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Abstract

The increasing use of zebrafish embryos as an alternative model for toxicological and pharmacological studies necessitates a better understanding of xenobiotic biotransformation in this species. As cytochrome P450 enzymes (CYPs) play an essential role in this process, *in vitro* drug metabolism of four human CYP-specific substrates, i.e. dextromethorphan (DXM), diclofenac (DIC), testosterone (TST) and midazolam (MDZ) was investigated in adult male and female zebrafish, and in zebrafish embryos and larvae up to 120 hours post-fertilization. Substrate depletion and production of their respective metabolites were measured using tandem quadrupole UPLC-MS/MS. Human liver microsomes were used as positive control. Adult zebrafish produced the two major human metabolites of DIC and DXM. For DIC the metabolite ratio was similar to that in man, whereas it was different for DXM. For TST, the major human metabolite could not be detected and MDZ was not metabolized. No sex-related differences were detected, except for the higher TST depletion rate in adult females. Zebrafish embryos and larvae showed no or only low biotransformation capacity. In conclusion, *in vitro* CYP-mediated drug metabolism in adult zebrafish shows differences compared to man and appears to be lacking in the early zebrafish life stages. As CYP-mediated drug metabolism in zebrafish may not be predictive for the one in man, we recommend including the zebrafish in metabolic stability testing of new compounds when considering non-clinical species for human risk assessment.

Highlights

- CYP activity in zebrafish has similarities but also differences compared to man.
- Zebrafish embryos have a much lower CYP activity compared to adults.
- Drug risk assessment in zebrafish necessitates metabolic stability test.

Keywords:

Zebrafish, Cytochrome, Microsomes, Ontogeny, Sex, LC-MS

1. Introduction

Zebrafish and in particular their embryos/larvae are increasingly used as a model for pharmacological and toxicological studies. Indeed, due to their small size, low compound needs, high fecundity and external embryonic development, zebrafish represent a promising tool for preclinical risk assessment. Furthermore, within Europe zebrafish embryos are not considered as laboratory animals until the stage of independent feeding at 120 hours post-fertilization (hpf), which makes them ideal candidates for alternative testing (EC, 2010). As such, the zebrafish embryo has already been accepted as a validated alternative for the acute fish toxicity test (OECD TG236). Several efforts are ongoing to develop the zebrafish embryo assay as an alternative for developmental toxicity testing in mammals (Beker van et al., 2013; Brannen et al., 2010; Panzica-Kelly et al., 2012), but to fully reach that objective, the model needs further characterization. One of the remaining issues is their biotransformation capacity (Saad et al., 2016a), as embryos from oviparous species lack the maternal metabolism of the dosed drug or chemical that is present in the *in vivo* mammalian studies. This is particularly important for compounds that require bioactivation to exert their toxic effect, i.e. biotransformation of the parent compound into one or more active metabolites. For developmental toxicity, these compounds are called proteratogens. So, if the zebrafish embryo lacks this bioactivation capacity of proteratogens, false negative results will occur in the zebrafish embryo assay and this will impact human risk assessment.

Cytochrome P450 enzymes (CYPs), a superfamily of mono-oxygenases, and in particular the CYP1, 2 and 3 families, are key in the biotransformation and bioactivation of xenobiotics. In humans, five CYP isoforms, namely CYP1A2, CYP2C9, CYP2D6 and CYP3A4/5, are responsible for the oxidative biotransformation of about 70% of most clinically used drugs (Zanger and Schwab, 2013) and comprise around 65% of the CYP content in the liver (Shimada et al., 1994). It is well-known that factors such as sex, age and inter-individual differences can affect CYP activity in mammals and may differ between species. Sex differences in CYP-related drug metabolism are less pronounced in humans, dogs, rabbits and monkeys compared to rats and mice (Waxman and Holloway, 2009). In contrast, inter-individual differences are more pronounced in man, which can be explained by the larger genetic variability compared to purpose-bred and more genetically uniform and defined laboratory animals (Shimada et al., 1994). In this respect, large groups are required to estimate a population effect of a compound when inter-individual differences exist (Waxman and Holloway, 2009). Age also appears to be an important factor, especially when considering the embryo-foetal development and risk assessments for the paediatric population. In several mammalian species including man, CYP-related drug metabolism tends to be significantly lower during early life stages (Hakkola et al., 1998; Hines and McCarver, 2002; Parkinson et al., 2004).

For the zebrafish, data on drug metabolism are scarce and fragmented. The majority of CYP-related studies have been focusing on gene expression profiles (reviewed in (Saad et al., 2016a)). However, as CYP activity is not necessarily correlated to its gene expression or even protein levels (Goldstein, 2001), more focus on the CYP activity is needed. In one of our previous studies, CYP1 activity in zebrafish was investigated using the *in vitro* ethoxyresorufin-o-deethylase (EROD) assay (Saad et al., 2016b), a fast, reproducible and easy screening tool. The

present study aims to provide a more comprehensive view on the ontogeny of CYP activity during zebrafish organogenesis and on the possible differences in the *in vitro* CYP-mediated drug metabolism between zebrafish and man. This was conducted using the microsomal stability assay that has the advantages of simplicity, speed and sensitivity when compared to other *in vitro* methods such as liver slices, cell lines or primary hepatocytes. In addition, this *in vitro* approach avoids potential confounding factors that are present in *in vivo* models, such as absorption and distribution issues. For this purpose, we chose four model substrates (dextromethorphan (DXM), diclofenac (DIC), testosterone (TST) and midazolam (MDZ)), that are directed towards four human CYPs considered key in human drug metabolism, including CYP2C9, CYP2D6 and CYP3A4/5 (EMA, 2012; FDA, 2016). In particular, the formation of 4′-hydroxydiclofenac (4-OHDIC) and dextrorphan (DRR) is known to be mediated by CYP2C9 and CYP2D6 (Zanger and Schwab, 2013), whereas the CYP3A4/5 pathway is mediating the formation of 5-hydroxydiclofenac (5-OHDIC), 6ß-hydroxytestosterone (6ß-OHTST), 1-hydroxymidazolam (1-OHMDZ), 4-hydroxymidazolam (4-OHMDZ) and 3-methoxymorphinan (3MM) (Bogaards et al., 2000; Jacqz-Aigrain et al., 1993; Patki et al., 2003; Shen et al., 1999). As such, this study will provide information that is relevant for human risk assessment.

2. Material and methods

2.1 Chemicals

Dimethyl sulfoxide (DMSO), DXM, DRR, 3MM, DIC, 4-OHDIC, TST, 6ß-OHTST, 1-OHMDZ, 4-OHMDZ and clonazepam (CLZ) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 5-OHDIC was obtained from Santa Cruz Biotechnology (Texas, USA), MDZ from Hoffmann-La Roche AG (Basel, Switzerland), and HPLC gradient water (H-water) from Fisher Scientific (Loughborough, UK). KH_2PO_4/K_2HPO_4 buffer and NADPH regenerating system

solution A and B were delivered by BD Gentest (Woburn, MA, USA). The Pierce bicinchoninic acid assay (BCA Assay) used for the determination of total protein content, and the Halt™ Protease Inhibitor Cocktail were obtained from Pierce Chemical (Rockford, IL, USA). All substrates were dissolved in DMSO to prepare the compound stock solutions and stored in aliquots at -20°C. The final concentration of the organic solvent was less than 0.5% (v/v) in all microsomal stability assays to prevent any effect on CYP activity (Chauret et al., 1998).

2.2 Animals and breeding

Zebrafish (*Danio rerio*, in-house wild-type AB zebrafish line) were housed as previously described (Saad et al., 2016b). Briefly, they were kept in a day-night rhythm of 14/10 hours while the temperature, conductivity and pH were 28 ± 1°C, 500 ± 40 $\mu S.cm^{-1}$ and 7.5 ± 0.3. Embryos were kept under the same environmental conditions. The Ethical Committee for Animal Experimentation from University of Antwerp (Belgium) approved the use of the animals (ECD 2015-49).

2.3 Microsomal proteins preparation

Adult fish were euthanized by decapitation followed by a rapid destruction of the brains (Recommendation 2007/526/EC, p89). In order to obtain sufficient microsomal proteins, each batch consisted of 10 adults or approximately 1500 embryos. Ten batches of adult zebrafish liver microsomes (ZLM), five of each gender, in addition to microsomes of a liver homogenate of 50 adult zebrafish were prepared as described previously (Saad et al., 2016b). Five batches of whole embryo microsomes (EM), harvested at different developmental stages, i.e. 5, 24, 48, 72, 96 and 120 hpf, were prepared as mentioned above. Additionally, insect cell control Supersomes (S) (BD Gentest, Woburn, MA, USA) and a pool of human liver microsomes (HLM) from 50 donors

(HMMCPL-PL050B, Life Technologies, Thermo Fisher Scientific, Rockford, USA) were used as negative and positive controls, respectively.

2.4 Microsomal stability assay

Each substrate was incubated with the microsomal proteins at 28.5°C in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 with 5% and 1% of NADPH regenerating system reagents A and B (Saad et al., 2016b). The initial experiments were conducted using 1 µM of substrate and a range from 10 to 500 µg microsomal proteins/ml of adult zebrafish to determine the optimal protein concentration. The latter concentration was incubated with a range from 0.1 to 80 µM substrate for estimation of the kinetic parameters, namely, maximum reaction velocity (V_{max}) and the substrate concentration corresponding to 50% of the V_{max} (K_m). Microsomal stability assays of female ZLM (fZLM), male ZLM (mZLM), and EM were conducted by incubation of K_m from each substrate with the optimal protein concentration. For identification of TST metabolites (Saad et al., 2016 In press) and separation of DIC metabolites, 40 µM of TST or DIC and 1000 µg/ml protein of HLM, mZLM, fZLM and EM (at 96 hpf) were used to attain high concentrations of metabolites to avoid measurement sensitivity issues.

2.5 Sample preparation

Immediately after the start of the microsomal stability assay, aliquots from the main reaction mixture were added at several time points to cooled ACN (4°C) (which already contained the internal standard (IS)) to stop the reaction and denature the microsomal proteins. IS was chosen for each substrate according to the best multiple reaction monitoring (MRM) where no interferences were observed (supplementary table 1).

Centrifugation at 10 000 ×g for 10 min was applied to precipitate the denatured proteins before the supernatants were diluted by H-water to reach the required percentage of ACN, which was 10% for all samples that were prepared for quantitative analysis. The same steps were applied for the calibration standard, which was diluted in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 to minimize matrix or extraction process effects. The samples for TST metabolite identification (qualitative analysis) were diluted to 30% ACN to reduce the dilution effect on the measurement sensitivity (Saad et al., 2016 In press).

2.6 Quantitative UPLC-MS/MS analysis

Microsomal stability assays and sample preparation were performed in duplicate at different days, and the analysis of each sample was also performed in duplicate at the same day. A volume of 5 µl of each sample was injected into a tandem quadrupole UPLC-MS/MS system (Waters® ACQUITY UPLC® – Xevo™ TQ MS, Milford, USA) fitted with an electrospray ionization probe (ESI). Data were acquired in positive ion mode using MRM. Nitrogen was used as nebulizer, desolvation and collision gas. Chromatographic separation was executed using a reversed phase Acquity® UPLC BEH C18 Column, 2.1 mm × 50 mm, 1.7 µm particle size (Waters, Milford, USA) with an Acquity® UPLC BEH C18 VanGuard Pre-column, 2.1 mm × 5 mm, 1.7 µm particle size (Waters, Milford, USA). The mobile phase consisted of a mixture of two solvents (Solvent A: 95:5 water:ACN + 0.1% formic acid, solvent B: ACN + 0.1% formic acid) with a flow rate of 0.6 ml/min and a gradient as described in supplementary table 2.

Applied temperatures for the source, desolvation, autosampler and column were 150, 600, 10 and 30°C, respectively. The gas flow rate was 1000 and 50 L/hour for desolvation and cone. MS parameters such as transitions, cone voltage, collision energy and retention time were optimized

for each analyte by conducting full scan acquisitions with a flow rate of 200 µl/min of 500 ng/ml of analyte in the mobile phase (supplementary table 3).

Since the above mentioned method did not allow separating 4-OHDIC and 5-OHDIC chromatographically, another UPLC-MS/MS method was developed using an ACQUITY UPLC TQ detector (Waters, Milford, USA). As a TQ Detector is less sensitive than a Xevo TQ MS, higher concentrations of DIC (40 µM) and 1000 µg/ml of HLM, ZLM and EM (at 96 hpf) were used for a microsomal stability assay for 60 minutes to reach higher concentrations of the metabolites. The samples were injected in full loop mode (10 µL) on an XSELECT CSH C18 2.5 µm 2.1 x 75 mm column XP (Waters Milford, USA) and thermostatically (40°C) eluted with mobile phase solvents consisting of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B), and a gradient as described in supplementary table 4. Data were acquired in ESI positive ion mode using MRM. Capillary voltage was set at 4.5 kV. Two transitions were monitored for DIC, namely 296 → 215 and 296 → 250 with cone voltage set at 26 V and collision energy at 19 V and 13 V, respectively. Two transitions were monitored for OHDIC, namely 312 → 231 and 312 → 266 with collision energy set at 19 V and 13 V and cone voltage at 26 V. The source and desolvation temperatures were set at 120 and 450°C. Nitrogen was used as nebulizer and desolvation gas. Argon was used as collision gas. The desolvation and cone gas flow rates were set at 850 and 50 L/h.

2.7 Qualitative ultra-performance liquid chromatography – accurate mass mass spectrometry (UPLC-amMS)

The protocol is described in *(Saad et al., 2016 In press)*.

2.8 Data processing following quantitative analysis

All experiments were performed in duplicate, and all microsomal stability assays samples, QC samples and blanks were measured two, four and 16 times, respectively. Data acquisition and analysis were conducted using Waters MassLynx™ Software, Version 4.1. The response of each sample was calculated by dividing the area under curve (AUC) of the analyte peak by the AUC of the corresponding IS. Limits of blank (LOB) and lower limits of detection (LLOD) were calculated according to Armbruster and Pry (2008) (Armbruster and Pry, 2008) and accuracy was calculated using the QC samples as:

$$Accuracy\ \% = \frac{mean\ measured\ concentration}{nominal\ concentration} \times 100$$

The coefficient of variation (CV) was used to express the precision percentage (SD: standard deviations):

$$CV = \frac{SD\ of\ the\ measured\ concentration}{mean\ of\ the\ measured\ concentration} \times 100$$

The data of accuracy, precision, LOB and LLOD are presented in Supplementary tables 5-8.

The velocity of all reactions was calculated within the linear part of the reaction curve and data were presented as mean ± SD (n=3). The kinetic parameters of all substrates were determined using non-linear least-squares regression analysis of untransformed data (GraphPad Software, La Jolla, CA, USA). To assess normal distribution of the data and homogeneity of variances, the one-sample Kolmogorov-Smirnov test and Levene's test were used, respectively. A one-way ANOVA test (embryos) or *t* test (adults) was applied for data that showed a normal distribution and no significant differences among variances. When data did not show a normal distribution, the Kruskal-Wallis test for non-parametric data was applied and post-hoc test was conducted to

detect differences. Differences were considered significant when $p < 0.05$. All statistical analyses were accomplished by means of SPSS statistics version 20 (IBM, Armonk, NY, USA).

3. Results

3.1 DXM

For ZLM, V_{max} and K_m for 3MM and DRR were 28.18 ± 1.48 pmol.mg^{-1}.min^{-1}, 9.43 ± 1.06 µM and 34.60 ± 5.70 pmol.mg^{-1}.min^{-1}, 10.11 ± 3.48 µM, respectively. Both metabolites followed Michaelis-Menten kinetics with R^2 of 0.98 and 0.89 (Figure 1). A concentration of 10 µM (≈ K_m) was chosen for the final experiments in adults and embryos.

No significant differences were observed for the production rates of 3MM and DRR between fZLM and mZLM with 20.73 ± 7.67 and 9.19 ± 4.13 pmol.mg^{-1}.min^{-1} for fZLM, and 14.88 ± 4.71 and 11.47 ± 4.78 pmol.mg^{-1}.min^{-1} for mZLM, respectively. Under these experimental conditions, HLM showed lower 3MM levels (4.57 ± 1.71 pmol.mg^{-1}.min^{-1}) and higher DRR levels (48.27 ± 1.50 pmol.mg^{-1}.min^{-1}) than ZLM (Figure 2).

Levels of DRR in EM at 5, 24 and 48 hpf were lower than the LLOD. EM at 96 hpf showed significantly higher DRR levels (0.37 ± 0.25 pmol.mg^{-1}.min^{-1}) than at 120 hpf (0.12 ± 0.10 pmol.mg^{-1}.min^{-1}) but not at 72 hpf (0.12 ± 0.06 pmol.mg^{-1}.min^{-1}) (Figure 3). For 3MM, concentrations were much lower than LLOD in all embryonic stages except for 96 hpf at which some levels of the metabolite could be observed but not quantified (higher than LOB but lower than LLOD).

3.2 DIC

It was not possible to distinguish the two metabolites of DIC, i.e. 4-OHDIC and 5-OHDIC from each other using the UPLC BEH C18 column, 2.1 mm × 50 mm, 1.7 µm particle size as both had a very close retention time and the same ionization products (312.04 → 166.02; 312.04 → 230.79 and 312.04 → 265.90). The abundant peak at *m/z* 230.79 was selected for quantitative assessment as it showed the best repeatability. V_{max} and K_m of OHDIC production were 659.10 ± 60.23 pmol.mg^{-1}.min^{-1} and 11.73 ± 5.65 µM with the Michaelis-Menten kinetic model and an R^2 of 0.92 (Figure 4A). A concentration of 12 µM (≈ K_m) of DIC was used for the final experiments. No significant differences were observed between mZLM (154.64 ± 63.15 pmol.mg^{-1}.min^{-1}) and fZLM (153.97 ± 30.25 pmol.mg^{-1}.min^{-1}) and lower levels of OHDIC were detected in HLM (58.06 ± 16.40 pmol.mg^{-1}.min^{-1}) under these experimental conditions (Figure 4B).

No metabolites were detected in EM, except in two 96 hpf-batches in which levels close to the LLOD were present. Using the ACQUITY UPLC-TQ Detector system and XSELECT CSH C18 2.5 µm 2.1x75mm column XP, both 4-OHDIC and 5-OHDIC could be separated. Increasing the concentrations of both substrate and microsomal proteins resulted in an increase in the concentrations of both metabolites that exceeded the LLOD in all samples, i.e. mZLM, fZLM, HLM and EM at 96 hpf. The concentrations of both metabolites in EM samples at 96 hpf were 10 times lower than those in ZLM and the ratio 4-OHDIC/5-OHDIC in HLM was very close to the ratio in ZLM (Figure 5).

MDZ

No depletion of MDZ was observed in fZLM and mZLM, although it was very pronounced in HLM (Figure 6). Therefore, higher substrate (0.1-10 µM) and microsomal protein concentrations (50-1000 µg/ml) were used to detect possible depletion of the substrate or formation of

metabolites. Even at these high concentrations no depletion of MDZ was observed in zebrafish samples (Figure 6A) and only very low, non-quantifiable, concentrations of 1-OHMDZ were observed in ZLM after 90 minutes of incubation (Figure 6B). High levels of 1-OHMDZ (Figure 6B) and much lower levels of 4-OHMDZ (Figure 6C) were detected in HLM.

3.3 TST

TST was metabolized by the zebrafish. However, the first experiments did not show any detectable concentration of 6ß-OHTST, which is the main metabolite of TST in humans. Therefore, an LC-amMS system (Saad et al., 2016 In press) was used in an attempt to identify other potential metabolites. No main metabolite(s) could be detected in ZLM. However, six minor metabolites with several isomers (three in positive and three in negative ionization mode) could be detected in ZLM samples after 120 minutes of incubation. These metabolites were absent at time zero and in blank samples as well (Saad et al., 2016 In press), but they could not be quantified because of the absence of standards. Therefore, the depletion of TST was used for the determination of the kinetic parameters of TST biotransformation. V_{max} and K_m were 301.70 ± 21.19 $pmol.mg^{-1}.min^{-1}$ and 9.70 ± 1.88 µM. The biotransformation of TST showed Michaelis-Menten kinetics with an R^2 of 0.94 (Figure 7A). A concentration of 10 µM of TST ($\approx K_m$) was used for the final experiments.

fZLM showed a significantly higher velocity (215.30 ± 42.09 $pmol.mg^{-1}.min^{-1}$) than mZLM (63.22 ± 21.07 $pmol.mg^{-1}.min^{-1}$), whereas the latter was comparable to the velocity in HLM (60.57 ± 11.26 $pmol.mg^{-1}.min^{-1}$) (Figure 7B). No substrate consumption was detected in any of the different embryonic stages.

4. Discussion

4.1 Adult zebrafish

In the present study, we detected several CYP-related reactions in zebrafish using human CYP2- and CYP3-specific substrates. Interestingly, the biotransformation rates were mostly higher in zebrafish compared to humans, except for CYP2D6-like activity. Sex-related differences were only present for TST.

DXM was metabolized by ZLM into DRR and 3MM. In man, DXM is O-demethylated by CYP2D6 to DRR and at a lower rate N-demethylated by CYP3A4 to 3MM. Both metabolites can be further N- or O-demethylated to 3-hydroxymorphinan (Jacqz-Aigrain et al., 1993). Although no ortholog of human *CYP2D6* has been found in zebrafish (Goldstone et al., 2010), the present study showed that zebrafish do metabolize DXM to DRR, in addition to 3MM, but the ratio between both metabolite concentrations was different than in man. DRR is produced with a much higher affinity than 3MM in humans (von Moltke et al., 1998) and in other mammals such as rats for example (Hendrickson et al., 2003). In zebrafish, both metabolites are characterized by similar K_m values while the total consumption rate of DXM showed no significant difference between ZLM and HLM under our experimental conditions. Furthermore, the present study failed to detect any sex-related difference in DXM metabolism in zebrafish. In man, the data are contradictory (Hagg et al., 2001; Labbe et al., 2000; McCune et al., 2001; Parkinson et al., 2004; Tamminga et al., 1999), which is most likely related to the genetic polymorphism of *CYP2D6* portrayed by more than 80 allelic variants (Teh and Bertilsson, 2012).

Also for DIC we showed high concentrations of 4-OHDIC in ZLM and 11 times lower concentrations of 5-OHDIC, despite the absence of any ortholog of *CYP2C9* in zebrafish (Goldstone et al., 2010). 5-OHDIC is most likely responsible for the formation of adducted proteins, which is the initial step in (idiosyncratic) hepatotoxicity of DIC in humans and rats

(Boerma et al., 2012; Kishida et al., 2012). This makes the identification of the DIC metabolism profile essential for a precise interpretation of toxic effects of this compound, also in the zebrafish. No significant differences were detected between fZLM and mZLM in 4- and 5-OHDIC production, which is in agreement with published reports on 4-OHDIC formation by HLM (Bogaards et al., 2000) and with the observation that *in vivo* CYP2C9 activity showed no sex-related differences in humans when other substrates such as losartan were used (Cabaleiro et al., 2013; Hatta et al., 2015). Interestingly, increasing DIC concentrations up to 4 x K_m in order to separate 4-OHDIC and 5-OHDIC showed smaller differences in the velocity of OHDIC formation between man and zebrafish than incubations with DIC at Km. This can be due to saturation of the CYP(s) in ZLM, as K_m values are different in both species, i.e. up to 52 µM in HLM (Spaggiari et al., 2014) and around 12 µM in ZLM.

In contrast to the former substrates, our study clearly showed differences in biotransformation of MDZ and TST between zebrafish and man. For MDZ, neither consumption nor metabolites could be detected in ZLM (above the LLOD), whereas this substrate was largely metabolized into 1-OHMDZ and, to a much lesser extent, into 4-OHMDZ in HLM. Also Alderton *et al.* (2010) could not detect any metabolism of MDZ in zebrafish larvae at 7 dpf. As a result, MDZ, which is a typical substrate for CYP3A in man, appeared to have no affinity for any of the zebrafish CYPs. For TST, the major human CYP3A metabolite, i.e. 6ß-OHTST, could not be detected (Saad et al., 2016 In press), although consumption of TST was evident in ZLM. This contrasts with the results obtained by Chng *et al.* (2012) who detected seven mono-hydroxylated metabolites of TST in fZLM of which 6ß-OHTST appeared to be predominant. Reschly *et al.* (2007) also detected this metabolite in addition to three different mono-hydroxylated metabolites in zebrafish hepatocytes. These authors reported that the levels of 6ß-OHTST increased after

exposure to different PXR inducers (Reschly et al., 2007). To ascertain that no 6ß-OHTST was formed in our zebrafish samples, we performed an experiment at very high concentrations of both microsomal proteins and substrate with a long incubation time (1000 µg/ml and 40µM, 120 minutes, respectively). No oxidation activity could be detected but several other abundant peaks were visible after biotransformation by ZLM (Saad et al., 2016 In press). Even if traces of 6ß-OHTST under the LLOD were present in ZLM, we can ensure that these presumptive levels are negligible when compared to those in humans (Saad et al., 2016 In press). As Chng *et al*. (2012) also noted much higher 6ß-OHTST levels in HLM than in fZLM, the relevance of this metabolite in zebrafish is questionable (Chng et al., 2012). Nevertheless, a remarkable depletion of TST was observed in ZLM in the microsomal stability assay and, in contrast to the other substrates, TST consumption showed large differences between fZLM and mZLM, which was also reflected in some of the observed metabolites peaks (Saad et al., 2016 In press). Our results are in accordance with another study on fathead minnows in which females biotransformed TST at higher rates compared to males (Parks and LeBlanc, 1998). These sex differences, which are also present in other species like rats and mice (Niwa et al., 1995; Wilson et al., 1999), are critical for the maintenance of androgen homeostasis, which is responsible for reproduction and sexual differentiation (Mangochi, 2010). Significant sex-related differences in TST biotransformation have not been reported in humans possibly due to high inter-individual variations.

So, although adult zebrafish showed for some CYP-probes reactions that also occur in man, e.g. dextromethorphan O-demethylation, dextromethorphan N-demethylation and 4- and 5-diclofenac hydroxylation, also clear differences between these two species were observed for other human CYP-probes. As such, CYP-mediated drug metabolism in zebrafish may not be

predictive for the one in man and should be investigated for each new compound when using this animal model for human risk assessment.

4.2 Zebrafish embryos/larvae

As zebrafish embryos are gaining interest as an alternative model for developmental toxicity studies, several research groups are evaluating their predictive potential for teratogenic compounds (Brannen et al., 2010; Panzica-Kelly et al., 2012). Weigt *et al.* (2010) showed that zebrafish embryos developed malformations when they were exposed to mammalian proteratogens in the absence of an external metabolizing system, suggesting intrinsic bioactivation of these compounds by the embryos (Weigt et al., 2011). However, as no metabolite analysis was performed, it cannot be excluded that the presumed proteratogens were teratogenic by themselves in the zebrafish and did not require bioactivation. This is in line with a study from Mattsson *et al.* (2012) who showed that albendazole, an anthelmintic drug, was very toxic for zebrafish embryos and that co-incubation with an external metabolizing system, i.e. rat liver microsomes, drastically decreased the toxicity due to consumption of the parent compound and formation of the non-toxic metabolite albendazole sulfoxide (Mattsson et al., 2012). Also for DXM, mortality and diverse morphological and physiological malformations were inversely correlated to the age of the embryos/larvae (Xu et al., 2011). The observed lower toxicity with increasing age might be, due to higher CYP activity at later stages, responsible for the detoxification of DXM. This is in accordance with our *in vitro* study, in which we observed a lack in biotransformation of DXM during the embryonic stages. Only at the larval stage (from 72 hpf onwards) and more specifically at 96 hpf, metabolites above the LLOD were detected for most CYP-probes. This is also in agreement with *in vivo* data in 96 hpf or 120 hpf larvae, which

showed *in vivo* EROD, ECOD and OOMR activities and TST metabolism (Jones et al., 2010). Alderton *et al.* (2010) also recorded several metabolic activities in 7 dpf larvae including glucuronidation of TST, N- and O-demethylation as well as hydroxylation of DXM, and sulphate conjugation of cisapride. Additionally, 12 metabolites of verapamil were detected in 7 dpf larvae and 10 of them could already be detected at 3 dpf. These authors also detected low levels of OHTST and OHDIC at 7 dpf, but exact determination of their chemical structure was unfeasible. However, even at this later stage of development the authors questioned the biological relevance of the detected metabolite concentrations as they were still very low (Alderton et al., 2010).

In summary, no *in vitro* biotransformation of the tested substrates was observed during zebrafish embryonic development. At 96 hpf, metabolic activation occurred for some CYP probes but, as this developmental stage is the end of organogenesis in this species, the embryo appears to lack the biotransformation and bioactivation potential during the sensitive period for developmental toxicants. As such, this limitation of the zebrafish embryo assay should be taken into account when setting-up and interpreting results from developmental toxicity studies.

4.3 Impact of the study

To our knowledge, this is the first study that assessed qualitative and quantitative differences in *in vitro* CYP-mediated drug metabolism between man, adult zebrafish and zebrafish embryos/larvae using human reference compounds. Four distinctive biotransformation patterns were observed in zebrafish compared to humans. The first pattern indicated similar biotransformation pathways and their potency as shown by the production of similar main metabolites at similar ratios in both species, i.e. DIC biotransformation into 4- and 5-OHDIC. This pattern represents the ideal situation for extrapolation of *in vitro* drug metabolism data from zebrafish to humans. In the second pattern, substrates were also biotransformed into similar main

metabolites as in humans, but with altered ratios as shown for DXM (DDR/3MM). The third pattern indicated different pathways as totally different metabolites were observed between zebrafish and man, e.g. for TST (6ß-OHTST). As such, different pharmacological/toxicological effects can be expected in both scenarios and especially in the third pattern. In the last pattern, in which the substrate was not metabolized by zebrafish, e.g. MDZ, there is the risk of overestimation of toxic effects by the parent compound and metabolite-mediated effects can be overlooked. The fourth scenario also applies for zebrafish embryos, as the lack of metabolic activity during a major part of organogenesis limits the extrapolation of developmental toxicity. So, from our study we can conclude that CYP-mediated drug metabolism can be substantially different in zebrafish compared with man. As such, results from toxicity studies in zebrafish should be interpreted with caution when used for human risk assessment. We recommend including zebrafish liver microsomes/hepatocytes in the *in vitro* drug metabolism testing battery during the non-clinical/discovery phase in addition to the currently used mouse, rat, dog, non-human primate and human liver microsomes/hepatocytes when considering this species for human risk assessment data to humans.

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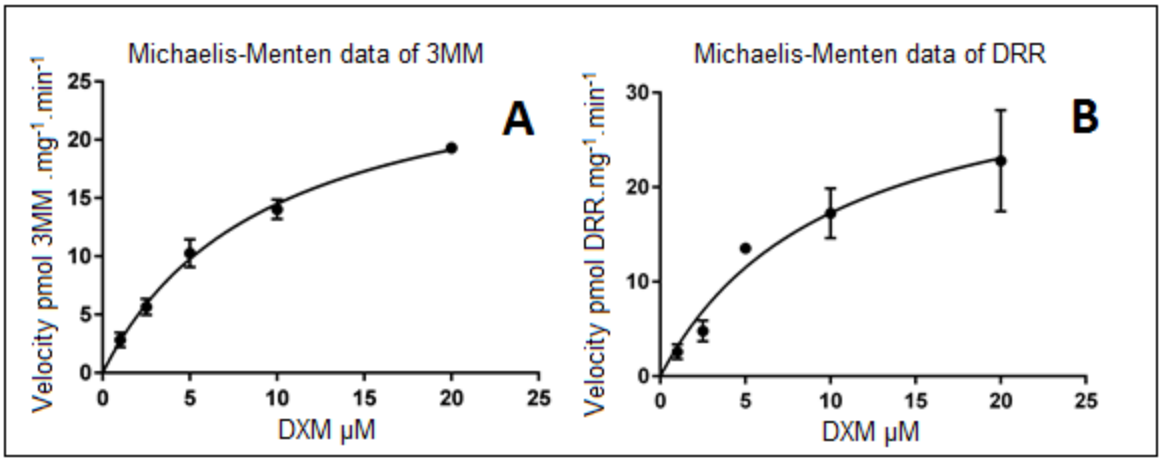

Figure 1: Velocity of 3-methoxymorphinan (3MM) (A) and dextrorphan (DRR) (B) formation at different concentrations of dextromethorphan (DXM) using 200 µg/ml ZLM. Each data point represents the mean of three independent experiments each with duplicate measurements.

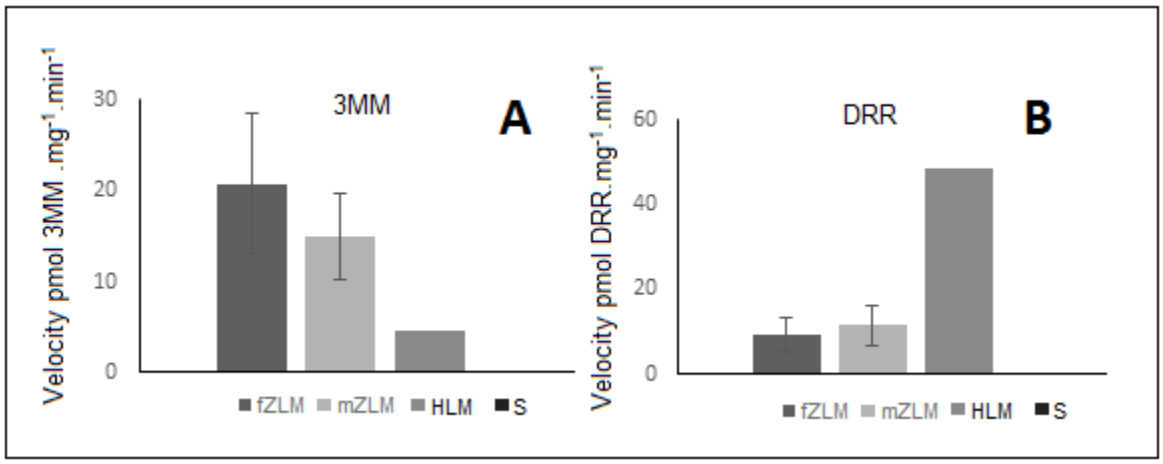

Figure 2: Velocity of 3-methoxymorphinan (3MM) (A) and dextrorphan (DRR) (B) formation in female zebrafish liver microsomes (fZLM) (five batches), male zebrafish liver microsomes (mZLM) (five batches), human liver microsomes (HLM) (one batch) and Supersomes (S) (one batch).

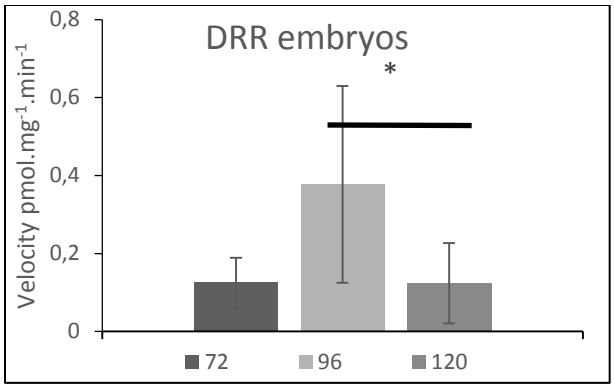

Figure 3: Velocity of dextrorphan (DRR) in microsomes of whole body homogenates of zebrafish embryos (EM) at 72, 96 and 120 hpf (five batches of each). Statistical differences (P <0.05) between developmental stages are indicated by (*).

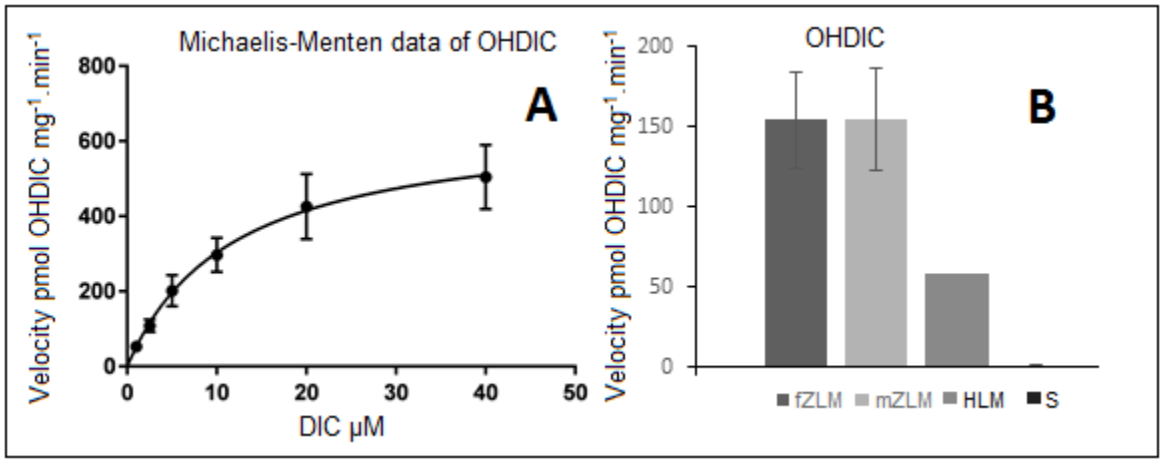

Figure 4: (A) Velocity of hydroxydiclofenac OHDIC formation at different concentrations of diclofenac (DIC) using 200 µg/ml of ZLM. Each data point represents the mean of three independent experiments, each with duplicate measurements. (B) Velocity of OHDIC formation in female zebrafish liver microsomes (fZLM) (five batches), male zebrafish liver microsomes (mZLM) (five batches), human liver microsomes (HLM) (one batch) and Supersomes (S) (one batch).

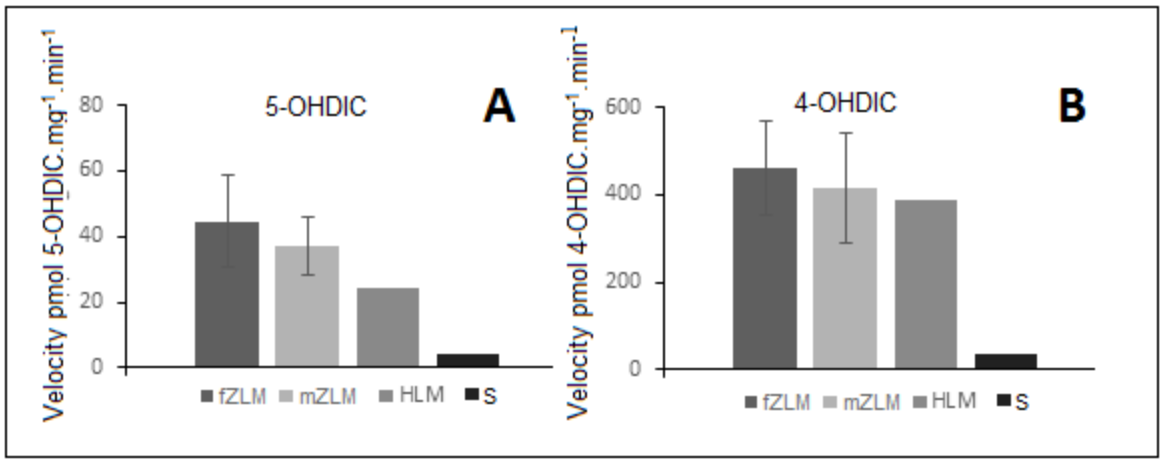

Figure 5: Velocity of 5-hydroxydiclofenac (5-OHDIC) (A) and 4′-hydroxydiclofenac (4-OHDIC) (B) formation in female zebrafish liver microsomes (fZLM) (five batches), male zebrafish liver microsomes (mZLM) (five batches), human liver microsomes (HLM) (one batch) and Supersomes (S) (one batch).

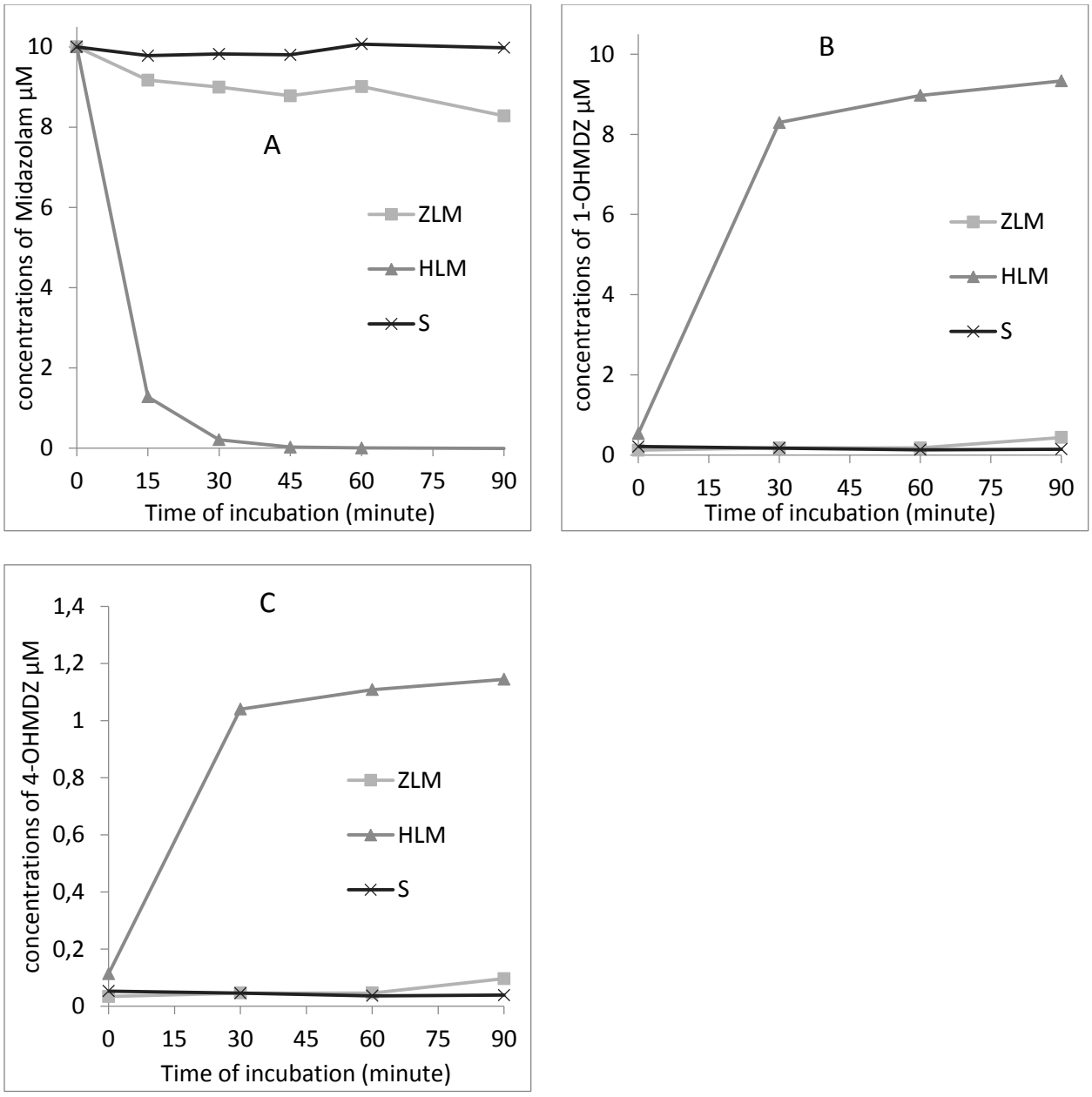

Figure 6: (A) Midazolam (MDZ) depletion, (B) 1-OHMDZ and (C) 4-OHMDZ formation in zebrafish liver microsomes (ZLM), human liver microsomes (HLM) and Supersomes (S). Protein concentration: 1000 µg/ml and MDZ concentration: 10 µM.

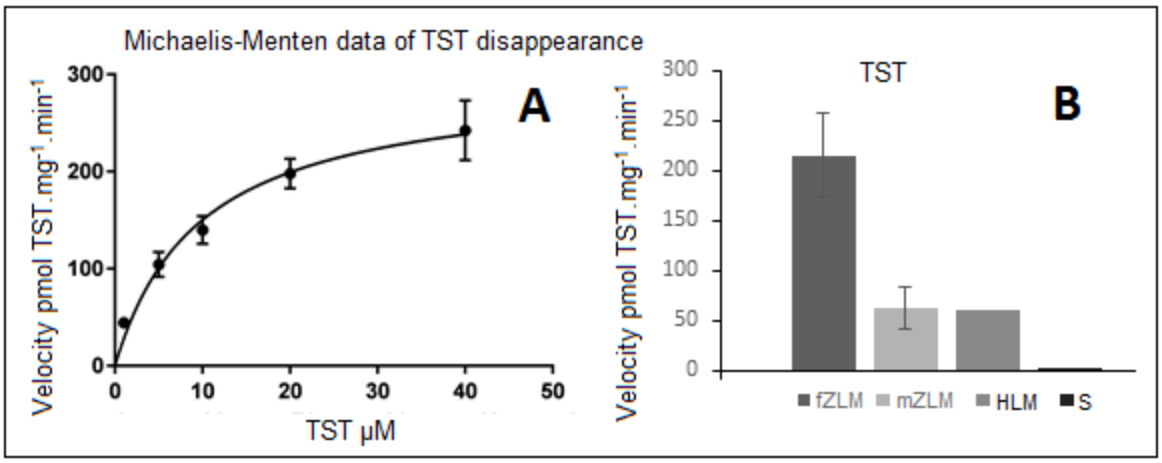

Figure 7: (A) Velocity of testosterone (TST) depletion at different concentrations of TST using 200 µg/ml of ZLM. Each data point represents the mean of three independent experiments each with duplicate measurements. (B) Velocity of TST depletion in female zebrafish liver microsomes (fZLM) (five batches), male zebrafish liver microsomes (mZLM) (five batches), human liver microsomes (HLM) (one batch) and Supersomes (S) (one batch).