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Cytochrome P450 inhibition in rainbow trout *in vitro*

Cytochrome P450 Inhibition by Antimicrobials and Their Mixtures in Rainbow Trout Liver Microsomes *In Vitro*

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Abstract: Antimicrobials are ubiquitous in the environment and can bioaccumulate in fish. In the present study, we determined the half-maximal inhibitory concentrations (IC50) of seven environmentally abundant antimicrobials (ciprofloxacin, clarithromycin, clotrimazole, erythromycin, ketoconazole, miconazole, and sulfamethoxazole) on the cytochrome P450 (CYP) system in rainbow trout (*Oncorhynchus mykiss*) liver microsomes, using 7-ethoxyresorufin O-deethylation (EROD, CYP1A) and 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylation (BFCOD, CYP3A) as model reactions. Apart from ciprofloxacin and sulfamethoxazole, all antimicrobials inhibited either EROD or BFCOD activities or both at concentrations below 500 µM. Erythromycin was the only selective and also time-dependent inhibitor of BFCOD. Compared with environmental

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concentrations, the IC50s of individual compounds were generally high (>mg/L), but as mixtures, the antimicrobials resulted in strong, indicatively synergistic inhibitions of both EROD and BFCOD at sub-micromolar (~µg/L) mixture concentrations. The cumulative inhibition of the BFCOD activity was detectable even at picomolar (~ng/L) mixture concentrations and potentiated over time, likely because of the strong inhibition of CYP3A by ketoconazole (IC50=1.7±0.3 µM) and clotrimazole (IC50=1.2±0.2 µM). The results suggest that if taken up by fish, the mixtures of these antimicrobials may result in broad CYP inactivation and increase the bioaccumulation risk of any other xenobiotic normally cleared by the hepatic CYPs even at biologically relevant concentrations.

Keywords: Environmental risk assessment, pharmaceuticals, cytochrome P450, xenobiotic metabolism, mixtures, bioaccumulation

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INTRODUCTION

Active pharmaceutical ingredients are ubiquitous in the aquatic environment (Vasquez 2014; Aus der Beek 2016). Remarkably high concentrations, exceeding mg per liter level, are reported especially for antimicrobials in the developing countries as the result of inadequate sanitation or pharmaceutical raw material production (Larsson 2014; Kairigo 2020). Population growth and ageing further increase the consumption of antimicrobials and their human excretions to the sewage. As a result, antimicrobial residues originating from human use represent an ever-increasing share of the total environmental pollution

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globally. Besides contributing to the evolution of global antimicrobial resistance, the residues of antimicrobial agents can be taken up by aquatic species. Measurable levels of several common antimicrobials have been detected in the tissues (kidney, liver, brain, bile, muscle, and gill) of various wild fish species in vivo (Table 1), and plasma concentrations in the range of low µg/L have been reported for, e.g., clarithromycin, clotrimazole, and miconazole (Cerveny 2021). If uptaken, the fish ability to metabolise the pharmaceuticals is a critical factor that determines whether the compound is effectively cleared or whether it bioaccumulates in the tissues. The superfamily of hepatic cytochrome P450 (CYP) enzymes is one of the most important xenobiotic elimination systems in practically all living species, including teleost fish (Celander 1996; Uno 2012; Nelson 2013). However, many pharmaceuticals may also inhibit the CYP activity and thereby interfere with the hepatic clearance of one another. Previous studies have revealed that antimicrobials may result in a broad, nonselective CYP inhibition in rainbow trout in vitro (Miranda 1998; Smith 2012; Burkina 2013) and in vivo (Hegelund 2009), but detailed mechanism-based enzyme inhibition studies are scarce. With a view to the bioaccumulation risk, irreversible CYP inactivation is the most severe form of enzyme inhibition in vivo, because it is long-lasting (~days) and requires de novo synthesis of new enzymes before the detoxification capacity is recovered. Reversible enzyme inhibition typically levels off more rapidly, although continuous exposure of aquatic species to a vast number of antimicrobials and other environmental chemicals concurrently complicates the prediction of the true in vivo effects.

In the present study, we determined the inhibitory concentrations of seven environmentally ubiquitous antimicrobials (ciprofloxacin, clarithromycin, clotrimazole,

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erythromycin, ketoconazole, miconazole, and sulfamethoxazole) toward the hepatic cytochrome P450 (CYP) system in rainbow trout in vitro. These compounds included three fungal CYP inhibitors and four bacterial inhibitors with different modes of action (Table 1). Four of them (ciprofloxacin, clotrimazole, miconazole, sulfamethoxazole) are also included in the European Commission's 3rd watch list of substances for Union-wide monitoring (EU 2020). In the present study, their half-maximal inhibitory concentrations (IC50) were determined separately toward CYP1A and CYP3A activities in rainbow trout liver microsomes. These enzymes were targeted because they are the two most abundant hepatic CYP isoforms in rainbow trout (Hegelund 2004; Jönsson 2006; Christen 2010). The time-dependency of the CYP inhibition was additionally evaluated based on IC50 shift assay, which is a high-throughput assay commonly used in the preclinical drug development for predicting the probability of irreversible CYP inhibition by new drug candidates (Obach 2007; Berry 2008). Time-dependent inhibition typically results from the formation of inhibitory, sometimes reactive, metabolites that are more potent than the parent compound. In the IC50 shift assay, the drug candidate is first preincubated with and without the CYP cofactor, nicotinamide adenine dinucleotide phosphate (NADPH), prior to addition of the enzyme-specific model substrate. If preincubation with NADPH potentiates the inhibition (IC50) by more than 1.5-fold (a typical threshold), the drug candidate is considered a time-dependent inhibitor (Berry 2008). In many cases, but not always, time-dependent inhibition is indicative of irreversible enzyme inhibition and therefore triggers more detailed, mechanism-based inhibition studies to explicitly distinguish whether the observed inhibition is reversible or irreversible. Here, we hypothesized that the IC50 shift assay could help identify the most

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risky compounds that are likely to cause time-dependent, possibly irreversible, enzyme inhibition in rainbow trout. In addition to antimicrobials, the feasibility of the IC50 shift assay for its intended purpose was examined with three other pharmaceuticals (Table 1), which are known to be selective and irreversible inhibitors of human CYP1A (furafylline) or CYP3A enzymes (diltiazem, verapamil).

The combined effects of the same antimicrobials on CYP1A and CYP3A activities were determined by incubating them as mixtures, at different relative ratios and different total concentrations, with rainbow trout liver microsomes. In the regulatory context, the mixture effects are typically examined using either concentration addition (similar mode of action) or independent action approaches (EC 2009). Here, we hypothesized that as mixtures, the pharmaceuticals could result in not only additive, but possibly even synergistic (1+1>2) CYP inhibition in rainbow trout. While the propensity of synergistic CYP inhibition is well-documented for human (Calvey 2005; Mishima 2017; Gupta 2018), fairly limited data is available of the combined impacts of pharmaceutical mixtures on fish CYP (apart from binary mixtures). In the present study, the propensity for synergistic inhibition was evaluated by comparing the measured inhibitory effects of pharmaceutical mixtures on CYP1A and CYP3A with corresponding predicted mixture effects, calculated based on the concentration addition approach.

MATERIALS AND METHODS

Enzyme sources and model enzyme activities

Commercially available rainbow trout (*Oncorhynchus mykiss*) liver microsomes, pooled from 35 male fish, were used as the enzyme source and purchased from Life technologies (Thermo Scientific). The total protein concentration of the stock solution was 20 mg/mL

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(according to the supplier). The activities of CYP1A and CYP3A enzymes were determined with 7-ethoxyresorufin O-deethylation (EROD) and 7-benzyloxy-4-trifluoromethylcoumarin O-debenzylation (BFCOD), respectively, as the model reactions. The enzyme-specificity of these model reactions has been well-established in previous studies (Hegelund 2004; Jönsson 2006; Christen 2010).

Chemicals

The prefluorescent CYP model substrates 7-ethoxyresorufin (ER) and 7-benzyloxy-4-trifluoromethyl coumarin (BFC) were purchased from Toronto Research Chemicals and Apollo Scientific Ltd, respectively. The corresponding metabolite standards resorufin and 7-hydroxy-4-trifluoromethyl coumarin were from Sigma Aldrich and Toronto Research Chemicals, respectively. The test pharmaceuticals included seven antimicrobials (ciprofloxacin, clarithromycin, clotrimazole, erythromycin, ketoconazole, miconazole nitrate, sulfamethoxazole) and three other method validation compounds (diltiazem, furafylline, verapamil hydrochloride), which were all purchased from Sigma Aldrich. Trizma® base (trishydroxy methylamino methane), β-nicotinamide-adenindinucleotide-2′-phosphate (reduced tetrasodium salt hydrate) (NADPH), and dimethyl sulfoxide were also from Sigma-Aldrich, dipotassium hydrogen phosphate was from Amresco, and potassium dihydrogen phosphate, acetone, acetonitrile, methanol, and hydrochloric acid were from Riedel-de-Haën. All reagents and solvents used were of HPLC or analytical grade (≥ 98.0%). Water was purified with Milli-Q water purification system (Merck Millipore).

Enzyme incubations and determination of the kinetics constants

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The EROD and BFCOD activities in rainbow trout liver microsomes were determined with separate incubations of the model substrates ER and BFC, respectively, in separate vials in total volume of 100 μL using NADPH as the cofactor. The reactions were initiated by the addition of NADPH to the reaction solution containing the substrate and the liver microsomes. All enzyme incubations were done in duplicate or triplicate. After desired incubation time, the reactions were terminated by adding 37.5 μL of the quenching solution (0.5 M Trizma base:acetonitrile, 20:80, v/v) to the incubation mixture, after which the quenched reaction solutions were kept on ice for minimum of 20 min and centrifuged (13000 rpm, 10-15 min) to precipitate the proteins. The supernatants were then analyzed by Varioskan LUX microplate reader (Thermo Fisher Scientific) using excitation and emission wavelengths of 570 nm/595 nm for resorufin detection (metabolite of EROD reaction) and 419 nm/501 nm for 7-hydroxy-4-trifluoromethyl coumarin detection (metabolite of BFCOD reaction) (Table 2). The enzyme activities were calculated by dividing the metabolite produced with incubation time and total protein amount (nmol/min/mg). To ascertain sufficient enzyme activity for IC50 determination, the incubation temperature (10±1 or 21±1°C) and the pH of the incubation buffer (from 7.4 to 8.0) were preliminarily optimized within the natural habitat conditions of rainbow trout (Raleigh 1984; Froese 2019). In addition, the microsomal total protein concentration (from 0.1 to 1.0 mg protein /mL) and the incubation time (from 10 to 60 min) were separately optimized for both model reactions to ensure that the metabolite production increases linearly with protein concentration and incubation time in accordance with the Michaelis-Menten steady-state assumption. NADPH concentration

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was adjusted to a saturation level. The optimized enzyme incubation conditions for EROD and BFCOD activity determinations are summarized in Table 2.

The enzyme kinetic constants, K_M (enzyme affinity) and V_{MAX} (maximal velocity), of the model activities were determined in 0.1M potassium phosphate buffer (pH 8.0) at 21±1°C using optimized, reaction-specific protein and NADPH concentrations and incubation times (Table 2). The substrate concentrations used were 0.5-16 μM in EROD assays and 25-1000 μM in BFCOD assays. All incubations were conducted in duplicate or in triplicate at each concentration level. The kinetic constants were derived from enzyme activities (pmol/min/mg total protein) calculated at each concentration level by fitting the data to Michaelis-Menten kinetics using Graphpad Prism software (version 8.2.1).

Determination of IC50 constants and the IC50 shift

The IC50 concentrations of test pharmaceuticals were determined in optimized enzyme incubation conditions (Table 2) separately toward EROD and BFCOD activities by incubating each pharmaceutical individually at six different concentrations, ranging from 0.5 to 500 μM, with either one of the model substrates at a time. In these reactions, the concentration of the model substrate was adjusted close to its K_M value and was 1 μM for ER and 75 μM for BFC (Table 2). The residual solvent content was dependent on the solubility of the test pharmaceutical and thus varied between compounds within 0.5-4.0% (v/v) (Table S1). However, the residual solvent content within each series was kept constant, so that the possible inhibitory impact of the solvent was identical at all pharmaceutical concentrations used, including control (zero pharmaceutical concentration).

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To evaluate the time-dependency of the enzyme inhibition based on IC50 shift, each test pharmaceutical and rainbow trout liver microsomes were preincubated for 30 minutes either together with the cofactor (+NADPH series) or without the cofactor (-NADPH series), before initiating the model reaction by adding the model substrate (+NADPH series) or the model substrate and the cofactor simultaneously (-NADPH series). The IC50 concentrations of both series (+NADPH and -NADPH) were determined with Graphpad Prism software (version 8.2.1) using nonlinear regression without weightings according to Eq. 1:

$$y = \frac{\mathbf{100}}{\mathbf{1+10}^{(\boldsymbol{LogIC50-x})\times\boldsymbol{HillSlope}}} \quad (1)$$

in which *y* is the relative activity (%) of the model reaction compared with control, *x* is the concentration of the test pharmaceutical (µM), and Hill Slope is the steepness of the curve (constant value of -1). The IC50 shift was calculated according to Eq. 2 and the corresponding standard error ($\Delta IC50_{shift}$) according to Eq. 3:

$$IC50_{shift,i} = \frac{IC50_{NADPH(-),i}}{IC50_{NADPH(+),i}} \quad (2)$$

$$\Delta IC50_{shift,i} = IC50_{shift,i} \times \sqrt{\frac{\Delta IC50_{NADPH(-),i}}{IC50_{NADPH(-),i}} + \frac{\Delta IC50_{NADPH(+),i}}{IC50_{NADPH(+),i}}} \quad (3)$$

where $IC50_{NADPH(-),i}$ and $IC50_{NADPH(+),i}$ are the IC50 values of compound *i* derived from -NADPH and +NADPH series, respectively, and $\Delta IC50_{NADPH(-),i}$ and $\Delta IC50_{NADPH(+),i}$ are the corresponding standard errors derived from the Hill model using 95% confidence level. The test compound was considered a time-dependent inhibitor, if the IC50 shift (Eq. 2) was ≥1.5 (Obach 2007; Berry 2008; Grimm 2009).

Determination of the inhibitory effects of pharmaceutical mixtures

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In addition to individual pharmaceuticals, the impact of pharmaceutical mixtures on EROD and BFCOD activities in rainbow trout liver microsomes were determined using four different mixture concentration levels with different relative molar fractions (p_i) of pharmaceuticals. These mixtures included the seven antimicrobials (n=7) and the method validation compounds, diltiazem and verapamil, but not furafylline, which is not in clinical use and has thus low environmental relevance.

Firstly, the pharmaceuticals were mixed at concentrations equivalent to their approximate individual IC50 values (IC50 mix). These IC50 mixtures were prepared separately for EROD and BFCOD assays accounting for the compound- and enzyme-specific inhibitory concentrations.

Secondly, the pharmaceuticals were mixed at concentrations that individually resulted in approximately 10% decrease in maximal enzyme activity (IC10 mix) and were approximately an order of magnitude lower than the corresponding IC50 concentrations (depending on the compounds' dose-response curves). Thus, in these mixtures, the relative molar ratios of pharmaceuticals resembled their inhibition strengths so that each pharmaceutical would cause approximately similar percentual inhibitory impact on EROD and BFCOD activities.

Thirdly, the pharmaceuticals were mixed at concentrations equivalent to their average influent concentrations (Influent mix), which were estimated based on data reported in the German Environment Agency database (Umweltbundesamt – UBA 2020). This mixture was considered to represent the upper end of environmental concentrations close to the wastewater treatment plants, assuming negligible degradation (0% removal) and no dilution. In this case, the relative molar ratios of pharmaceuticals (p_i) resembled their

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relative proportions in the influents (uneven inhibitory impact) and the concentrations were the same in both EROD and BFCOD assays.

Lastly, the pharmaceuticals were mixed at an equimass ratio (uneven inhibitory impact) using a concentration of 1 ng/L for each of the seven antimicrobials, diltiazem, and verapamil (total concentration 9 ng/L). This mixture was considered to represent the low end of the reported environmental concentrations (Vasquez 2014; Aus der Beek 2016) and its inhibitory impact was determined on the BFCOD activity only.

The predicted mixture concentrations that would theoretically result in half-maximal enzyme inhibition ($IC50_{mix}$) were calculated based on the relative molar ratios (p_i) of pharmaceuticals in each of the mixtures, using the concentration addition approach (EC 2009), according to Eq. 4:

$$IC50_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{IC50_i}\right)^{-1} \quad (4)$$

in which $IC50_i$ is the half-maximal inhibitory concentrations of an individual compounds *i* and p_i is its relative molar fraction in the mixture. In the calculation of the $IC50_{mix}$, only those compounds that inhibited EROD or BFCOD activities were accounted for, but in the mixture assays, also non-inhibitory pharmaceuticals were included at indicated concentrations.

All mixture assays were performed under optimized enzyme incubation conditions (Table 2), in a total initial volume of 1000 μL in triplicate or quadruplicate. In this case, the model substrate (ER or BFC), the cosubstrate (NADPH), and the inhibitors were first mixed together and the reaction was initiated by the addition of the rainbow trout liver microsomes. One hundred μL aliquots of the reaction solution were then withdrawn at eight different time points (5, 10, 20, 30, 60, 90, 120 and 180 min), and their enzymatic

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activity was quenched by adding 37.5 µL of the quenching solution. The residual EROD and BFCOD activities (%) of the pharmaceutical mixtures were compared with corresponding control activities determined similarly at each time point without the inhibitory pharmaceuticals (in duplicate). Residual solvent concentrations (dimethyl sulfoxide, acetonitrile, methanol, and acetone) in the mixture assays varied between 0.63 and 1.2 % (v/v) based on the mixture type (Table S2), but was identical between the control incubations (no inhibitors) and incubations including the inhibitory pharmaceuticals.

RESULTS

Method development and validation

As the CYP activity in rainbow trout liver microsomes can be fairly low (Han 2009), the enzyme incubation conditions were preliminarily optimized within the natural habitat conditions (pH, temperature) of rainbow trout, so as to ensure that even residual EROD and BFCOD activities could be measured. Enzyme incubation at pH 8.0 and room temperature (21±1°C) was found sufficient to yield detectable amounts of the model metabolites, resorufin (CYP1A) and 7-hydroxy-4-trifluoromethyl coumarin (CYP3A). Under these conditions, with 0.5 mg/mL microsomal total protein concentration, the metabolite formation was linear up to 20 min (EROD) and 30 min (BFCOD). The enzyme kinetic parameters determined for the model reactions under optimized incubation conditions (Table 2) are given in Figures 1A and 1B. On the basis of these results, the concentrations of the model substrates in the enzyme inhibition studies were adjusted slightly above the K_M values of the model reactions, that is 1 µM ER (K_M=0.8±0.3 µM) and 75 µM BFC (K_M=48±9 µM). The enzyme kinetic constants as

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well as the subsequent IC50 concentrations were defined in moles per liter to enable straightforward comparison of the inhibitory concentrations of pharmaceuticals in rainbow trout with those reported for human CYP.

Next, the IC50 concentrations and the IC50 shifts of three method validation compounds (furafylline, diltiazem, and verapamil) were determined separately toward both EROD and BFCOD activities in optimized enzyme incubation conditions (Table 2). As expected, furafylline (irreversible inhibitor of human CYP1A) inhibited the EROD activity in rainbow trout liver microsomes in a time-dependent manner with IC50 shift of approximately 5.9-fold and IC50 of 23±12 µM, when preincubated with the microsomes and NADPH before initiation of the model reaction (Table 3). In addition, furafylline showed weak, but not time-dependent inhibition toward the BFCOD activity with IC50 of 171±45 µM. Similarly, the second validation compound, diltiazem (time-dependent inhibitor of human CYP3A) resulted in an indicatively time-dependent inhibition of the BFCOD activity with IC50 shift of approximately 1.5-fold and IC50 of 91±19 µM (Table 3). Diltiazem also inhibited EROD activity, although not in a time-dependent manner, with IC50 of 78±32 µM. The third validation compound, verapamil (time-dependent inhibitor of human CYP3A) was incoherently not a time-dependent inhibitor of neither EROD nor BFCOD activity in rainbow trout liver microsomes (IC50 shift ca. 0.9-fold in both cases), even if it showed moderate inhibition toward both EROD (IC50 70±12 µM) and BFCOD (IC50 33±2 µM) activities (Table 3).

Cytochrome P450 inhibition by antimicrobials in rainbow trout liver microsomes

From the seven antimicrobials, all except for ciprofloxacin and sulfamethoxazole, inhibited either EROD or BFCOD activities or both in rainbow trout liver microsomes at

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concentrations below 500 μM (Table 3). Clotrimazole and ketoconazole were the strongest inhibitors with IC50s <10 μM, whereas others exhibited moderate to weak inhibition with IC50s ranging from 20 to 200 μM (Table 3). Erythromycin was shown to selectively inhibit BFCOD only, indicatively in a time-dependent manner (IC50 shift 1.5±0.7), whereas others were nonselective inhibitors of both EROD and BFCOD activities in rainbow trout in vitro. However, ciprofloxacin and sulfamethoxazole inhibited neither EROD nor BFCOD at concentrations <500 μM and thus they were considered noninhibitory compounds in the context of mixture assays. Apart from erythromycin, none of the other antimicrobials were time-dependent inhibitors according to the IC50 shift assay. The dose-response curves for the EROD and BFCOD inhibition by all of the test pharmaceuticals are given in the Supplemental data (Figure S1).

Cytochrome P450 inhibition by pharmaceuticals' mixtures in rainbow trout liver microsomes

As a proof of concept, the combined effects of the pharmaceuticals' mixtures on the EROD and BFCOD activities were determined by incubating them with rainbow trout liver microsomes for total of 3 hours at four different total concentration levels, using different relative molar fractions of individual compounds in each mixture. According to the IC50 shift assays, these mixtures included two time-dependent inhibitors of BFCOD activity (diltiazem, erythromycin), but not any time-dependent inhibitors of EROD activity. As expected, when the pharmaceuticals were mixed at concentrations equivalent to their compound specific IC50 concentrations (Table S3), only residual EROD activity, about 1% of that of the control incubation, was observed (Figure 2A). The corresponding inhibitory effect of the IC50 mixture on the BFCOD activity was shown to be somewhat

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less than that of EROD, but the inhibition clearly potentiated over time yielding ca. 10% residual activity of that of the control after the 3-hour incubation (Figure 2B). On this basis, it was concluded that the inhibitory impacts of these pharmaceutical mixtures on fish CYP were cumulative in nature.

To examine whether the cumulative enzyme inhibition was additive or synergistic, the pharmaceuticals' concentrations were reduced below the IC50 concentrations of individual pharmaceuticals. First, the pharmaceuticals' concentrations were adjusted close to those that individually resulted in somewhat even inhibition of approximately 10% of the control, as indicated in Table 4 (IC10 mix). In this case, the total concentration of pharmaceuticals that inhibited the EROD activity (excluding ciprofloxacin, erythromycin, and sulfamethoxazole) was 33 µM and it resulted in ca. 10% residual EROD activity compared with control incubation without any pharmaceuticals (Figure 2A). The theoretical concentration addition approach (Eq. 4) does not allow the prediction of the percentual inhibitory level of this mixture. However, the model allows calculation of the predicted mixture concentration ($IC50_{mix}$) that would theoretically result in half-maximal enzyme inhibition, when the pharmaceuticals are mixed in the same relative molar ratios. This predicted inhibitory mixture concentration ($IC50_{mix}$) toward EROD activity was 67 µM (i.e., about two-fold higher than the 33 µM concentration used in the experiments). On this basis, it was concluded that the inhibitory impact of the IC10 mixture on EROD activity was synergistic in nature.

In case of BFCOD assays, the time-dependent inhibitors (diltiazem and erythromycin) were excluded from the IC10 mixture (Table 4) to examine if they were critical to the observed potentiation of BFCOD inhibition over time. As a result, the total concentration

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of pharmaceuticals that inhibited the BFCOD activity (excluding ciprofloxacin, diltiazem, erythromycin, and sulfamethoxazole) in the IC10 mixture assays was 23 µM, which was somewhat close to their predicted $IC50_{mix}$ concentration toward BFCOD (17 µM) calculated based on the relative molar ratios of the inhibitory pharmaceuticals (Table 4). Nevertheless, the mixture assays resulted in substantially lower (than 50%) residual BFCOD activity with plateau at ca. 25% compared with control (Figure 2B). On this basis, it was concluded that the inhibitory impact of the IC10 mixture on BFCOD activity was at least indicatively synergistic in nature. It should also be noted that, similar to IC50 mixtures, the inhibitory effect of the IC10 mixture on the BFCOD activity was less in the beginning, but potentiated over time, even if the time-dependent BFCOD inhibitors were excluded from the IC10 mixture (Figure 2B).

The combined impacts of the pharmaceuticals' mixtures on CYP activities were additionally determined at two different, environmentally relevant concentration levels. Firstly, the pharmaceuticals were mixed in a ratio that mimicked their relative molar fractions in the influents of the wastewater treatment plants (Table 4, Influent mix), estimated based on the UBA database (UBA 2020). When mixed in these molar ratios (uneven inhibition), the predicted inhibitory mixture concentrations for half-maximal enzyme inhibition ($IC50_{mix}$) were 11 µM toward EROD and 1.8 µM toward BFCOD, whereas the total concentration of inhibitory pharmaceuticals in the assays was 0.11 µM, i.e., about 100-fold (EROD) or 10-fold (BFCOD) lower than the predicted $IC50_{mix}$ concentration. Nevertheless, the measured inhibitory effect of the Influent mix was substantial toward both enzyme activities resulting in ca. 75% residual EROD activity and ca. 60% residual BFCOD activity compared with the corresponding controls (Figures

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2C and D). Similar to IC50 and IC10 mixtures, the inhibitory impact of the Influent mix toward BFCOD potentiated over time with and without the time-dependent BFCOD inhibitors, diltiazem and erythromycin (Figure 2D).

Lastly, to examine if the inhibitory effect of the pharmaceuticals' mixture toward BFCOD levels off at low concentrations, the pharmaceuticals were mixed at an equimass ratio of 1ng/L of each of the seven antimicrobials, diltiazem, and verapamil. When mixed in these ratios (uneven inhibition), the predicted inhibitory mixture concentration for half-maximal ($IC50_{mix}$) toward BFCOD was 3.7 µM, whereas the total concentration of BFCOD inhibitors (excluding ciprofloxacin and sulfamethoxazole) was as low as 14 pM (7 ng/L). Nevertheless, the equimass mixture resulted in detectable drop of about 10% in the BFCOD activity, i.e., 90% residual activity compared with control (Figure 2D). In this case, however, there was no significant potentiation of the BFCOD inhibition over time similar to what was observed at higher mixture concentrations.

DISCUSSION

In the present study, we determined the inhibitory impacts on seven widely used, and thus environmentally abundant, antimicrobials on the CYP activity in rainbow trout in vitro. All of the tested antimicrobials are also known to be potent or weak inhibitors of human CYP1A or CYP3A enzymes or both (Table 3). Two of the antimicrobials (clarithromycin and erythromycin) are additionally time-dependent, irreversible inhibitors of human CYP3A (Polasek 2006; Grimm 2009; Burt 2010). All of these antimicrobials have been detected in measurable amounts in the tissues of wild fish, and some also in fish plasma (Table 1), giving reason to believe that they may interfere with the hepatic clearance of other uptaken chemicals via CYP inhibition.

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Apart from ciprofloxacin and sulfamethoxazole (no inhibition) and erythromycin (selective BFCOD inhibition), all other antimicrobials caused nonselective inhibition of both EROD and BFCOD activities in rainbow trout in vitro at concentrations below 500 μM (the maximum concentration used in this study). This result is well in line with the previous findings about broad CYP inhibition by clotrimazole, erythromycin, ketoconazole, and miconazole in rainbow trout in vitro and in vivo (Miranda 1998; Hegelund 2009; Smith 2012; Burkina 2013). However, fairly little is known about the critical inhibitory concentrations (IC50) of these antimicrobials, or their inhibition mechanisms, toward the fish CYP system, which was the scope of the present study. According to our data, most antimicrobials tested were moderate to weak CYP inhibitors (IC50s ranging from 20 to 200 μM) and erythromycin was the only indicatively time-dependent inhibitor of BFCOD, but not EROD, activity in rainbow trout in vitro. In addition, same as with human CYP, two of the method validation compounds, furafylline (CYP1A) and diltiazem (CYP3A), were time-dependent inhibitors of these CYP orthologs in rainbow trout in vitro. From the seven antimicrobials, the strongest CYP inhibition in rainbow trout liver microsomes was observed toward BFCOD/CYP3A by clotrimazole (IC50 1.2±0.2 μM) and ketoconazole (IC50 1.7±0.3 μM), both of which are also strong inhibitors of human CYP3A (Table 3). In addition, ketoconazole was a fairly strong inhibitor of CYP1A in rainbow trout in vitro with IC50 of 9.9±3.4 μM, which is somewhat smaller than that reported for human CYP1A (60±13 μM, Eagling 1998). However, considering that the model substrate used may also have a minor impact on the exact IC50 concentration, it was concluded that the antimicrobials' inhibitory concentrations in rainbow trout in vitro (ER and BFC as model substrates) were overall

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somewhat close to those reported for human CYPs by using FDA approved drugs as the model substrates (Table 3). Nevertheless, our data suggests that direct read-across from human data may not be feasible, because of substantial differences in enzyme selectivity between human and fish, as also previously concluded by others (Smith 2012; Connors 2013; Baron 2017). In addition, the inhibition mechanisms may vary between human and fish, which was the case of verapamil, a time-dependent inhibitor of human, but not fish, CYP3A. These differences may arise, for instance, from environmental factors (pH, temperature) that impact the ionization and binding of the inhibitor to the enzyme's active site, or from functional differences in the active sites between human and fish CYP orthologs.

Compared with measured environmental concentrations of the tested antimicrobials, the IC50 concentrations were generally many orders of magnitude greater (in the range of mg/L) than their reported concentrations in fish plasma (~μg/L, Cerveny 2021) or in surface waters (~ng-μg/L, UBA 2020). However, when incubated as mixtures, together with diltiazem and verapamil, strong cumulative inhibitions of both EROD and BFCOD activities were observed in vitro even at submicromolar (~μg/L) mixture concentrations (Influent mix, Figures 2C and D), i.e., well below the IC50 values of individual compounds. In case of BFCOD, detectable enzyme inhibition (ca. 10%) by the pharmaceutical mixture was observed even at picomolar (~ng/L) mixture concentration. Interestingly, the BFCOD inhibition potentiated over time during the 3-hour enzyme incubation, whereas the EROD inhibition levelled off soon after initiation of reaction. Initially, it was speculated that the potentiation of the BFCOD inhibition results from the time-dependent inhibition of erythromycin and diltiazem toward BFCOD, but not EROD

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(first research hypothesis). This initial hypothesis was, however, abolished by the fact that similar potentiation over time was observed even in the absence of the time-dependent BFCOD inhibitors. Instead, it was re-hypothesized that potentiation of the BFCOD inhibition over time could result from the high affinity (strong inhibition) of ketoconazole and clotrimazole toward rainbow trout CYP3A. It should also be noted that the basal CYP activity also drops over time even without the inhibitors. In the present study, this was accounted for by normalizing the enzyme activities measured with inhibitors at each timepoint to control activities determined at the same timepoints without the inhibitors, so as to eliminate the impact of the inherent CYP activity decrease. From the risk assessment viewpoint, the strong and broad (nonselective) CYP inhibition by ketoconazole as well as its relatively high (predicted) bioconcentration factor (Table 1) and high abundance in the influents of wastewater treatment plants (Table 4, Influent mix) are likely to increase the risk for ketoconazole-induced deficiencies in hepatic clearance of pharmaceuticals in fish in vivo. However, the strong CYP3A inhibition by clotrimazole, together with its extremely high (predicted) bioconcentration factor (Table 1), may also result in substantial interferences in pharmaceuticals' clearances in fish in vivo, even if clotrimazole is much less abundant in the wastewaters compared with ketoconazole (Table 4, Influent mix). It may be hypothesized that the uptake of these two pharmaceuticals in fish in vivo could increase the bioaccumulation of any other pharmaceutical in case their clearances are critically dependent on CYP metabolism. Even if ketoconazole is also known to induce CYP1A expression and activity at low concentrations in vivo, for instance in juvenile rainbow trout, its inhibitory impacts have been reported to dominate at high concentrations (Hegelund 2009). Moreover, the

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inhibitory impacts are usually immediate, whereas enzyme induction typically takes several days, which emphasizes the relevance of more detailed CYP inhibition studies in fish.

To test the second initial hypothesis about the propensity for synergistic CYP inhibition in rainbow trout, we examined whether the observed cumulative inhibition by pharmaceutical mixtures was in accordance with the concentration addition approach (additive inhibition) or whether it was greater than the additive sum of the inhibitory effects of individual pharmaceuticals (synergistic inhibition). For this purpose, the predicted mixture concentrations for half-maximal (50%) inhibition were calculated, based on the concentration addition approach, for pharmaceuticals mixed at different relative molar ratios including IC10 mixtures (even inhibitory potential) and Influent and equimass (each 1 ng/L) mixtures (uneven inhibitory potential). On the basis of IC10 mixtures, it could be concluded that at micromolar concentrations, equivalent to approximately 10% enzyme inhibition of individual pharmaceuticals, the combined inhibitory effects of these pharmaceuticals toward both EROD and BFCOD were clearly synergistic in nature. In the Influent mix, ketoconazole was the dominating inhibitor, with relative molar fraction (p_i) of 0.92, because of its high abundance in the wastewaters (Table 4). Therefore, the predicted inhibitory mixture concentrations ($IC50_{mix}$) of the Influent mix toward EROD and BFCOD activities (11 μM and 1.8 μM, respectively, Table 4) were close to those of ketoconazole's individual IC50 values (Table 3). However, substantial drop in the EROD activity (reaching a plateau at 75% of the control) was observed already at a 100-fold lower total concentration of inhibitory pharmaceuticals (0.11 μM), which gave further evidence of the synergistic inhibitory

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impacts of pharmaceuticals toward CYP1A in rainbow trout in vitro. Even greater drop was observed in BFCOD activity (residual activity ca. 60% of the control) at the same total concentration of the inhibitory pharmaceuticals (0.11 µM). In this case, the predicted $IC50_{mix}$ concentration (1.8 µM) was about 10-fold higher than the concentration used in the experiments. Although our assay design does not allow for direct comparison of the predicted and measured inhibitory effects of pharmaceutical mixtures, the results suggest that the observed CYP inhibition by pharmaceutical mixtures in rainbow trout in vitro is more than the predicted additive sum of the inhibitory effects of individual compounds. Therefore, the propensity for synergistic inhibition of the hepatic CYPs in rainbow trout cannot be ignored, when making predictions of the combined effects of pharmaceuticals in fish. This conclusion was further confirmed with an equimass mixture of pharmaceuticals at 1 ng/L concentration (each), which was considered to represent the low end of the measured environmental concentrations. For this mixture, the predicted half-maximal inhibitory concentration ($IC50_{mix}$) was 3.7 µM (Table 4). Nevertheless, detectable drop of ca. 10% in BFCOD activity compared with control was observed (Figure 2D), even if the total mixture concentration was about five orders of magnitude less (14 pM) compare with the predicted $IC50_{mix}$. These concentrations of pharmaceuticals (ng/L) are already much below the reported plasma concentrations of antimicrobials in wild fish (~µg/L, Cerveny 2021), suggesting that synergistic CYP inhibition could also occur at biologically relevant concentrations.

Overall, our results call for more detailed examination of the combined effects of pharmaceutical mixtures on the fish CYP system. The results also support the previous arguments about the necessity of addressing the mixture effects in the context of

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ecotoxicity risk assessment (Backhaus 2016). Incorporation of more detailed assessment factors that better address not only the additive, but also synergistic effects of pharmaceuticals could also shed light on the occasionally contradictory in vivo findings. For example, erythromycin, which is fairly hydrophilic (logK_{OW} =2.48) and effectively cleared in cultured fish under controlled exposure conditions (Rai 2014) has been shown to bioconcentrate in wild (Muir 2017) and cultured fish when in mixtures (Liu 2017). In human, erythromycin is cleared via demethylation by CYP3A4, suggesting that its elimination in fish could also be dependent on the activity of corresponding fish CYP3A ortholog. According to our data, this CYP3A ortholog in rainbow trout is highly sensitive to synergistic, time-dependent inhibition by pharmaceuticals mixtures in vitro, which could theoretically explain the contradictory findings about the elimination/bioaccumulation of erythromycin in fish.

CONCLUSIONS

In the present study, the inhibitory impacts of seven environmentally ubiquitous antimicrobials on the hepatic CYP1A (EROD) and CYP3A (BFCOD) enzymes were assessed using rainbow trout liver microsomes. Most of the tested antimicrobials (ciprofloxacin, clarithromycin, clotrimazole, ketoconazole, and miconazole) caused nonselective inhibition of both enzyme activities in rainbow trout in vitro, which was cumulative, when the antimicrobials were incubated as mixtures (including also the method validation compounds diltiazem and verapamil). The fact that cumulative inhibition was also observed at mixture concentrations well below the IC50 values of individual compounds suggests that the CYP inhibition by these pharmaceuticals in rainbow trout is likely synergistic in nature and thus of biological relevance considering

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the high environmental abundance of antimicrobials and their proven uptake in wild fish in vivo.

Interestingly, the combined inhibitory effect of pharmaceutical mixtures toward rainbow trout BFCOD activity was shown to potentiate over time (during a 3-hour experiment), whereas the cumulative inhibition of EROD activity levelled off soon after initiation of the experiment. Even if some of the tested pharmaceuticals (erythromycin and the method validation compound, diltiazem) were time-dependent inhibitors of BFCOD in rainbow trout in vitro, the potentiation of the BFCOD inhibition could not be explicitly associated with these compounds. Instead, it was hypothesized that the potentiation of BFCOD inhibition by pharmaceutical mixtures could result from the high affinity (strong inhibition) of ketoconazole and clotrimazole toward rainbow trout CYP3A. This together with the propensity for synergistic CYP inhibition in vitro could theoretically result in strong, time-dependent inhibition of fish CYP3A in vivo and explain some of the previously reported contradictions with respect to bioaccumulation of CYP3A-cleared pharmaceuticals (such as erythromycin) in wild fish exposed to a vast number of environmental pharmaceutical residues. Overall, our data emphasizes the need for more detailed, mechanism-based CYP inhibition studies in the context of the ecotoxicity risk assessment of pharmaceutical mixtures in fish.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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FIGURE CAPTIONS

Figure 1. The Michaelis-Menten kinetic constants of (A) 7-ethoxyresorufin O-deethylation (EROD) and (B) 7-benzyloxy-4-trifluoromethyl coumarin O-deethylation (BFCOD) activities in rainbow trout liver microsomes (*Oncorhynchus mykiss*): enzyme affinity (K_M) and maximal velocity (V_{MAX}). ER=7-ethoxyresorufin; BFC=7-benzyloxy-4-trifluoromethyl coumarin. The error bars represent the variation between n=3 parallel incubations at each concentration level (technical replicates).

Figure 2. Normalized, residual 7-ethoxyresorufin O-deethylation (EROD) and 7-benzyloxy-4-trifluoromethyl coumarin O-deethylation (BFCOD) activities in rainbow trout liver microsomes (*Oncorhynchus mykiss*) incubated simultaneously with seven antimicrobials and two other pharmaceuticals at different mixture concentrations and relative fractions for 180 min, as indicated in Tables 4 and S3. The error bars represent the variation between n=3 or 4 parallel incubations (technical replicates). (A) Residual EROD activities in IC50 and IC10 mixtures including all nine pharmaceuticals. The concentrations of individual pharmaceuticals in these mixtures were equivalent to their half-maximal (IC50) inhibitory concentrations (Table S3) or the concentrations that resulted in ca. 10% decrease in the enzyme activity (IC10 mix, Table 4), respectively. (B) Corresponding residual BFCOD activities in IC50 and IC10 mixtures over time. In this case, all nine pharmaceuticals were included in the IC50 mix (Table S3), but the time-dependent inhibitors (TDIs) of BFCOD, diltiazem and erythromycin, were excluded from the IC10 mixture (Table 4). (C) Residual EROD activity in artificial Influent mixture

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including all nine pharmaceuticals. In this mixture, the concentrations of individual pharmaceuticals were equivalent to their average influent concentrations at the wastewater treatment plants based on the UBA database and their total molar concentration was 0.11 µM. (D) Residual BFCOD activities in the similarly prepared artificial Influent mixtures, including all nine pharmaceuticals (Influent mix w/ TDIs) and excluding the time-dependent inhibitors of BFCOD (Influent mix w/o TDIs), and residual BFCOD activity in a pharmaceuticals' mixture including all nine pharmaceutical at 1 ng/L concentration each (total molar concentration 14 pM).

Graphical abstract. Synergistic and time-dependent inhibition of cytochrome P450 3A activity by antimicrobials' mixtures was observed in rainbow trout liver microsomes in vitro even at trace-level concentrations.

Table 1. Overview of the pharmaceuticals examined in the present study including their physicochemical properties affecting bioavailability in fish, the predicted bioconcentration factors, their tissue and plasma concentrations in different fish species as reported in the previous studies, and their primary mode of action.

	Physicochemical properties [a]				**Bioconcentration factor (predicted)** [b]	**Reported tissue concentrations in vivo (literature)**	**Mode of action** [c]
	pK_a	$LogK_{OW}$	Log D (pH 8.0)	Water solubility (mg/L, pH 8.0)			
Antifungals							
Clotrimazole (344.8 g/mol)	6.26 (b)	5.84	5.83	0.7	436-469	*Oncorhynchus mykiss* (cultured): liver 1140±700 ng/g, kidney 110±40 ng/g (Burkina 2016); *Rutilius rutilus* (wild) plasma 0.66±0.613 µg/L (Cerveny 2021); *Squalius cephalus* (wild): plasma 0.73±0.727 µg/L (Cerveny 2021)	Fungal CYP (lanosterol 14α-demethylase) inhibitors

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Ketoconazole (531.4 g/mol)	6.42 (b)	4.19	4.18	1.0	35.8	*Carassius auratus* (cultured): liver 40-60 ng/g, muscle 20-25 ng/g, brain 10-15 ng/g, gill ~10 ng/g (Liu 2016)	
Miconazole (416.1 g/mol)	6.48 (b)	5.96	5.95	0.3	1 240	*Rutilius rutilus* (wild): plasma 0.93±2.108 µg/L (Cerveny 2021)	
Antibiotics							
Ciprofloxacin (331.3 g/mol)	5.56 (a) 8.77 (b)	1.24	-0.91	1 890	2.90-11.0	*Ctenopharyngodon idella* (cultured): liver ~50 µg/kg, bile ~100 µg/L (Chen 2018)	Bacterial DNA gyrase/topo-isomerase inhibitor
Clarithromycin (748.0 g/mol)	12.46 (a) 9.00 (b)	3.24	2.20	31 280	4.08	*Squalius cephalus* (wild): plasma 2.44±3.4 µg/L (Cerveny 2021)	Bacterial 23S ribosomal RNA inhibitors
Erythromycin (733.9 g/mol)	12.45 (a) 9.00 (b)	2.6	1.55	43 270	3.37	*Cyprinus carpio* (wild): plasma 0.35-0.79 ng/g (Muir 2017); *Carassius auratus* (cultured): muscle ≤252.7 ng/g, gill ~10 ng/g (Liu 2014)	
Sulfamethoxazole (253.3 g/mol)	6.16 (a) 1.97 (b)	1.24	-0.91	1 890	2.74-17.1	*Ctenopharyngodon idella* (cultured): liver ~15 µg/kg, bile ~25 µg/L, muscle 50 µg/kg (Chen 2018)	Bacterial dihydrofolic acid synthesis inhibitor
Validation compounds							
Furafylline (260.3 g/mol)	8.27 (a) -0.26 (b)	3.06	-0.02	7 320	3.23-4.14	n/a	Selective human CYP1A2 inhibitor
Diltiazem (414.5 g/mol)	12.86 (a) 8.18 (b)	2.73	2.33	20	6.32-10.7	*Oncorhynchus mykiss:* Kidney 9.7 ± 4.2 µg/kg (Steinbach et al. 2016). *Micropterus salmoides:* Liver 0.7 ng/g (Ramirez et al. 2009)	Selective human CYP3A4 inhibitors; Calcium channel inhibitors
Verapamil (454.6 g/mol)	9.68 (b)	5.05	3.36	1 400	197-201; experim. 6.6-16.6 (Steinbach 2013)	*Ictalurus punctatus:* liver ~100 ng/g, kidney ~500 ng/g, gill ~100 ng/g (Nallani et al. 2016)	

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[a] The physicochemical properties, including acid dissociation constants (pKa) for the strongest acidic (a) and basic (b) functional groups, water-oil partitioning coefficients (LogK_{OW}) and distribution coefficients at pH 8.0 (Log$D$), and water solubilities at pH 8.0, were derived from ChemAxon Chemicalize database, [b] the predicted bioconcentration factors from CompTox Chemicals Dashboard (comptox.epa.gov), and [c] the modes of action from DrugBank database (drugbank.com).
n/a=not available.

Table 2. Optimized enzyme incubation conditions used for determination of EROD and BFCOD activities in rainbow trout (*Oncorhynchus mykiss*) liver microsomes in vitro.

Model activity	**EROD assays**	**BFCOD assays**
Microsomal protein	0.5 mg/mL	0.5 mg/mL
Model substrate	7-ethoxy resorufin (ER), 1 µM	7-benzyloxy-4-trifluoromethyl coumarin (BFC), 75 µM
Cofactor	NADPH[a], 1 mM	NADPH[a], 2 mM
Incubation time	10 min	20 min
Incubation buffer	0.1M potassium phosphate (pH 8.0) / dimethyl sulfoxide (99.5:0.5, v/v)	0.1M potassium phosphate (pH 8.0) / acetonitrile (98:2, v/v)
Temperature	21±1°C	21±1°C
Metabolite	Resorufin	7-hydroxy-4-trifluoromethyl coumarin (HFC)
Detection wavelengths (excitation/emission)	570 nm / 595 nm	419 nm / 501 nm

NADPH = β-Nicotinamide adenine dinucleotide 2′-phosphate; EROD=7-ethoxyresorufin O-deethylation; BFCOD=7-benzyloxy-4-trifluoromethylcoumarin O-debenzylation.

Table 3. The half-maximal inhibitory constants (IC50) of the test pharmaceuticals toward CYP1A and CYP3A in rainbow trout (*Oncorhynchus mykiss*) liver microsomes (present study), and in human as reported in scientific literature. The IC50 concentrations in rainbow trout liver microsomes were determined in accordance with IC50 shift assay

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using 95% confidence level, using EROD (CYP1A) and BFCOD (CYP3A) as the model activities. The CYP inhibition mode was determined based on the ratio of IC50 concentrations obtained without (-) and with (+) preincubation of the test pharmaceuticals with the CYP cofactor (NADPH) prior to addition of the model substrate. IC50 shift ≥1.5 was used as the threshold for time-dependent inhibition.

Half maximal inhibitory concentrations (IC50, µmol/L)	**CYP1A**			**CYP3A**		
	Rainbow trout (EROD)		**Human[a]**	**Rainbow trout (BFCOD)**		**Human[a]**
Validation compounds						
Diltiazem	78 ± 32 (-) nd (+)	Shift: -	n/a	130 ± 23 (-) 91 ± 19 (+)	**Shift:** **1.5±0.4**	110-127 (TDI)
Furafylline	140 ± 45 (-) 23 ± 12 (+)	**Shift:** **5.9 ± 3.6**	0.48 ± 0.23 (irreversible)	170 ± 45 (-) 370 ± 230 (+)	Shift: 0.5±0.3	>100
Verapamil	66 ± 32 (-) 70 ± 12 (+)	Shift: 0.9 ± 0.5	n/a	31 ± 2 (-) 34 ± 2 (+)	Shift: 0.9±0.1	23-26 (TDI)
Antifungals						
Clotrimazole	71 ± 37 (-) 92 ± 63 (+)	Shift: 0.8 ± 0.7	n/a	1.2 ± 0.2 (-) 1.3 ± 0.1 (+)	Shift: 0.9±0.2	0.18 (reversible)
Ketoconazole	9.9 ± 3.4 (-) 17.2 ± 8.3 (+)	Shift: 0.6 ± 0.3	60 ± 13 (reversible)	1.7 ± 0.3 (-) 2.2 ± 0.3 (+)	Shift: 0.8±0.1	0.0117-1.2 (reversible)
Miconazole	54 ± 8 (-) 58 ± 19 (+)	Shift: 0.9 ± 0.3	2.9 (reversible)	42 ± 12 (-) 43 ± 12 (+)	Shift: 1.0±0.4	1.7 ± 0.1 (reversible)
Antibiotics						
Ciprofloxacin	>500 (-) >500 (+)	Shift: -	220 ± 80	>500 (-) nd (+)	Shift: 0.2±0.1	n/a
Clarithromycin	110 ± 40 (-) nd (+)	Shift: -	n/a	29 ± 6 (-) 34 ± 10 (+)	Shift: 0.9±0.3	56 ± 5 (TDI)
Erythromycin	nd (-) nd (+)	Shift: -	n/a	69 ± 21 (-) 47 ± 17 (+)	**Shift:** **1.5±0.7**	33 ± 6 (TDI)
Sulfamethoxazole	nd (-) nd (+)	Shift: -	n/a	>500 (-) nd (+)	Shift: -	n/a

[a] Human IC50 values, and CYP inhibition modes from Burt 2010, Eagling 1998, Godamudunage 2018, Grimm 2009, Karjalainen 2008, Niwa 2005, Quinney 2010, Sakaeda 2005, Wen 2002, Yeo 2001, Zhao 1999, and Zimmerlin

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CYP=cytochrome P450; NAPDH=β-Nicotinamide adenine dinucleotide 2-phosphate; EROD=7-ethoxyresorufin O-deethylation; BFCOD=7-benzyloxy-4-trifluoromethylcoumarin O-debenzylation; TDI=time-dependent inhibitor; nd=not detected; n/a=not available.

Table 4. The concentrations of pharmaceuticals used in the mixture assays and the predicted, theoretical mixture concentrations for half-maximal inhibitory concentrations ($IC50_{mix}$) by these mixtures toward EROD and BFCOD.

IC10 mixture	**Concentration**	**EROD assays [a]**		**BFCOD assays [b]**	
	(μM)	**p_i**	**$p_i/IC50_i$ (μM^{-1})**	**p_i**	**$p_i/IC50_i$ (μM^{-1})**
Pharmaceutical					
Diltiazem [d]	(10) [d]	0.303	3.9E-03	*not included (TDI of BFCOD)*	
Verapamil	2	0.061	9.2E-04	8.7E-02	2.8E-03
Clotrimazole	0.5	0.015	2.1E-04	2.2E-02	1.8E-02
Ketoconazole	0.5	0.015	1.5E-03	2.2E-02	1.3E-02
Miconazole	10	0.303	5.6E-03	4.3E-01	1.0E-02
Ciprofloxacin [c]	100	*not EROD inhibitor*		*not BFCOD inhibitor*	
Clarithromycin	10	0.303	2.8E-03	4.3E-01	1.5E-02
Erythromycin [c,d]	(50) [d]	*not EROD inhibitor*		*not included (TDI of BFCOD)*	
Sulfamethoxazole [c]	100	*not EROD inhibitor*		*not BFCOD inhibitor*	
Total conc. of inhibitory pharmaceuticals [c,d]			**33 μM**		**23 μM**
Predicted inhibitory conc. (IC50mix) [c,d]		**$\Sigma (p_i/IC50_i)^{-1}$**	**67 μM**	**$\Sigma (p_i/IC50_i)^{-1}$**	**17 μM**

Influent mixture	**Concentration**	**EROD assays [a]**		**BFCOD assays [b]**	
	(nM)	**p_i**	**$p_i/IC50_i$ (μM^{-1})**	**p_i**	**$p_i/IC50_i$ (μM^{-1})**
Pharmaceutical					
Diltiazem [d]	(0.4) [d]	1.3E-02	1.6E-04	3.7E-03	2.8E-05
Verapamil	0.4	3.7E-03	5.6E-05	3.7E-03	1.2E-04
Clotrimazole	0.03	2.3E-04	3.2E-06	2.3E-04	1.9E-04
Ketoconazole	100	9.2E-01	9.3E-02	9.2E-01	5.4E-01
Miconazole	7	6.4E-02	1.2E-03	6.5E-02	1.5E-03
Ciprofloxacin [c]	11	*not EROD inhibitor*		*not BFCOD inhibitor*	
Clarithromycin	0.3	2.7E-03	2.5E-05	2.8E-03	9.5E-05
Erythromycin [c,d]	(0.3) [d]	*not EROD inhibitor*		2.8E-03	4.0E-05
Sulfamethoxazole [c]	910	*not EROD inhibitor*		*not BFCOD inhibitor*	
Total conc. of inhibitory pharmaceuticals [c,d]			**0.11 μM**		**0.11 μM**
Predicted inhibitory conc. (IC50mix) [c,d]		**$\Sigma (p_i/IC50_i)^{-1}$**	**11 μM**	**$\Sigma (p_i/IC50_i)^{-}$**	**1.8 μM**

Equimass 1 ng/L mix	**Concentration**	**BFCOD assays [b]**	
	(pM)	**p_i**	**$p_i/IC50_i$ (μM^{-1})**
Pharmaceutical			
Diltiazem	2.5	1.7E-01	1.3E-03
Verapamil	2.1	1.4E-01	4.6E-03
Clotrimazole	3.0	2.1E-01	1.7E-01
Ketoconazole	2.0	1.4E-01	8.2E-02

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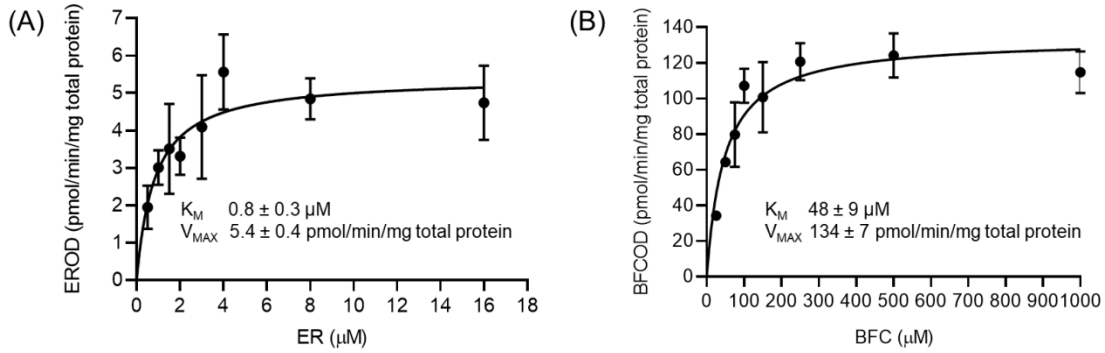
Miconazole	2.1	1.5E-01	3.5E-03
Ciprofloxacin [c]	3.3	*not EROD inhibitor*	
Clarithromycin	1.4	9.4E-02	3.3E-03
Erythromycin	1.4	9.6E-02	1.4E-03
Sulfamethoxazole [c]	4.0	*not EROD inhibitor*	

Total conc. of inhibitory pharmaceuticals [c]		**14 pM**
Predicted inhibitory conc. (IC50mix) [c]	**$\Sigma (p_i/IC50_i)^{-1}$**	**3.7 µM**

[a,b] The predicted $IC50_{mix}$ concentrations were calculated according to Eq. 4 by dividing the relative molar fraction (p_i) by the pharmaceuticals' inhibitory concentrations ($IC50_i$) toward EROD[a] and BFCOD[b] as reported in Table 3.
[c] Some pharmaceuticals did not inhibit the EROD (ciprofloxacin, erythromycin, and sulfamethoxazole) and BCFOD (ciprofloxacin, sulfamethoxazole) activities at concentrations below 500 µM and thus, they were not included in the total concentration of inhibitory pharmaceuticals nor in the calculation of the predicted (theoretical) inhibitory concentrations of the mixtures.
[d] Diltiazem and erythromycin were time-dependent inhibitors of BFCOD (IC50 shift ≥1.5) and therefore they were excluded in some of the BFCOD mixture assays (IC10 and Influent) to be able to examine their impact on the potentiation of the BFCOD inhibition by pharmaceutical mixtures.
EROD=7-ethoxyresorufin O-deethylation; BFCOD=7-benzyloxy-4-trifluoro-methylcoumarin O-debenzylation.



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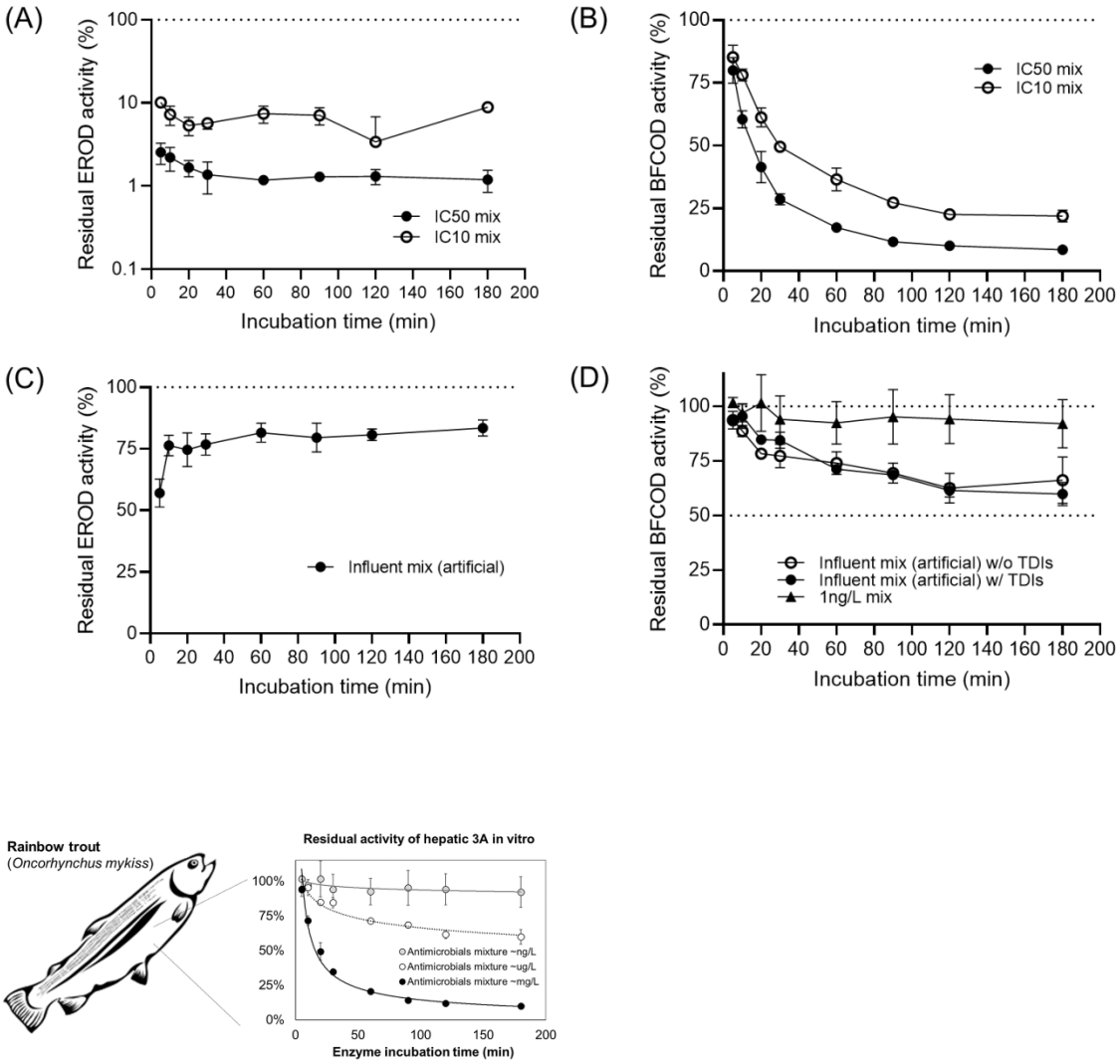

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