in very diverse types of enzyme kinetics and poorly predictable drug-drug interactions. Dependent on the substrate used, for all CYP3A-isoforms enzyme kinetics can appear as normal Michaelis-Menten kinetics, or atypical kinetics such as autoactivation and substrate inhibition (Atkins *et al*., 2005; Williams *et al*., 2002). As possible explanations for this complex behavior, multiple binding sites in the active-site allowing accommodation of multiple substrate molecules or the presence of an effector binding site has been proposed (Ekins *et al*., 2003; Houston *et al*., 2005). Because of the multiple binding sites in CYP3A-enzymes, also the type of drug-drug interactions appears complex and strongly substrate dependent (Ekins *et al*., 2003; Stresser *et al*., 2000). Therefore to predict the ability to cause CYP3A4-inhibition the use of multiple CYP3A4 substrates is recommended (Stresser *et al*., 2000). The inhibition experiments in the present study seem to confirm the complicated inhibition pattern of CYP3A-reactions. The established CYP3A4-inhibitors ketoconazole, troleandomycin and miconazole showed relatively low degree of inhibition at the concentrations used. Remarkably, sulfaphenazole, which is considered to be a highly selective inhibitor of CYP2C9 (Khojasteh *et al*., 2011) showed almost strong inhibition of FLX-hydroxylation by pooled HLM, CYP3A4 and CYP3A7, whereas the 6-beta-hydroxylation of testosterone was not affected. These results suggest that FLX has a unique binding mode to the active site to CYP3A4, explaining the relatively weak inhibition by 1 µM ketaconazole and an unexpected strong inhibition by sulfaphenazole. A similar situation has been described recently in a study where sulfaphenazole was able to significantly inhibit bioactivation of hydroxylapatinib by HLM, although recombinant CYP2C9 showed only very low activity (Towles *et al.*, 2016). Therefore, CYP3A family in rare occurrences can be inhibited by sulfaphenazole in a substrate dependent manner. The results of the present study contrast with the results of Lakehal et al. which reported no inhibition of HLM-catalyzed hydroxylation of FLX by 25 µM sulfaphenazole (Lakehal *et al*., 2001). The reason for the discrepancy between these results remains to be elucidated. A possible explanation might be the different sources of enzymes used, since the ratio between CYP and reductase as well as the membrane composition can affect specific activity of CYPs (Kim *et al*,. 2003).

In conclusion, the results of this present study confirm the involvement of CYP3A4 in the hydroxylation of FLX, whereas also CYP3A7 and CYP2C9 may contribute significantly in individuals expressing high hepatic levels. The large variability of both hepatic CYP3A

and CYP2C9 levels may contribute to the variability of plasma-levels of 5'-HM-FLX, which is suspected to play a role in FLX-induced DILI by causing cytotoxicity to BEC. The strong inhibition of CYP3A-catalyzed hydroxylation of FLX by the commonly used CYP2C9-inhibitor SFZ suggest that unanticipated drug-drug interactions can occur with coadministered drugs.

Acknowledgements

This work was supported by the European Community under the Innovative Medicines Initiative (IMI) program through Grant Agreement number 115336.

Author contributions

S.J.D. and F.D. performed the experiments; S.J.D. and J.N.M.C. contributed to the writing; S.J.D., N.P.E.V. and J.N.M.C. contributed to the study design.

Conflict of Interest.

All authors declare that they do not have conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

References

Achour B, Barber J, Rostami-Hodjegan A (2014). Expression of hepatic drug-metabolizing cytochrome P450 enzymes and their intercorrelations: A meta-analysis. Drug Metab Dispos 42: 1349–1356.

Adam D, Koeppe P, Heilmann HD. (1983). Pharmacokinetics of amoxicillin and flucloxacillin following the simultaneous intravenous administration of 4 g and 1 g, respectively. Infection. 11: 150-154.

Andrews E, Daly AK (2008). Flucloxacillin-induced liver injury. Toxicology 254:158-163

Atkins WM (2005). Non-Michaelis-Menten kinetics in cytochrome P450-catalyzed reactions. Annu Rev Pharmacol Toxicol. 45: 291-310.

Burk O, Tegude H, Koch I, Hustert E, Wolbold R, Glaeser H, et al. (2002). Molecular mechanisms of polymorphic CYP3A7 expression in adult human liver and intestine. J Biol Chem. 277: 24280-24288.

Comuth WJ, Comuth JA, Hauer RNW and Malingré MM (2012) Interaction of flucloxacillin and quinidine. Eur J Clin Pharmacol 68: 891-893.

Curtis MJ, Bond RA, Spina D, Ahluwalia A, Alexander SP, Giembycz MA, et al. Experimental design and analysis and their reporting: new guidance for publication in BJP. Br J Pharmacol. 2015 ;172:3461-3471.

Cynke E, Binet I, Haefeli WE and Thiel G (1999) Flucloxacillin & cyclosporine A: an unrecognized but relevant interaction in renal transplant recipients. Kidney Int. 55: 1156-1157.

Daly AK, Donaldson PT, Bhatnagar P, Shen Y, Pe'er I, Floratos A, et al. (2009). DILIGEN Study; International SAE Consortium. HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin. Nat Genet. 41: 816-819.

De Abajo FJ, Montero D, Madurga M, Garcia Rodriguez LA. (2004) Acute and clinically relevant drug-induced liver injury: a population based case-control study. Br. J. Clin. Pharmacol. 58: 71-80.

Den Braver-Sewradj SP, den Braver MW, van Dijk M, Zhang Y, Dekker SJ, Wijaya L. (2018) Inter-individual variability in activity of the major drug metabolizing enzymes in liver homogenates of 20 individuals. Curr. Drug Metab. *(E-pub ahead of print)*

Derby LE, Jick H, Henry DA, Dean AD (1993). Cholestatic hepatitis associated with flucloxacillin. Med. J. Aust. 1581: 596-600.

Dijkmans AC, den Hartigh J, van Dissel JT, Burggraaf J (2012).A simplified oral flucloxacillin absorption test for patients requiring long-term treatment. Therap Drug Monit 34: 356–358

Eckstein RP, Dowsett JF, Lunzer MR (1993). Flucloxacillin induced liver disease: histopathological findings at biopsy and autopsy. Pathology 25: 223–228

Ekins, S., Stresser, DM and Williams JA. In vitro and pharmacophore insights into CYP3A enzymes (2003). TIPS 24: 161-166.

Fairley CK, McNeil JJ, Desmond P, Smallwood R, Young H, Forbes A, et al. (1993) Risk factors for development of flucloxacillin associated jaundice. Br Med J. 306: 233–235

Gath J, Charles B, Sampson J, Smithurst B (1995). Pharmacokinetics and Bioavailability of Flucloxacillin in Elderly Hospitalized Patients. J Clin Pharmacol 35: 31–36.

Harding SD, Sharman JL, Faccenda E, Southan C, Pawson AJ, Ireland S et al. (2018). The IUPHAR/BPS Guide to PHARMACOLOGY in 2018: updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. Nucl Acids Res 46: D1091-D1106.

Houston JB, Kenworthy KE (2000). In vitro-in vivo scaling of CYP kinetic data not consistent with the classical Michaelis-Menten model. Drug Metab Dispos. 28: 246-54.

Houston JB, Galetin A (2005). Modelling atypical CYP3A4 kinetics: principles and pragmatism. Arch Biochem Biophys. 433: 351-360.

Huwyler J, Wright M, Gutmann H, Drewe J (2006). Induction of Cytochrome P450 3A4 and P-Glycoprotein by the isoxazolyl- penicillin antibiotic flucloxacillin. Curr Drug Metab 7: 119–126.

Jenkins RE, Meng X, Elliott VL, Kitteringham NR, Pirmohamed M, Park BK (2009). Characterisation of flucloxacillin and 5-hydroxymethyl flucloxacillin haptenated HSA in vitro and in vivo. Proteomics Clin Appl 3: 720–729.

Khojasteh SC, Prabhu S, Kenny JR, Halladay JS, Lu AYH (2011). Chemical inhibitors of cytochrome P450 isoforms in human liver microsomes: A re-evaluation of P450 isoform selectivity. Eur J Drug Metab Pharmacokinet 36: 1–16.

Kim K, Ahn T, Yun C (2003) Membrane Properties Induced by Anionic Phospholipids and Phosphatidylethanolamine Are Critical for the Membrane Binding and Catalytic Activity of Human Cytochrome P450 3A4. Biochemistry 42: 15377-15387

Koek GH, Stricker BHC, Blok APR, Schalm SW, Desmet VJ (1994). Flucloxacillin-associated hepatic injury. Liver 14: 225–229

Kumar V, Wahlstrom JL, Rock DA, Warren CJ, Gorman LA, Tracy TS (2006). CYP2C9 inhibition: impact of probe selection and pharmacogenetics on in vitro inhibition profiles. Drug Metab Dispos 34: 1966-1975.

Lakehal F, Dansette PM, Becquemont L, Lasnier E, Delelo R, Balladur P, et al. (2001) Indirect cytotoxicity of flucloxacillin toward human biliary epithelium via metabolite formation in hepatocytes. Chem Res Toxicol. 14: 694-701.

Landersdorfer CB, Kirkpatrick CMJ, Kinzig-Schippers M, Bulitta JB, Holzgrabe U, Drusano, GL et al. (2007), Population pharmacokinetics at two dose levels and pharmacodynamic profiling of flucloxacillin. Antimicrob Agents and Chemother. 51: 3290-3297.

Leder K, Turnidge JD, Korman TM, Grayson ML (1999). The clinical efficacy of continuous-infusion flucloxacillin in serious staphylococcal sepsis. J. Antimicrob. Chemother. 43: 113-118.

Luirink RA, Dekker SJ, Capoferri L, Janssen LFH, Kuiper CL, Ari ME, Vermeulen NPE, Vos JC, Commandeur JNM, Geerke DP (2018) A combined computational and experimental study on selective flucloxacillin hydroxylation by cytochrome P450 BM3 variants. J. Inorg. Biochem. 184:115-122.

Maier-Salamon A, Elgendy SA, Meyer B, Vossen M, Thalhammer T, Thalhammer F, et al. (2017). Pharmacokinetics of flucloxacillin and its metabolites in patients with renal failure: impact on liver toxicity. Int J Clin Pharmacol Ther 55: 701-711.

Monshi MM, Faulkner L, Gibson A, Jenkins RE, Farrell J, Earnshaw CJ, et al. (2013). Human leukocyte antigen (HLA)- B*57:01-restricted activation of drug-specic T cells provides the immunological basis for flucloxacillin-induced liver injury. Hepatology 57: 727-739.

Muilwijk EW, Dekkers BGJ, Henriet SSV, Verweij PE, Witjes B, Lashof AMLO, Groeneveld GH, van der Hoeven J, Alffenaar JWC, Russel FGM, van de Veerdonk F2, Brüggemann RJM. (2017) Flucloxacillin results in suboptimal plasma voriconazole concentrations. Antimicrob Agents Chemother. 6: e00915-17.

Niwa T, Shiraga T, Takagi A (2009). Effect of antifungal drugs on cytochrome P450 (CYP) 2C9, CYP2C19, and CYP3A4 activities in human liver microsomes. Biol Pharm Bull 28: 1805-1808.

Ohtsuki S, Schaefer O, Kawakami H, Inoue T, Liehner S, Saito A, et al (2012). Simultaneous absolute protein quantification of transporters, cytochromes P450, and UDP-glucuronosyltransferases as a novel approach for the characterization of individual

human liver: comparison with mRNA levels and activities. Drug Metab Dispos 40: 83-92.

Røder BL, Frimodt-Møller N, Espersen F, Rasmussen SN (1995). Dicloxacillin and flucloxacillin: pharmacokinetics, protein binding and serum bactericidal titers in healthy subjects after oral administration. Infection 23: 107–112.

Russmann S, Kaye JA, Jick SS, Jick H (2005). Risk of cholestatic liver disease associated with flucloxacillin and flucloxacillin prescribing habits in the UK: Cohort study using data from the UK General Practice Research Database. Br J Clin Pharmacol 60: 76–82.

Schuetz JD, Beach DL, Guzelian PS (1994), Selective expression of cytochrome P450 3A mRNAs in embryonic and adult human liver. Pharmacogenetics 4: 11-20.

Sim SC, Edwards RJ, Boobis AR, Ingelman-Sundberg M (2005). CYP3A7 protein expression is high in a fraction of adult human livers and partially associated with the CYP3A7*1C allele. Pharmacogenet Genomics 15: 625-631.

Stresser DM, Blanchard AP, Turner SD, Erve JCL, Dandeneau AA, Miller VP, et al (2000). Substrate-dependent modeulation of CYP3A4 catalytic activity: analysis of 27 test compounds with four fluorimetric substrates. Drug Metab Dispos 28: 1440–1448.

Sutherland R, Croydon EAP, Rolinson GN (1970). Flucloxacillin, a New Isoxazolyl Penicillin, Compared with Oxacillin, Cloxacillin, and Dicloxacillin. Br Med J 4: 455–460.

Tailor A, Faulkner L, Naisbitt DJ, Park BK (2015). The chemical, genetic and immunological basis of idiosyncratic drug-induced liver injury. Hum Exp Toxicol 34: 1310-1317.

Thijssen HHW and Wolters J (1982). The metabolic disposition of flucloxacillin in patients with impaired kidney function. Eur J Clin Pharmacol 22: 429–434

Towles JK, Clark RN, Wahlin MD, Uttamsingh V, Rettie AE, Jackson KD (2016). Cytochrome P450 3A4 and CYP3A5-catalyzed bioactivation of lapatinib. Drug Metab Dispos 44: 1584-1597.

Uetrecht J (2007). Idiosyncratic drug reactions: current understanding. Annu Rev Pharmacol Toxicol 47: 513–539.

Williams JA, Ring BJ, Cantrell VE, Jones DR, Eckstein J, Ruterbories K, et al (2002). Comparative metabolic capabilities of CYP3A4, CYP3A5, and CYP3A7. Drug Metab Dispos 30: 883-891.

Wuillemin N, Adam J, Fontana S, Krähenbühl S, Pichler WJ, Yerly D (2013). HLA haplotype determines hapten or p-i T cell reactivity to flucloxacillin. J Immunol. 190: 4956-4964.

Wuillemin N, Terracciano L, Beltraminelli H, Schlapbach C, Fontana S, Krahenbuhl S, et al. (2014). T cells infiltrate the liver and kill hepatocytes in HLA-B(*)57:01-associated oxacillin-induced liver injury. Am J Pathol 184: 1677-1682.

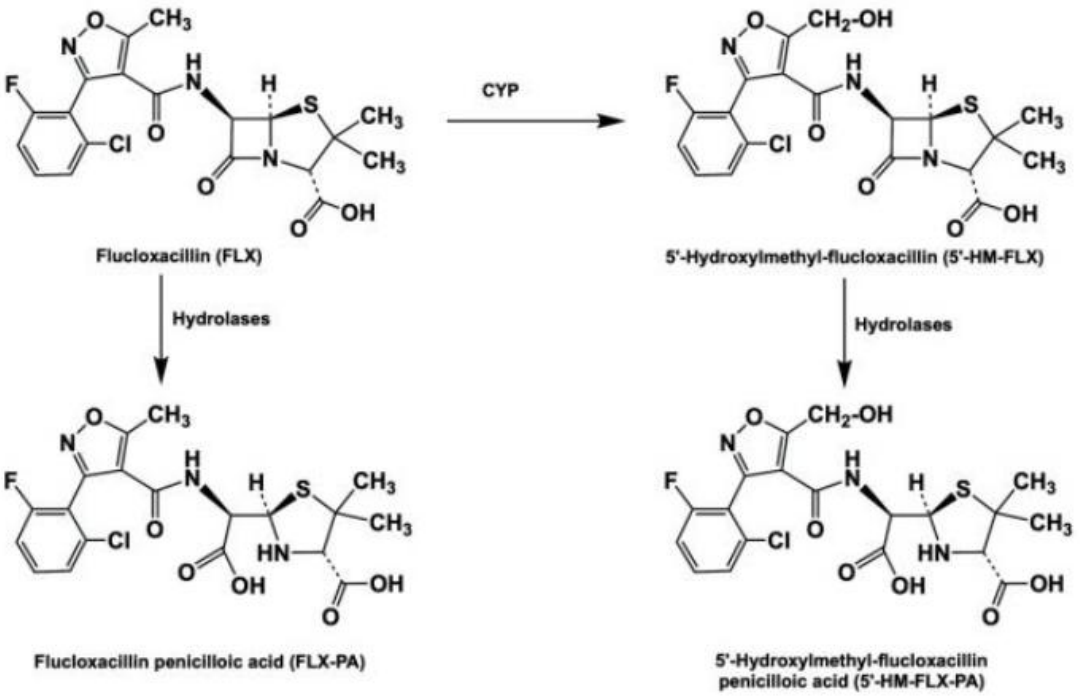

Figure 1: Oxidative metabolism of flucloxacillin (FLX) to 5'-hydroxymethyl fluclocaxillin (5'-HM-FLX) and hydrolysis of FLX and 5'-HM-FLX to their corresponding penicilloic acids.

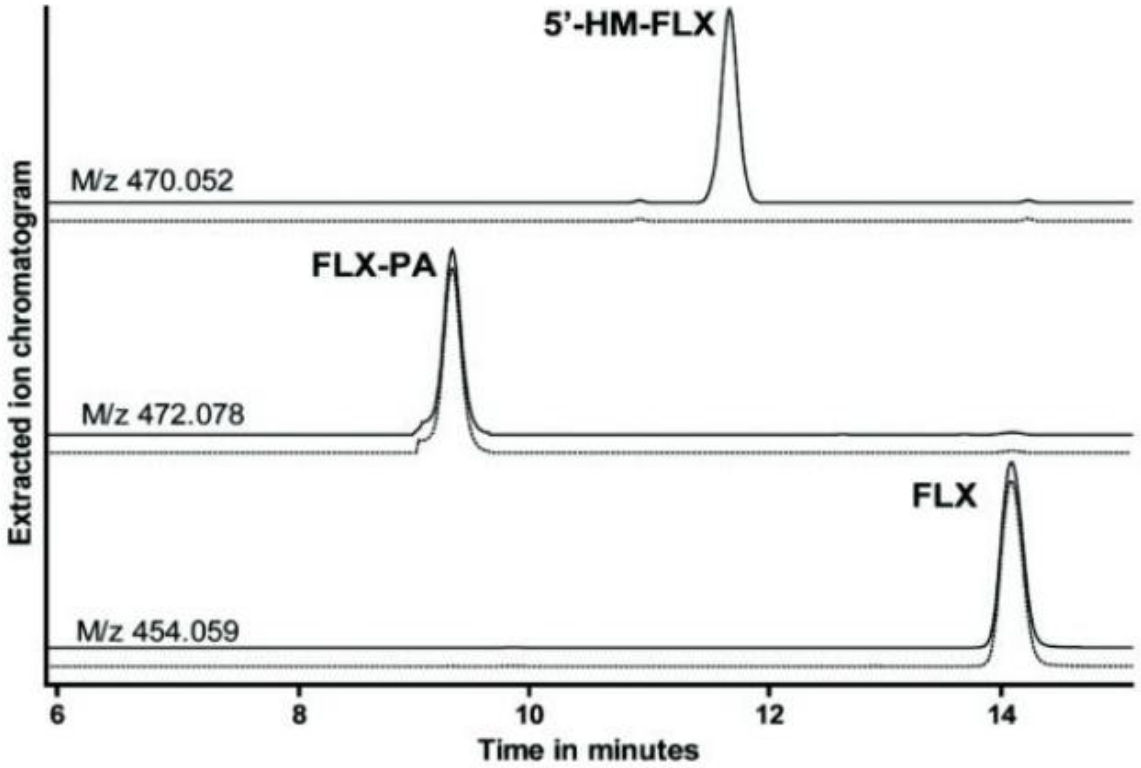

Figure 2: LC-MS-analysis of an incubation of 100 µM FLX incubated for 60 minutes with 0.5 mg/mL pooled human liver microsomes with (solid line) or without (dotted line) NADPH-regenerating system. (**A**) Extracted ion chromatogram of m/z 470.052 corresponding to the $[M+H]^+$ of 5'-HM-FLX; (**B**) extracted ion chromatogram of m/z 472.078 corresponding to the $[M+H]^+$ of the penicilloic acid of 5'-HM-FLX; (**C**) extracted ion chromatogram of m/z 454.059 corresponding to the $[M+H]^+$ of FLX.

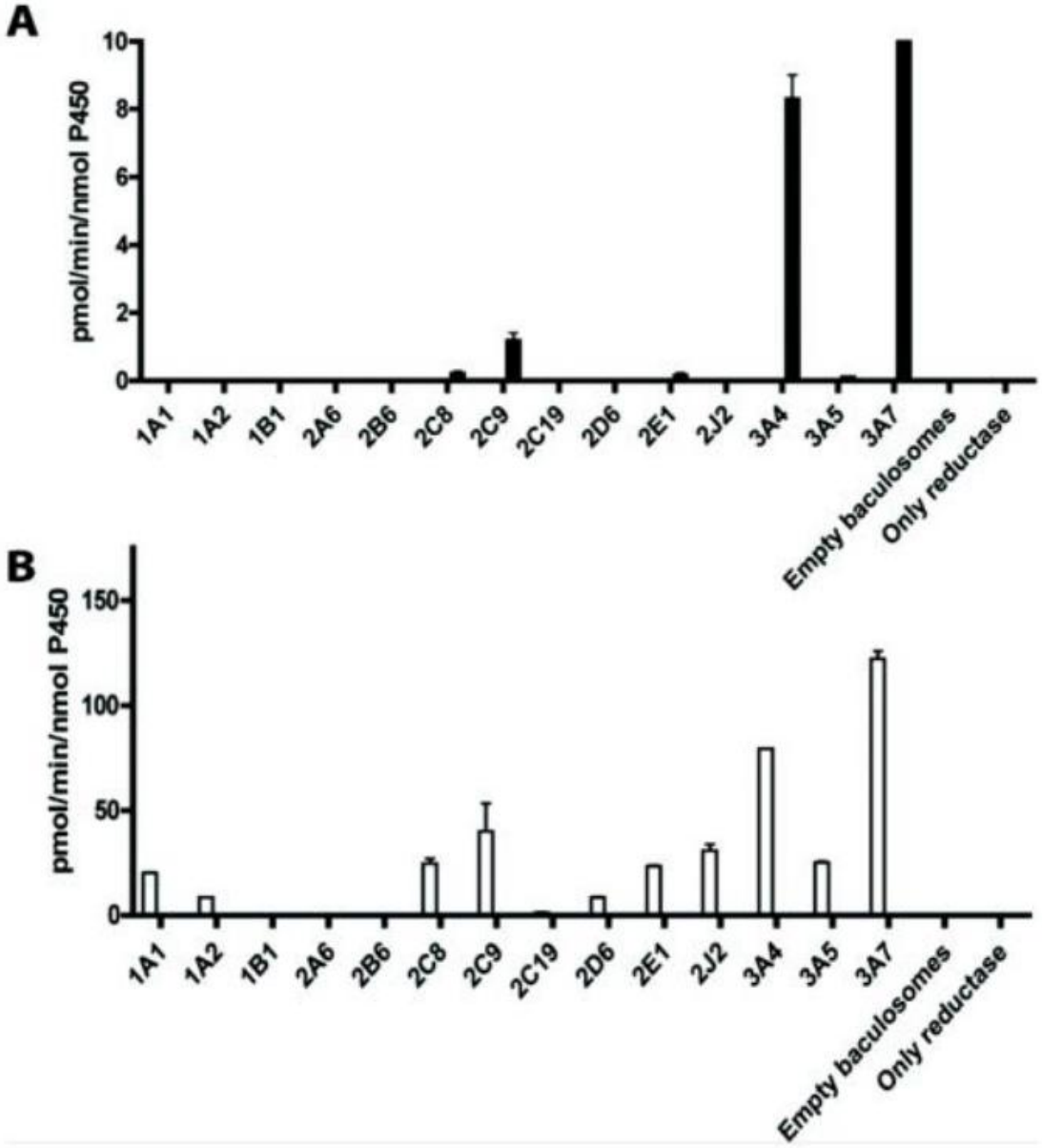

Figure 3: Concentration dependency of the rate of FLX-hydroxylation as catalyzed by 0.5 mg/mL pooled HLM (**A**), 100 nM CYP3A4 Supersomes (**B**), 100 nM CYP3A7 Supersomes (**C**) and 100 nM CYP2C9 supersomes (**D**). Each condition was incubated in duplicate and each data point represent as mean whereas error bars represent the range of duplicate measurements. Enzymes kinetic parameters obtained by non-linear regression according to the Michaelis Menten-equation were: (**A**) HLM: K_m 284 ± 38 µM and V_{max} 30 ± 2 pmol/min/mg protein; (**B**) CYP3A4 Supersomes: K_m 124 ± 15 µM and V_{max} 197 ± 9

pmol/min/nmol P450, (**C**) CYP3A7 Supersomes: K_m of 65 ± 8 µM and a V_{max} 193 ± 7 pmol/min/nmol P450 and (**D**) CYP2C9 supersomes: K_m of 508 ± 82 µM and a V_{max} 67 ± 7 pmol/min/nmol P450.

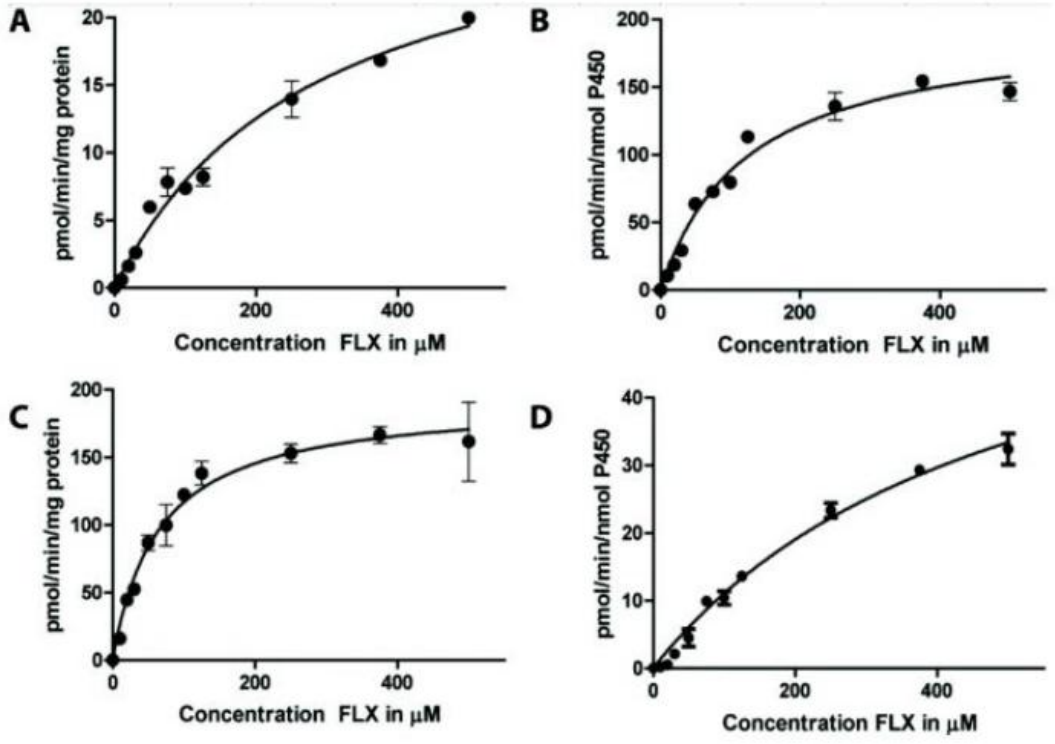

Figure 4: Specific activities of formation of 5'-HM-FLX in incubations of FLX with CYP-containing Supersomes (100 nM). (**A**) Incubations in presence of 10 µM FLX; (**B**) incubations in presence of 100 µM FLX. Data represent mean of duplicate experiments; the error bars represent the range of duplicate measurements.

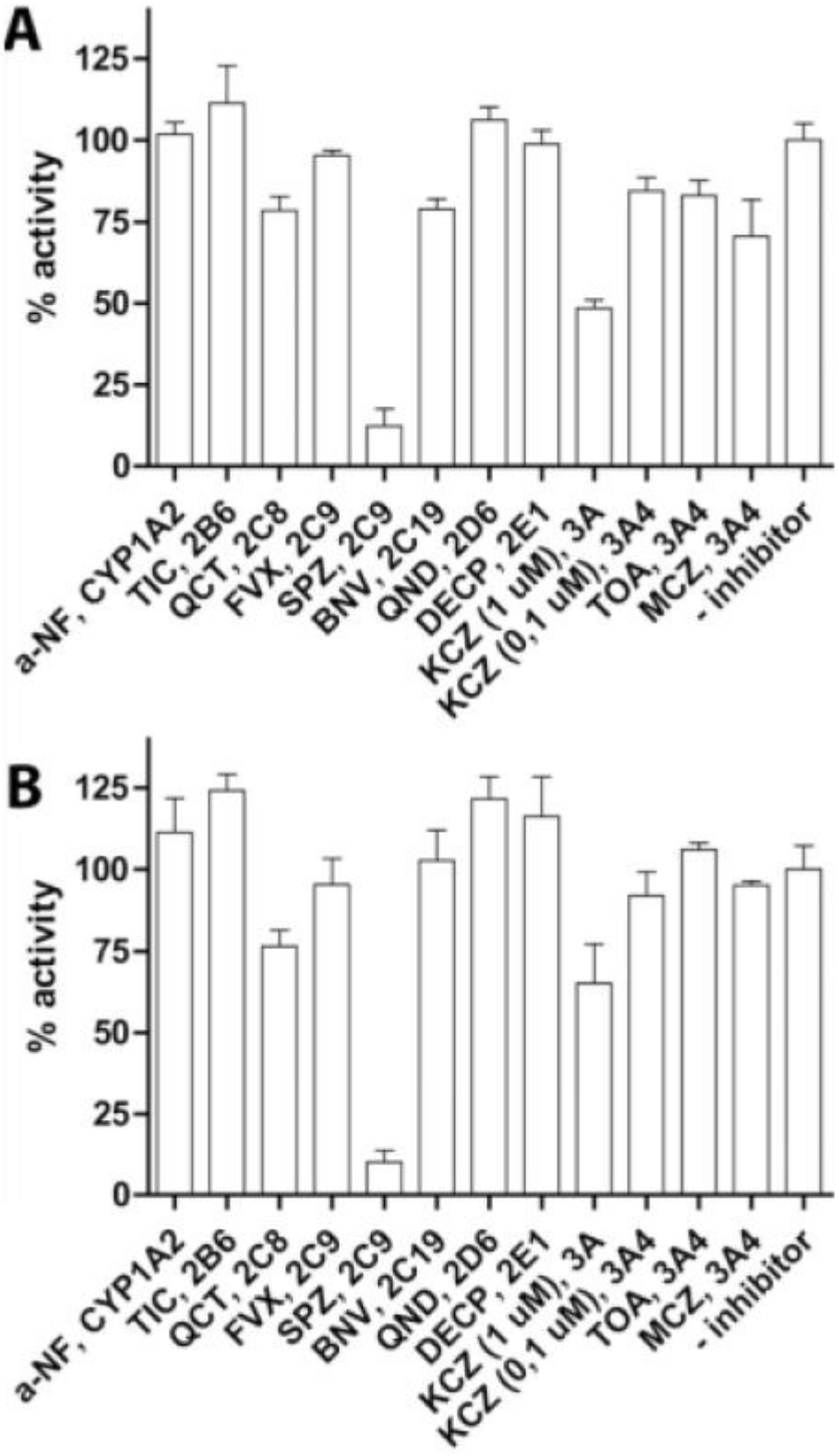

Figure 5: The inhibition of 5'-HM-FLX formation by pooled HLM (0.5 mg/mL) by isoform-specific inhibitors of CYPs. (**A**) Incubations performed in presence of 10 µM FLX; (**B**)

incubations performed in presence of 100 μM FLX. Activities are relative to the non-inhibited reactions which showed specific activities of 0.52 pmol/min/mg protein (**A**) and 7.4 pmol/min/mg protein (**B**), respectively. 100% was defined as the mean of enzyme activities in absence of inhibitor and 0% was defined as no enzyme activity. Data represent the averages of a three independent experiments in with each condition was performed in duplicate; error bars represent the standard deviation of the six individual measurements.

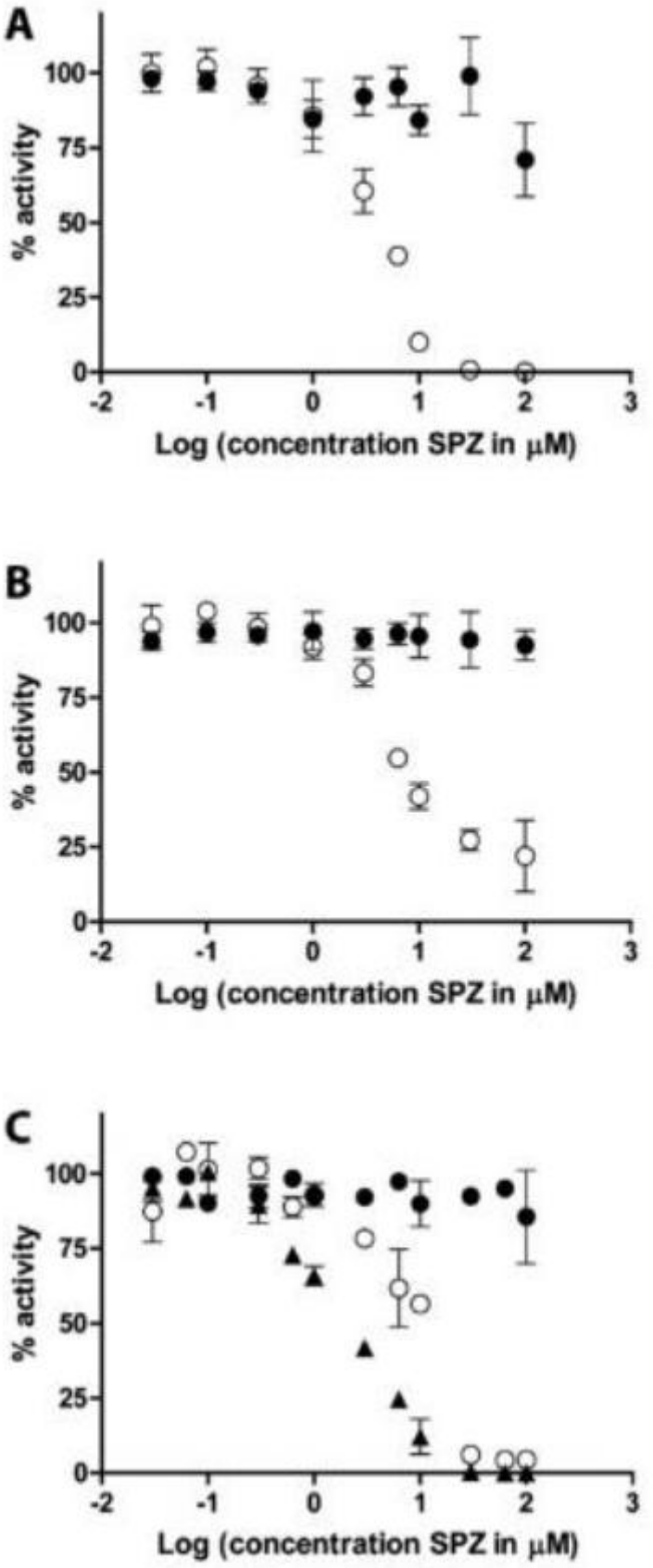

Figure 6: Concentration dependent inhibition by sulfaphenazole (SPZ) of 5'-hydroxylation of FLX (open circles), 6ß-hydroxylation of testosterone (closed circles) and 4'-hydroxylation of diclofenac (closed triangles) as catalyzed by CYP3A4 Supersomes (**A**), CYP3A7 Supersomes (**B**) and HLM (**C**). 100% was defined as the mean of enzyme activities in absence of inhibitor and 0% was defined as no enzyme activity (e.g. full inhibition). Each condition was incubated in duplicate and results are depicted as mean with the range of duplicate measurements as error bar.

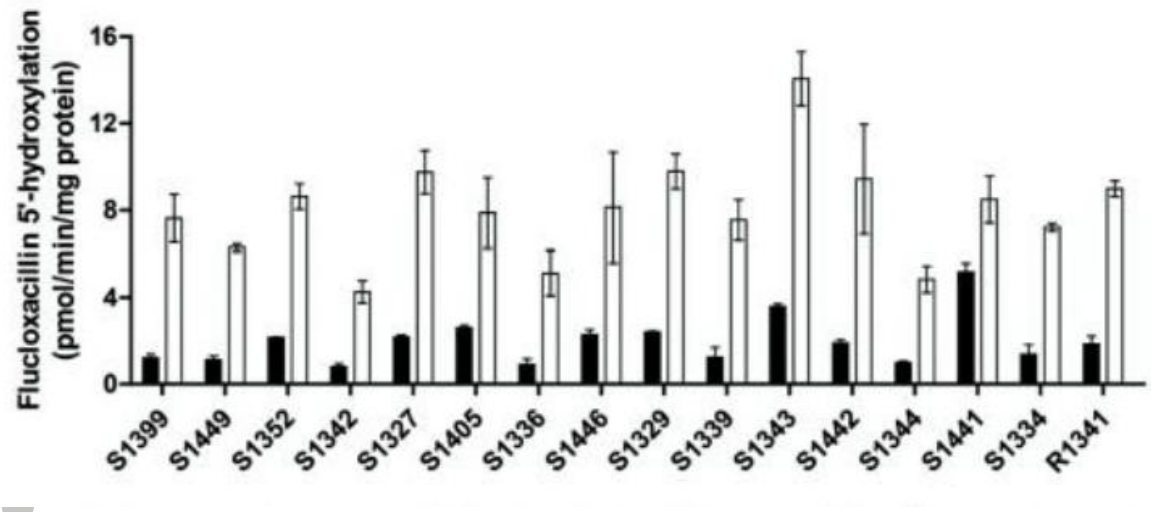

Figure 7: Variability in specific activity of 5'-hydroxylation of FLX as catalyzed by HLM from 16 individual donors. Black bars represent specific activities obtained in presence of 10 µM FLX; white bars represent specific activities obtained in presence of 100 µM FLX. Each data point was measured in duplicate and depicted as mean with the range of duplicate measurements.

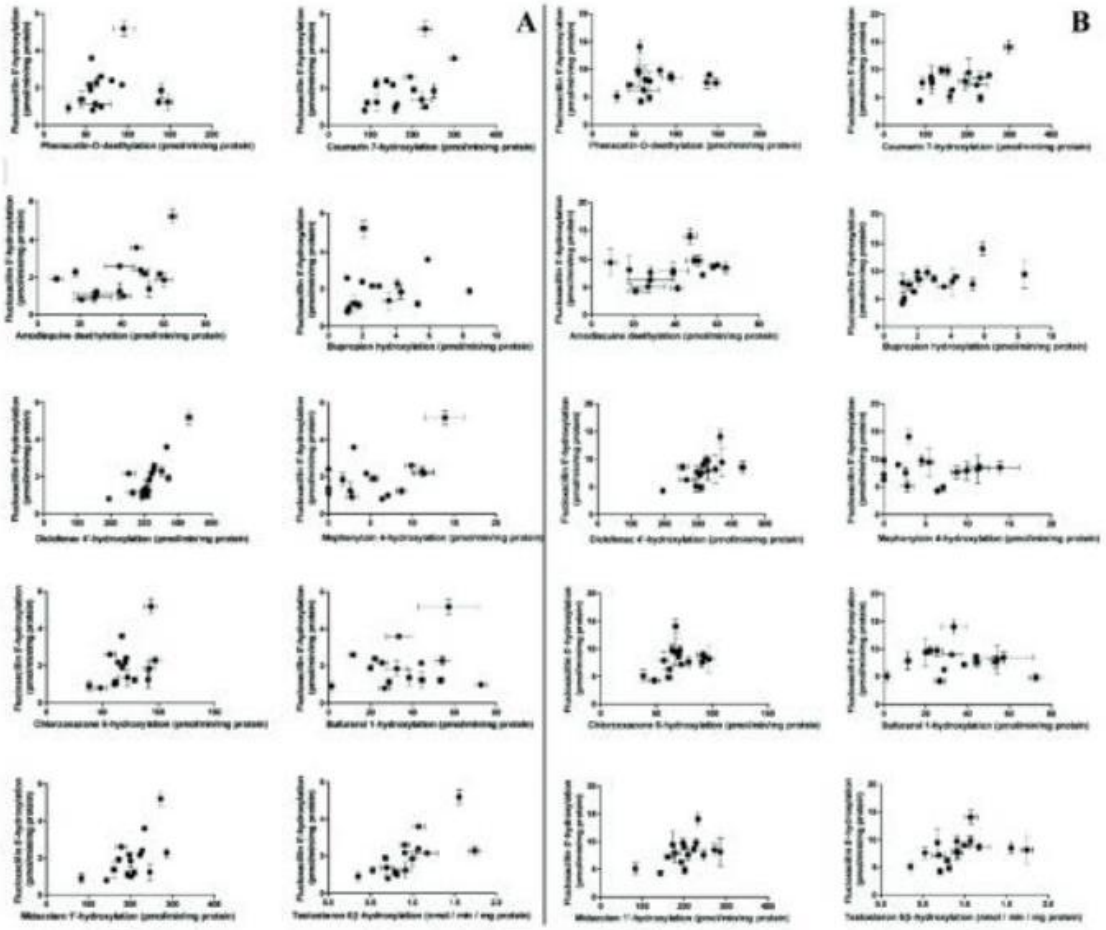

Figure 8: Correlation analysis of CYP-specific reactions and FLX hydroxylation at 10 µM FLX (A) or 100 µM FLX (B) using HLM of 16 different donors. Each data point was measured in duplicate and depicted as mean with the range of duplicate measurements. Pearson correlation coefficients and corresponding P-values are tabulated in Table 1.

Table 1: Statistical analysis of the correlation between activities of FLX hydroxylation and isoenzyme specific reactions
catalyzed by HLM from 16 different donors.

Isoenzyme-specific reaction	CYP	10 µM FLX		100 µM FLX	
		Pearson R	P-value	Pearson R	P-value
phenacetin O-deethylation	CYP1A2	0.04	0.87	0.10	0.70
coumarin 7-hydroxylation	CYP2A6	0.45	0.078	0.45	0.093
buproprion hydroxylation	CYP2B6	0.16	0.54	0.49	0.16
amodiaquine N-deethylation	CYP2C8	0.50	0.051	0.044	0.87
diclofenac 4'-hydroxylation	CYP2C9	0.78	0.0004	0.58	0.019
mephenytoin 4'-hydroxylation	CYP2C19	0.45	0.079	-0.059	0.83
bufuralol 1-hydroxylation	CYP2D6	0.15	0.59	-0.069	0.80
chlorzoxazone 6-hydroxylation	CYP2E1	0.42	0.10	0.36	0.17
midazolam 1'-hydroxylation	CYP3A4	0.58	0.012	0.48	0.061
testosterone 6ß-hydroxylation	CYP3A4	0.69	0.003	0.39	0.14