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Ibuprofen Metabolism in the Liver and Gill of Rainbow Trout, *Oncorhynchus mykiss*

C. F. Gomez · L. Constantine · M. Moen · A. Vaz · W. Wang · Duane B. Huggett

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Abstract The presence of pharmaceuticals in the environment has become an important topic of discussion with respect to pharmaceutical absorption, metabolism and elimination in fish. This study investigates the metabolism of ibuprofen by rainbow trout (*Oncorhynchus mykiss*). In vitro metabolic loss of parent compound was measured in gill and liver S9 and microsomal fractions. Metabolite analysis found 2-hydroxy-ibuprofen as the major metabolite in uninduced S9 fractions. Supplementing S9 fractions with UDPGA did not significantly enhance metabolism. Additionally, assays involving the induction and inhibition of specific CYP isozymes support CYP1A2 as a possible metabolic pathway in fish.

Keywords Metabolic biotransformation · In vitro tests · Pharmaceuticals · Ibuprofen

Ibuprofen is a nonsteroidal anti-inflammatory drug (NSAID) found in over-the-counter and prescription pharmaceuticals. Ibuprofen is typically administered as a racemic mixture of (S)-(+)- and (R)-(−)-enantiomers [Rodrigues 2005], with (R)-(−)-ibuprofen undergoing unidirectional chiral inversion to (S)-(+)-ibuprofen [Davies, 1998]. Both enantiomers are readily metabolized through Phase I and Phase II pathways. In human and rat liver, cytochrome P450 (CYP) 2C9 and 2C8 contribute to the majority of Phase I biotransformation of ibuprofen [Jacqz-Aigrain and Anderson 2006]. Uridine diphosphate glucuronyl-transferase (UGT) 1A9, 1A3, 2B1 and 2B7 are known to conjugate ibuprofen [Ritter 2000]. Studies have shown direct glucuronidation to account for approximately 14% of (S)-(+)-ibuprofen metabolism with 2-hydroxy-ibuprofen (2-OH-IBP) and 3-hydroxy-ibuprofen (3-OH-IBP) metabolites accounting for 28% and 45%, respectively in human microsomes [Rudy et al. 1991]; [Davies 1998]; [Rodrigues 2005]. 3-OH-IBP is quickly biotransformed to 3-carboxy ibuprofen via cytosolic dehydrogenases [Rudy et al. 1991]; [Davies 1998]. As fish do not contain the same CYP2C isoforms as mammals [Buhler and Wang-Buhler 1998], it cannot be assumed that piscine biotransformation will be equivalent to that of mammals.

Aquatic organisms may be exposed to chronic, low doses of ibuprofen in the environment. Ibuprofen has been identified in the environment with concentrations observed as high as 4,239 ng L^{-1} in wastewater effluent and 2,370 ng L^{-1} in surface water [Ternes 2001]; [Roberts and Thomas 2006]. Toxicity studies with *Daphnia magna* indicate 14-day EC_{50} concentrations at several orders of magnitude greater than environmentally relevant concentrations (survival 80 mg L^{-1}; reproduction 13.4 mg L^{-1}) [Heckmann et al. 2007]. Acute toxicity studies in fish reflect similar results [Kim et al. 2009]. However, other species appear to be more sensitive, such as the cnidarian *Hydra attenuata*, which has a calculated toxicity threshold of 320 μg L^{-1} [Quinn et al. 2008]. Thus, understanding the ability and mechanism of organisms to metabolize ibuprofen will assist in further elucidating the effects of ibuprofen in the aquatic environment.

Materials and Methods

Chemicals used in the following studies were purchased from Sigma–Aldrich Corp. (St. Louis, MO) unless noted

C. F. Gomez · D. B. Huggett (✉)
Department of Biological Sciences, University of North Texas, 1704 West Mulberry, Room 215, Denton, TX 76208, USA
e-mail: dbhuggett@unt.edu

L. Constantine · M. Moen · A. Vaz · W. Wang
Pfizer Global Research and Development, Eastern Point Road, Bldg 118D/D218, Groton, CT 06340, USA

below. Alamethicin and uridine diphosphate glucuronic acid (UDPGA) were generously provided by Pfizer Inc. (Groton, CT). The Bradford Protein Assay was procured from Bio-Rad Laboratories (Hercules, CA). Uninduced, male Sprague–Dawley rat liver S9 and uninduced, male CD-1 mouse S9 were purchased from Moltox (Boone, NC). Ibuprofen and d^3-ibuprofen were sourced from Toronto Research Chemicals (North York, ON, Canada).

Gill and liver tissue were harvested from anesthetized sexually mature male rainbow trout (Greers Ferry National Fish Hatchery, Heber Springs, AR). Maturity was determined via visual inspection. Fish were maintained in dechlorinated tap water at 16°C in Frigid Units Living Streams under fluorescent lights with a 16 h light/8 h dark photoperiod. Fish were fed ground Purina Trout Chow. All studies were conducted in accordance with University of North Texas animal use protocols.

For uninduced fractions, tissues from five to ten fish were composited and homogenized on ice using an electric homogenizer. The homogenate was centrifuged at 9,000 g for 20 min at 4°C. The S9 supernatant was carefully separated from the pellet and stored at −80°C. To obtain microsomal fractions, S9 was centrifuged at 100,000 g for 60 min at 4°C. The supernatant was discarded and the pellet resuspended in homogenization buffer. For induced fractions, rainbow trout (n = 3) were injected with beta-naphthoflavone (BNF, 100 mg (kg bw)$^{-1}$) and sacrificed after 24-hours. Microsomal fractions were prepared from the composited gill and liver tissue.

S9 and microsomal fractions were diluted to 2.0 and 1.0 mg protein mL^{-1} with 0.01 M phosphate buffer (pH 7.4), respectively. The Bradford Protein Assay determined the protein content of each sample. Samples (650 μL total volume) included an NADPH regeneration system and 10 μM ibuprofen. Samples were run in duplicate or triplicate. S9 matrix controls and solvent controls were run with each assay to assure loss of parent was not due to binding effects. Samples were equilibrated for 10 min (trout at 15°C; mammalian at 37°C) in a temperature controlled shaker. To initiate the reactions, 6.5 μL of 50 mM NADPH in phosphate buffer was added to each sample. Samples were then incubated for 60–120 min. Aliquots of 100 μL were removed throughout the course of the incubation and placed in equal volume cold methanol to stop biological activity. A deuterated internal standard was then added. Samples were centrifuged at 2,500 g for 10 min to pellet the denatured protein. The supernatant was then stored at 4°C until instrumental analysis. GC/MS was used to analyze loss of parent compound (see below).

To further investigate UGT specific activity, the above procedure was modified to include the addition of the cofactor UDPGA. Alamethicin was added at 50 μg mg^{-1} protein to S9 samples prior to equilibration. To initiate the reaction, 5 mM UDPGA was added to the sample along with NADPH. To evaluate the influence of Phase I biotransformation on ibuprofen metabolism, alpha-naphthoflavone (ANF), a CYP1A2 inhibitor, was added prior to incubation at 10 μM to BNF-induced microsomes. Uninhibited samples were run for comparison. Samples of uninduced S9 and induced, uninhibited microsomes were collected after 60 min for metabolite analysis. Metabolites were analyzed via LC/MS (see below).

For GC/MS analysis, samples were derivatized with boron trifluoride (BF3)-methanol and analyzed with an Agilent 6890N Network GC System coupled with an Agilent 5973 inert Mass Selective Detector and an Agilent autosampler (Agilent Technologies, Inc.). Analytes were detected and quantified in selective ion monitoring (SIM) mode with the quantitative ions 161 m/z for ibuprofen and 164 m/z for deuterated ibuprofen. For LC/MS analysis, chromatography was performed with an Agilent solvent delivery system and autosampler on a Phenomenex Synergi Hydro RP-C18 (4.6 mm x 150 mm, 4 μm) column. Metabolite identification was performed using a Thermo LTQ equipped with an electrospray interface operating in negative ion mode (Thermo Electron).

Statistical analysis was conducted using GraphPad Prism 5 (GraphPad Software, Inc.). Loss of parent data was found to be homoscedastic within each S9 and microsomal fraction type (i.e., trout liver S9, etc.). Two-way repeated measures ANOVA confirmed statistical significance of loss of parent compound over time while Bonferroni post tests were used to evaluate the differences between tissue homogenate batches at each sample time point. CL_m values were analyzed either via an unpaired t-test or one-way ANOVA with Bonferroni's multiple comparison post test. Previous studies have shown that compositing tissue reduces the variability in metabolic activity that may be found between individual fish [Gomez et al. 2010]. The uninduced S9 and microsomes used in these studies come from the same pools of liver and gill tissues which have been used to evaluate metabolic activity of other compounds with confidence. Thus, although some assays are replicates of single tissue pools, it is assumed that their activity is in line with that of other similarly prepared tissue composites.

Results and Discussion

In the S9 metabolism study, first order kinetics was assumed for each reaction. Remaining parent compound (C) was plotted versus time (Fig. 1). Loss of ibuprofen parent compound was observed in all trials, indicating the occurrence of metabolism.

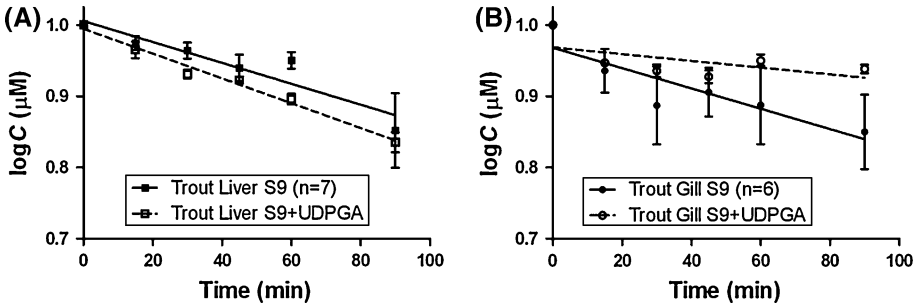

Fig. 1 Loss of parent compound as a function of incubation time in trout liver and gill S9 in the presence and absence of UDPGA cofactor. Mean and standard error displayed. n indicates number of tissue pools assayed. Assays including UDPGA were performed with a single pool of composited tissues

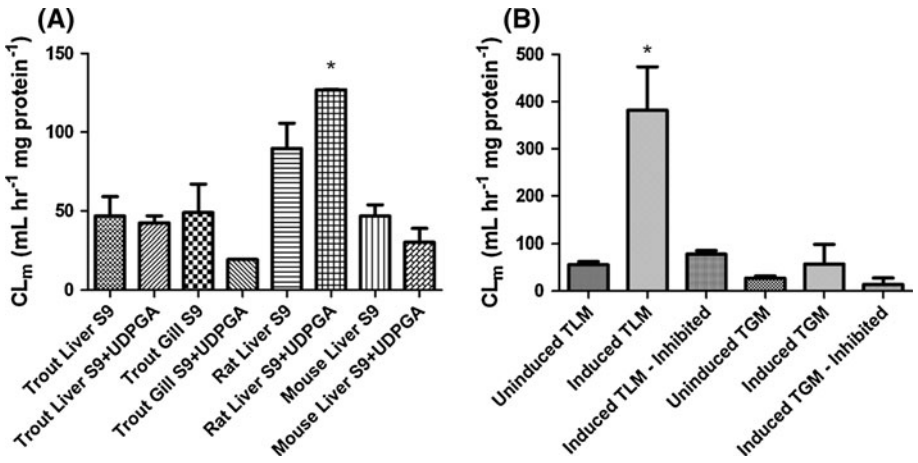

Fig. 2 **a** Mean and standard error of CL_m of uninduced S9 fractions. CL_m of rat liver S9+UDPGA was significantly greater (*) than other tissue fractions +UDPGA. Significance was not found between species or tissue types in samples without cofactor. **b** Mean and standard error of CL_m of BNF-induced, ANF-inhibited and uninduced/uninhibited trout liver microsomes (TLM) and trout gill microsomes (TGM). CL_m of BNF-induced TLM was significantly greater (*) than all other tissue fractions

The intrinsic clearance rate (CL_m, mL hr^{-1} (mg protein)$^{-1}$) was calculated from loss of parent material normalized to protein content, according to the equation based upon [Han et al. (2009)]: CL_m = [Rate loss of parent (μmol hr^{-1} (mg protein)$^{-1}$) * Vol of test solution (mL)]/ Initial parent concentration (μmol). Initial parent concentrations were analytically confirmed and not based on nominal concentrations.

Significant differences in CL_m were not found between species or tissue type of uninduced S9 fractions without UDGPA (Fig. 2a). Rat liver CL_m, on average, were higher than trout or mouse CL_m. With addition of cofactor, rat liver CL_m was significantly greater than other tissue fractions with UDPGA. Metabolite analysis of S9 samples at test termination identified 2-OH-IBP as the primary metabolite in all samples (Fig. 3a). 3-OH-IBP was not detected as it was most likely converted to 3-carboxy ibuprofen during incubation [Chang et al. 2008]. A significant difference was not observed between the relative frequency of 2-OH-IBP from mouse and trout liver S9. However, the relative frequency of 2-OH-IBP produced by trout gill S9 was significantly more than mouse liver S9.

Glucuronide metabolites were not detected in the trout gill S9 samples. This indicates that direct glucuronyl conjugation may not play a major role in biotransformation in gill tissues. Moreover, the addition of UDPGA did not significantly increase CL_m. As UDPGA is often found at levels below the K_m of UGTs in uninduced environments [Ritter 2000], CLm was expected to increase with the addition of the cofactor. The lack of a significant increase reinforces that glucuronidation is not a major ibuprofen pathway in the gill. Glucuronide metabolites were found in mouse liver S9 at expected relative frequencies [Chang et al. 2008]. No significant difference was found between the relative frequency of trout liver and mouse liver S9 glucuronide metabolites.

As Phase I biotransformation appears to be the major pathway in ibuprofen metabolism, studies with induced and/or inhibited microsomes were conducted to further delineate the mechanism behind trout biotransformation of

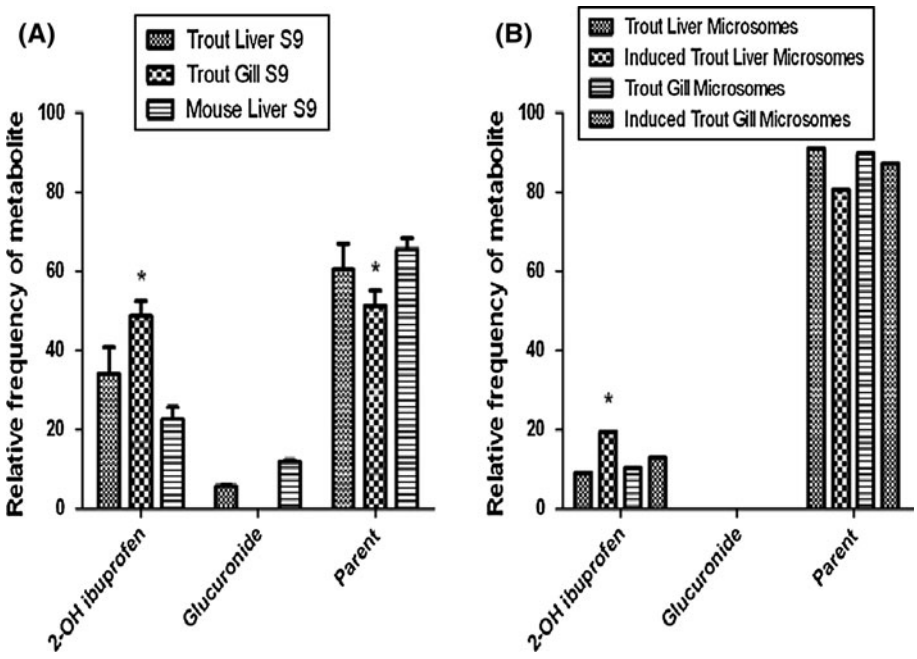

Fig. 3 Relative frequencies of ibuprofen and its metabolites. **a** Significant differences (*) noted between fish tissue fraction and mouse tissue fraction, indicating potential difference in metabolic mechanism of trout gill and rat liver. **b** Significant increase (*) in 2-OH metabolite formation and significant decrease (*) of parent compound observed in BNF-induced trout liver microsomes versus uninduced trout liver microsomes

ibuprofen. Metabolic activity was greatly enhanced in BNF-induced trout liver microsomes (mean 19.6% loss of parent compound within 20 min). This may be due to increased CYP1A activity [Buhler and Wang-Buhler 1998]. Metabolite analysis indicated 2-OH-IBP levels in BNF-induced liver microsomes to be double that of uninduced liver microsomes (Fig. 3b). Glucuronide metabolites were not detected in induced liver microsomes. Moreover, ANF inhibited metabolic activity in induced liver microsomes (Fig. 2b). The remaining activity is assumed to be glucuronide conjugation. BNF did not enhance metabolic activity in gill microsomes, nor did ANF significantly inhibit activity. Metabolite analysis found similar relative frequencies of 2-OH-IBP in gill microsomes, while glucuronide metabolites were not detected as in gill S9 fractions.

The enhanced metabolism in BNF-induced microsomes and subsequent inhibition by ANF suggests a fish CYP1A homolog, rather than a CYP2C homolog, as a metabolic pathway for ibuprofen. Naproxen, another NSAID, is known to undergo CYP1A2 biotransformation along with CYP2C9 in humans [Miners et al. 1996]; [Rodrigues et al. 1996]. In fish, CYP1A may oxidize ibuprofen in a similar manner as naproxen is metabolized in humans.

These data suggest that ibuprofen can be metabolized in fish at similar rates and with similar end metabolites as mammals. The intrinsic clearance in mouse and rat liver S9 factions was not significantly different than trout liver CL_m. The mechanism responsible for ibuprofen metabolism may be different (e.g. CYP1A) than known mammalian mechanisms (e.g. CYP2C9), yet it may be feasible to read-across some information from mammalian literature since intrinsic clearance rates were similar.

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