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Exposure to the synthetic FXR agonist GW4064 causes alterations in gene expression and sublethal hepatotoxicity in eleutheroembryo medaka (*Oryzias latipes*)

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Abstract

The small freshwater teleost, medaka (*Oryzias latipes*), has a history of usage in studies of chronic toxicity of liver and biliary system. Recent progress with this model has focused on defining the medaka hepatobiliary system. Here we investigate critical liver function and toxicity by examining the *in vivo* role and function of the farnesoid X receptor alpha (FXR α , NR1H4), a member of the nuclear receptor superfamily that plays an essential role in the regulation of bile acid homeostasis. Quantitative mRNA analysis of medaka FXR α demonstrates differential expression of two FXR α isoforms designated Fxr α 1 and Fxr α 2, in both free swimming medaka embryos with remaining yolk (eleutheroembryos, EEs) and adults. Activation of medaka Fxr α *in vivo* with GW4064 (a strong FXR α agonist) resulted in modification of gene expression for defined FXR α gene targets including the bile salt export protein, small heterodimer partner, and cytochrome P450 7A1. Histological examination of medaka liver subsequent to GW4064 exposure demonstrated significant lipid accumulation, cellular and organelle alterations in both hepatocytes and biliary epithelial cells of the liver. This report of hepatobiliary injury following GW4064 exposure extends previous investigations of the intrahepatic biliary system in medaka, reveals sensitivity to toxicant exposure, and illustrates the need for added resolution in detection and interpretation of toxic responses in this vertebrate.

Keywords

Farnesoid X receptor; Liver; Medaka; Toxicity; Eleutheroembryo

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Introduction

The medaka (*Oryzias latipes*), a small aquarium fish, is being increasingly used in developmental biology (Shima and Mitani, 2004; Takeda, 2008); mutagenesis (Winn et al., 2005); carcinogenesis (Hawkins et al., 1990; Teh and Hinton, 1993; Hawkins et al., 1995, 1998; Teh and Hinton, 1998; Reddy et al., 1999; Hawkins et al., 2003); and as an OECD screen for compounds with endocrine disruptive potential (Kinoshita et al., 2009; Padilla et al., 2009). Recent work analyzing mechanisms of toxicity with medaka and other model fishes has been reviewed (Hahn and Hestermann, 2008; Hinton et al., 2009). Briefly, this work involves two distinct rationales, in which fish may serve as targets or as models. With respect to the former, epizootics of fish liver cancer, reported in contaminated harbors (Myers et al., 1998) or at specific historical, industrial sites (Vogelbein et al., 1990) pointed to degradation of environmental quality and led to development of biomarkers of effect which were later used to verify remediation processes (Johnson et al., 2008). In addition, responses to low concentrations of endocrine disrupting compounds (EDCs) in surface waters illustrate the important roles of fish as environmental sentinels. As animal models in toxicological research, the ultimate goal is to answer questions concerning the potential human health effects of chemical exposure (Hinton et al., 2009). Both field observations and mechanistic laboratory experiments are essential to unravel the complexities of these risks (see review by Hahn and Hestermann, 2008).

Using both comparative genomics and conventional molecular approaches, our laboratory has developed methodologies to screen, clone and assess molecular function of nuclear receptors (NRs) in teleost fish. Recently we identified and characterized two isoforms of the farnesoid X receptor alpha (FXR α , NR1H4), a member of the nuclear receptor superfamily that plays an essential role in the regulation of bile acid homeostasis (unpublished observations this laboratory). Extensive investigations using mammalian models have demonstrated that FXR α functions through modulating expression of genes involved in both bile acid synthesis and transport. These genes include, but are not limited to, the bile salt export pump (BSEP), small heterodimer partner (SHP), sodium taurocholate cotransporting peptide (NTCP, indirectly through SHP), multidrug resistance protein 2 (MRP2), and the cytochrome P450s 7A1 and 8B1 (CYP7A1 and CYP8B1, indirectly through SHP) (Forman et al., 1995; Makishima et al., 1999; Wang et al., 1999; Goodwin et al., 2000).

Much of the above research exploring mammalian FXR α function has occurred using the potent, synthetic FXR α agonist, GW4064. Created by GlaxoSmithKline, GW4064 has proven a useful tool for exploring FXR α biology (Maloney et al., 2000; Bass et al., 2009). The greatest benefit of this agonist is its selectivity/fidelity for FXR α . This is critical when examining FXR α biology given that previous agonists such as bile acids activate multiple NRs including the pregnane X receptor (PXR, NR1I2) and vitamin D receptor (VDR, NR1I1) (Xie et al., 2001; Makishima et al., 2002; Wang et al., 2002). This explains the wide usage of GW4064 to further elucidate FXR α function (Liu et al., 2003; Stayrook et al., 2005; Cariou et al., 2006; Frankenberg et al., 2006; Inagaki et al., 2006; Zhang et al., 2006). The similarity of FXR α protein sequences among diverse species suggests that these regulatory proteins are well-conserved throughout vertebrate evolution. As demonstrated previously, GW064 is a potent agonist in transactivation assays using fish FXR α sequences (Reschly et al., 2008).

With REACH legislation that went into force in 2008, efforts to replace, reduce, and refine the use of animals in toxicity testing are being demanded (Lammer et al., 2009). Embryos as well as eleutheroembryos (EEs, free swimming forms of fish with yolk sac unresorbed) may prove viable alternatives (OECD, 1998). Recent research in this laboratory has performed whole body in vivo brightfield and fluorescence microscopy on embryos and EEs of the orange-red (OR)

medaka and a transparent mutant, the STII medaka (Wakamatsu et al., 2001). Our in vivo, non-invasive imaging studies showed that the medaka intrahepatic biliary system is an interconnected network of canaliculi and bile-ductules (Hardman et al., 2007). Medaka canaliculi, like their mammalian counterparts, are delimited solely by hepatocyte plasma membranes. However, a major difference exists with respect to abundant passageways that connect the canaliculi to larger passageways in the biliary hierarchy. In the mammal, such transitional passageways are restricted to the periphery of the hepatic lobule; however, they occur throughout the parenchyma in medaka and other teleosts including trout (Hampton et al., 1988; Rocha et al., 1997), bullhead catfish (*Ameiurus nebulosus*) (Hampton et al., 1987) and tilapia (*Oreochromis niloticus*) (Figueiredo-Fernandes et al., 2007). When we first described these cells in fish, we adopted terminology from previous work (Carruthers and Steiner 1961; Steiner and Carruthers, 1961) and named these transitional passageways lined by both hepatocytes and biliary epithelial cells “bile ductules”. Similarly, small biliary epithelial cells of the passageways were named “bile ductular epithelial cells” (BPDECs). In whole body, non-invasive investigations using confocal fluorescence microscopy, induction of CYP1A by use of an aqueous bath exposure to β -naphthoflavone, resulted in metabolic conversion of ethoxyresorufin, taken up from water, to resorufin and fluorescence was detected in bile passageways (Hardman et al., 2007). After embryos/EEs were exposed to the reference hepatotoxicant, α -naphthylisothiocyanate (ANIT), the fluorescence within intrahepatic bile passageways showed linear attenuation alternating with expansion (Hardman et al., 2008). We then used high-resolution light microscopy and transmission electron microscopy in fixed, static preparations to demonstrate that swelling and shrinkage of BPDECs accounted for the luminal fluorescent pattern in exposed fish. From these investigations, a portion of our emphasis in the present study became to better understand molecular mechanisms underlying hepatobiliary function and toxic response in this model organism. The present study couples molecular and histological approaches to definitively characterize responses observed after GW4064 exposure in this organism.

Methods

Animals

Orange-red (OR) medaka fish were maintained at the Duke University Aquatic Research Facility under standard recirculating water conditions. Animal care and maintenance protocols were approved by the Duke University Institutional Animal Care and Use Committee (DUIACUC). Water temperature and pH were monitored daily and maintained at $\sim 25^{\circ}\text{C}$ and ~ 7.4 , respectively, and broodstock were under a strict light/dark cycle of 16:8 h. Dry food (Otohime B1, Reed Mariculture, Campbell, CA) was fed several times per day via automated feeders with once daily supplementation of newly hatched *Artemia* nauplii. Adults reared under the above conditions were used in initial studies of Fxr α isoform expression (see below). Under the care and culture conditions described above, medaka spawned daily and masses of embryonated eggs (20–30/mass) were collected, and separated (disruption of attachment filaments joining individual eggs) by gentle rolling on a moistened surface. Groups of individual embryonated eggs of specified collection date and developmental stage (Iwamatsu, 2004) were suspended in a 2% saline solution made using Instant Ocean (Aquatic Ecosystems, Apopka, FL) in a 100 $\times$ 20 mm tissue culture dish (BD Biosciences, Franklin Lakes, NJ) and housed at 26°C under gentle movement. After 5 days, marine water was replaced with 1 $\times$ embryo rearing medium (ERM) containing: 17.1 mM NaCl; and, in μM s, 272 $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 402 KCl, and 661 $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ until hatching (Kirchen and West, 1976), usually 9 days post-fertilization. This regime provided embryos and EEs for Fxr α isoform expression analyses and for acute toxicity studies completed within 4 days following hatch. For the latter, EEs were not fed during exposure.

Fxr α isoform expression analyses in untreated adults

Five male and five female medaka approximately 6 months of age were anesthetized by placement in ice-cold 1 $\times$ ERM and killed by severing the spinal cord in the cervical region. Brain, gill, gut, heart, kidney, liver, skeletal muscle, spleen, ovary, and testis were dissected individually and snap frozen in liquid nitrogen. Following homogenization, total organ RNA was extracted using RNA Bee (Tel-Test, Friendswood, TX) according to the manufacturer's instructions using a Kinematica Polytron homogenizer (Lucerne, Switzerland). RNase ZAP (Sigma, St. Louis, MO) was used throughout all isolations to prevent RNA degradation. RNA quality (260/280 ratio) and quantity were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA was reverse transcribed into cDNA using the high-capacity cDNA master kit from Applied Biosystems (Foster City, CA) as per the manufacturer's instructions with random hexamer as primer and processed using quantitative, real-time PCR (qPCR).

Fxr α isoform expression in untreated embryos and EEs

Individual embryos were staged and collected daily until 12 dpf, then snap frozen directly (embryos) or after anesthesia (EEs) as above. Pools of three to six embryos and EEs per sample with three samples per each development stage (Iwamatsu, 2004) were used. Whole homogenate RNA was isolated using the Qiagen RNeasy Mini Kit as per the manufacturer's instructions. DNase digestion was performed on-column using RNase-free DNase (Qiagen, Valencia, CA). RNA analysis and cDNA synthesis were as described above. All samples were subsequently processed using quantitative, real-time PCR.

GW4064 aqueous bath exposures

For dose response studies, GW4064 in DMSO (HPLC-grade, Mallinckrodt Chemicals, Phillipsburg, NJ,) was suspended in each well of a 6-well tissue culture plate (Corning, Corning, NY) containing 4 ml of 1 $\times$ ERM. GW4064 was added to a final concentration of 0.01, 0.1, 1.0, 5.0, or 10.0 μ M from a stock solution of 10 mM GW4064 in DMSO. Fifteen EEs between 10 and 12 days post fertilization (dpf) and ranging in weight from 0.8 to 0.9 mg each were placed in each individual well, maintained in a 26 $^{\circ}$ C incubator on a rotating shaker to allow for adequate oxygenation of the ERM. EEs were observed repeatedly and were collected after 24 h of exposure, anesthetized by immersion in ice cold water and processed for quantitative, real-time PCR. GW4064 was graciously provided by Dr. Steven Kliewer, University of Texas Southwestern Medical Center, Dallas, TX.

Time course study

Using the protocol as above, 15 EEs were placed in each well of a 6-well tissue culture plate and exposed to 0 (DMSO carrier control), 5 μ M, or 10 μ M GW4064 in 1 $\times$ ERM for 6, 24, 48, or 72 h. RNA isolation and cDNA synthesis are as described previously and samples processed using qPCR.

qPCR for FXR expression studies

Relative levels of medaka Fxr α 1, Fxr α 2, and 18S rRNA were measured in various adult organs and in developing medaka embryos and EEs using the Applied Biosystems ABI PRISM 7300 Real-Time PCR System. Medaka specific qPCR primers for 18S RNA, Fxr α 1, and Fxr α 2 were constructed using PrimerQuest (Integrated DNA Technologies, Coralville, IA) and are listed in Table 1. Each transcript was amplified in duplicate for each cDNA sample in a 96-well PCR plate. For each 25- μ l real-time PCR reaction, first-strand cDNAs were amplified using 2 μ l (100–300 ng) first-strand cDNA, 9 μ l RNase-free water, 0.75 μ l 10 μ M forward primer (0.3 μ M), 0.75 μ l 10 μ M reverse primer (0.3 μ M), and 12.5 μ l 2 $\times$ QuantiTect SYBR Green PCR Master Mix (Qiagen). Real-time PCR reaction conditions were: 95 $^{\circ}$ C for 15 min followed by

40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min. Relative quantitation of gene expression within each reaction was calculated as described (Fu et al., 2005). Additionally, Ct values of *Fxrα1* and *Fxrα2* were subjected to correction by their respective primer efficiencies and corrected values were then normalized to the corresponding 18S values for each sample.

qPCR for GW4064 dose-response and time-course

Relative levels of bile salt export pump (Bsep), cholesterol 7 α hydroxylase (Cyp7a1), small heterodimer partner (Shp), multidrug resistance protein 2 (Mrp2), sodium taurocholate cotransporting peptide (Ntcp), and β -actin transcripts in EEs from each dosing regimen were measured using quantitative real-time PCR. Sequences were identified as above and medaka specific real-time primers for Bsep, Shp, Cyp7a1, Mrp2, Ntcp, and β -actin were designed using PrimerQuest (Integrated DNA Technologies). All primer sequences are listed in Table 1; and, predicted gene locations listed in Supplementary Table 1. qPCR targets including Bsep, Shp, Cyp7a1, Mrp2, Ntcp, and β -actin were amplified separately in duplicate using QuantiTect SYBR Green PCR Kit (Qiagen) as described above. Relative quantitation of gene expression was calculated using the comparative cycle threshold (Ct) method. Each cDNA sample was run in duplicate, their Cts averaged, and normalized to their respective β -actin Ct to calculate the Δ Ct (Δ Ct = Gene_{Ct} - Actin_{Ct}). Changes in gene expression were determined by subtracting the average Δ Ct for control (DMSO) from each treatment, yielding the $\Delta\Delta$ Ct, and then converted to fold induction (fold induction = $2^{-\Delta\Delta$ Ct}).

Whole mount fat staining using Oil Red O

From the dose response study above, a total of 12 randomly selected EEs (0.8–0.9 mg body weight) were maintained in the appropriate well of the tissue culture plate and exposed for an additional 24 h (total duration = 48 h) to 0 (DMSO carrier control- *N* = 3) or to 1, 5, or 10 μ M GW4064 (*N* = 3 for each concentration). At the termination of exposure, EEs were transferred to ice cold 1 $\times$ ERM for anesthesia and then immersed in 10 volumes of fixative (4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2–7.4) and held at 4 °C until processing (Passeri et al., 2009). Fixed individuals were rinsed 3 $\times$ in PBS, infiltrated via a graded propylene glycol series, and stained overnight at 4 °C in a 0.5% Oil Red O/100% propylene glycol solution. After staining, EEs were cleared of propylene glycol via graded series of glycerol, rinsed 5 $\times$ in PBS and stored in 80% glycerol. Individuals were placed in a depression slide in 80% glycerol, and imaged and photographed at 200 $\times$ magnification. The abdominal lipid vacuole, a normal structure of medaka EEs (Iwamatsu, 2004) served as an indwelling control for each individual. Images were recorded and stored electronically using equipment and methods described below.

Paraffin based histological analysis

As described above, randomly selected EEs (*N* = 3 per concentration, vehicle control; 10 μ M GW4064) were removed from the appropriate wells after 48 h exposure (based on qPCR results), anesthetized, fixed as above, transported to the Histopathology Laboratory, College of Veterinary Medicine, North Carolina State University, Raleigh, NC and processed using standard methods. Following sectioning (5 μ m thickness), mounting on glass histological slides, staining with hematoxylin and eosin (H&E), and coverslipping, specimens were imaged as described below.

Glycol methacrylate (GMA) embedment

Individual EEs from the GW4064 exposures (as described above) (*N* = 3 per exposure), were fixed, transported and processed by the same staff as above. In order to increase resolution in liver morphologic assessment, the water-soluble monomer GMA in Technovit 7100 GMA Kit from EBSciences East Granby, CT was used. Dehydration, infiltration, and hardening followed

the manufacturer's recommendations. A Leica 2065 Supercut Microtome (Leica Microsystems Inc., Bannockburn, IL) with a knife holder for triangular glass knives was used for sectioning (2.5 μ m thickness). Mounted sections were stained by H&E as above permitting comparison of higher resolution preparations with those of paraffin methods.

Transmission electron microscopy (TEM)

TEM was used to provide optimal resolving power. EEs were anesthetized and immediately fixed by immersion in 4% paraformaldehyde/1% glutaraldehyde, pH 7.2–7.4 (4F/1G) (McDowell and Trump, 1976) at 4 °C until processing. After rinsing (2 $\times$) in 0.1 M sodium phosphate buffer (pH 7.2–7.4), samples were post-fixed in 1% osmium tetroxide/0.1 M sodium phosphate buffer (pH 7.2–7.4) for 1 h at room temperature. Resultant secondarily fixed EEs were then rinsed 2 $\times$ in distilled water, dehydrated in graded ethanol solutions, rinsed 2 $\times$ in 100% acetone, placed in a 1:1 mixture of Spurr's resin and acetone for 30 min, and then transferred to 100% Spurr's resin for 1 h. After an initial incubation, EEs were incubated in a new solution of 100% Spurr's resin for 1 h, placed in molds and polymerized at 70 °C overnight. Semithin sections (500 nm thick) were cut with glass knives and stained with 1% toluidine blue O in 1% sodium borate. These sections served two purposes. First, to determine presence of liver in section plane; second, to provide the highest resolution light microscopy. Once liver was shown to be present in semithin sections, ultrathin sections (70–90 nm thick) were cut with a diamond knife, stained with methanolic uranyl acetate and lead citrate, and examined using a FEI/Philips EM 208S transmission electron microscope at 80 kV accelerating voltage. All TEM processing, analysis, and imaging were performed at the Laboratory for Advanced Electron and Light Optical Methods (LAELOM), College of Veterinary Medicine, North Carolina State University.

Imaging

All oil red O stained EEs, paraffin- and GMA-embedded H&E stained sections, as well as toluidine blue stained Spurr's semithin sections were surveyed for presence of liver alterations and imaged with a Nikon Eclipse E600 light microscope, a Nikon DXM 1200 digital camera, and EclipseNet imaging software (Nikon, Melville, NY). Morphometric analyses of images were performed using ImageJ (National Institutes of Health, Bethesda, MD). Enumeration of cellular organelles, size of organelles (calculated based on the original image scale and presented as per unit area), and calculations of circularity (0.01–1.00), where 1.0 equates to absolute circularity, were conducted.

Statistics

All qPCR data are shown as the mean mRNA level $\pm$ SEM. Quantitative morphometric data are shown as mean value $\pm$ SEM. Statistical analyses for GW4064 dose-response and time-course comparisons were determined by analysis of variance ANOVA using StatView 9.0 for Mac and JMP for PC (The SAS Institute, Cary, NC). Post hoc analyses of differences were determined using Fisher's PLSD post-hoc test and Tukey–Kramer comparison of means test. Assumptions of normality and homogeneity of variance were tested using box plots and Bartlett's test, respectively. A *p* value <0.05 was considered significant.

Results

Expression of Fxr α 1 and Fxr α 2 in adult and developing medaka

Medaka Fxr α isoforms were differentially expressed within adult and developing animals, Figs. 1A and B, respectively. Quantitative, real-time PCR data demonstrated significant expression of Fxr α 1 in liver and spleen, while Fxr α 2 was expressed predominantly in the gut of both genders. Moderate levels of expression for Fxr α 1 was additionally observed in heart, testis and

brain, whereas Fxr α 2 transcripts were additionally observed in heart, liver, muscle and testis. Sexual dimorphic expression was also observed for Fxr α 1 levels in liver and spleen where expression was more abundant in males than females. In developing medaka, Fig. 1B, little expression of either Fxr α isoform was noted at the blastula stage (0 days post-fertilization, or dpf) however with Fxr α 2 a low abundance transcript could be measured. Expression for both forms steadily increased throughout development with appreciable levels of Fxr α 1 occurring on day 3 and Fxr α 2 occurring on day 1. Expression of both Fxr α 1 and Fxr α 2 were maximal at 9 dpf when medaka embryos normally hatch. From 3 days post hatch Fxr α 1 mRNA levels were consistently higher than Fxr α 2 at all time points. The levels noted in developing medaka were lower than most organs of the adult organism, even at times of highest expression (9 and 10 dpf).

qPCR: GW4064 dose-response

Expression of FXR α gene targets were altered as a result of GW4064 exposure, Figs. 2A–E. Expression of Bsep and Shp increased and Cyp7a1 decreased significantly in a dose-responsive fashion (Figs. 2A–C, respectively). Bsep expression increased most robustly, with an ~80 fold increase in mRNA levels at the highest dose of GW4064 (10 μ M). Shp expression was also increased but to a lesser extent (~10 fold increase at 10 μ M). Cyp7a1 expression was markedly reduced at 1, 5, and 10 μ M GW4064 to levels that proved much lower than those in control EEs. No significant change in gene expression was observed for both Mrp2 and Ntcp, following GW4064 exposure at any dose examined (Figs. 2D, E).

qPCR: GW4064 time-course

Changes in Bsep and Shp expression after aqueous exposure to 5 μ M GW4064 for 6, 24, 48, and 72 h is shown in Figs. 3A and B. Bsep and Shp expression were increased markedly at 24, 48, and 72 h while expression at 6 h was not significantly different from time-matched untreated controls. After 24 h there was no significant difference in Bsep or Shp expression between exposure groups, indicating that maximal induction was achieved after 24 h of treatment. BSEP expression in exposed EEs at 48 and 72 h was not significantly different from treated individuals at 6 h due to large SEM values.

Oil red O whole mount staining

A significant increase in lipid accumulation was noted in medaka EEs using the oil red O stain 48 h after GW4064 exposure (Fig. 4). Morphometric studies confirmed a dose response by illustrating a consistent increase in lipid accumulation with increasing GW4064 concentration. Whereas control liver (first panel) lacked red droplets, red staining was noted only in the oil droplet, which is slowly resorbed by EEs (Iwamatsu, 2004) (inset of first panel). EEs exposed to 1, 5 or 10 μ M GW4064 showed increasing amounts of lipid inclusions in liver (second, third, and fourth panels, respectively). Using morphometric analyses, we demonstrated that the diameter of individual lipid droplets was similar across all concentrations. However, the numerical density of lipid droplets, measured morphometrically as number of droplets/100 μ m², differed significantly ($p < 0.001$) and increased in a dose-response fashion (i.e., control (0 ± 0 SEM) < 1 μ M (59.7 ± 06.9 SEM) < 5 μ M (351.0 ± 19.16) < 10 μ M (479.5 ± 12.5 SEM)). In intact EEs, the lipid droplet or vesicle lies adjacent to the liver (Iwamatsu, 2004), is slowly metabolized; and, for our purposes served as an internal control. These findings enable rapid visualization of lipid-specific alterations to medaka liver after GW4064 exposure and demonstrate that significant lipid accumulation within the liver occurs at low micromolar concentrations.

Paraffin-based morphology

Normal liver structure was observed in control medaka EEs at 48 h and examples of this are illustrated in Fig. 5A. Hepatocyte arrangement, in longitudinal array, was revealed as cord-like structures typically two cells thick and separated from its nearest neighbor by sinusoidal lumens lined with endothelial cells, and occasionally containing nucleated red blood cells. When the latter were not present, sinusoidal lumens were difficult to image in paraffin sections. A representative image from an EE exposed to 10 μ M GW4064 for 48 h is shown in Fig. 5B. H&E-stained paraffin sections showed that GW4064 administration resulted in vacuolation of hepatocytes. However, since alcohol dehydration used in paraffin processing removed lipids, the nature and exact cellular location of the vacuolation was not apparent from these sections. For this reason morphometric analysis was not performed on paraffin sections.

GMA sections

Those stained with H&E are shown in Fig. 6. With the added resolution afforded by this approach, biliary epithelial cells, specifically bile preductular epithelial cells (BPDECs), were readily seen in the hepatic parenchyma and differentiated from hepatocytes. Normal liver morphology in control individuals was observed but in much more detail due to the thinner sections. Biliary passageways lined by BPDECs were clearly visible in control animals, as were nucleoli of hepatocyte nuclei. Cytoplasm of hepatocytes contained granular basophilic material sometimes forming linear arrays. This contrasted with pink-staining circular regions shown later in electron micrographs to be glycogen depots. Exposure to 10 μ M GW4064 for 48 h (Fig. 6B) produced alterations of BPDECs, most notably attenuation. Vacuolation was observed after 48 h of GW4064 exposure and was restricted largely to hepatocytes. Glycogen depletion by GW4064 exposure was apparent as a loss of eosinophilic glycogen depots that were prominent in hepatocytes of control individuals. However, to confirm these observations, higher resolution techniques were employed.

Toluidine blue staining

Control morphology is shown in Fig. 7A. Adjacent sinusoids were separated by cords of hepatocytes (two cells thick). Small BPDECs were apparent in the field and no large lipid vacuoles were noted. Sections from medaka EEs exposed to 10 μ M GW4064 showed distinct, aquamarine-stained lipid inclusions at 48 h (Fig. 7B). A significant increase ($p < 0.01$) in the number of lipid vacuoles/ $100 \mu\text{m}^2$ was identified between DMSO (0 ± 0 SEM), 1 μ M GW4064 (14.5 ± 3.5 SEM), and 10 μ M GW4064 (74.0 ± 13.0 SEM) treatments. Other clear vacuoles were present throughout the fields. Some of these were small and apparently empty, while larger ones contained membranous debris. BPDECs in GW4064 exposed treatment groups demonstrated expanded cytoplasm with an abundance of dense inclusions and clear vacuoles. Hepatocyte nuclei exhibited altered morphology compared to controls (Fig. 7A). While nucleoli of controls were rounded and generally occupied central nuclear regions, those of exposed individuals were attenuated and often located at the nuclear periphery. Hepatocyte nuclear conformation appeared altered by treatment. Whereas control nuclei were round suggesting a spherical shape, those of exposed individuals (48 h 10 μ M GW4064) were elliptical relative to controls. Measures of nuclear circularity indicated no significant mean differences ($p < 0.0001$) between DMSO (0.903 ± 0.013 SEM) and 1 μ M GW4064 (0.866 ± 0.018 SEM) treatments; but were different from the 10 μ M GW4064 (0.682 ± 0.018 SEM) treatment ($p < 0.0001$).

Transmission electron microscopy

Normal BPDEC and hepatocyte morphology was apparent in micrographs from control medaka EEs (Fig. 8A). Numerous hepatocellular mitochondria were clearly visible near parallel arrays of rough endoplasmic reticulum. Little to no vacuolation was observed

confirming previous observations of adjacent sections stained with toluidine blue (Fig. 7A above). TEM images of medaka EEs exposed to 10 μ M GW4064 showed marked alterations to liver ultrastructure at 48 h, and confirmed and extended observations seen with high resolution light microscopy. For example, vacuoles noted in BPDECs in the toluidine blue-stained sections at 48 h exposure (Fig. 7B) often presented as phagocytosis of neighboring apoptotic cellular fragments (Fig. 8B). Lipid inclusions were also readily apparent in the BPDECs and their phagocytosed fragments. Together, these alterations resulted in cytoplasmic expansion in BPDECs. Lipid inclusions and clear vacuoles were also apparent in hepatocytes, some containing membranous debris as seen in the toluidine blue-stained sections. Enlarged mitochondria with ruptured inner mitochondrial membranes were seen throughout the field, as well as disrupted endoplasmic reticulum showing swollen and vesicular forms in hepatocytes. Glycogen depletion was also observed. Significant increase ($p < 0.001$) in % mitochondrial area relative to nuclear area was identified between DMSO (3.56 ± 0.82 SEM) and 1 μ M GW4064 (6.17 ± 0.56 SEM) treatments, and 10 μ M GW4064 (18.23 ± 3.53 SEM) treatments.

Discussion

This *in vivo* study was undertaken to characterize medaka Fxr α function in vivo through the use of a potent FXR α agonist, GW4064. Herein, we show the expression of two Fxr α isoforms within adult and developing medaka (Fig. 1) and activation by the synthetic agonist, GW4064, through altered expression of known FXR α gene targets (Figs. 2 and 3). The two isoforms in medaka, Fxr α 1 and Fxr α 2, are differentially expressed within organs of the adult organism. Expression of Fxr α 1 predominates in liver and spleen, while Fxr α 2 was greater in gut and kidney. A slight difference in mRNA levels between genders was noted in liver, gut and spleen (male > female) that, to our knowledge, has not been demonstrated in other species. The underlying reason for and significance of these differences are presently unknown. In developing medaka, mRNA levels of Fxr α 1 were higher than α 2 at all timepoints, and both peaked at 9 dpf when medaka embryos undergo hatching from the external chorion.

Alteration in expression of known Fxr α gene targets such as Shp, Bsep, and Cyp7a1, but not Ntcp and Mrp2, was observed subsequent to treatment with GW4064 as low as 1 μ M. Changes in gene expression as a result of GW4064 exposure reached maximum levels after 24 h. The alterations in Bsep, Shp, and Cyp7a1 expression were consistent with *in vitro* findings with mammalian Fxr (Goodwin et al., 2000; Lu et al., 2000; Plass et al., 2002). Furthermore results of our *in vivo* study correlated strongly with observations in rat and mouse (Goodwin et al., 2000; Liu et al., 2003; Inagaki et al., 2006). These results demonstrate a conserved regulatory function of FXR between teleosts and mammals. In contrast medaka Mrp2 and Ntcp were non-responsive to GW4064 exposure differing from responsive mammalian systems (Denson et al., 2001; Kast et al., 2002; Zollner et al., 2003). We can thus say, for the first time, that select molecular aspects of hepatobiliary function in medaka are quite similar to those of mammalian models.

Studies from this and other laboratories have shown that hepatocytes and biliary epithelial cells are sensitive responders under conditions of acute and chronic toxicity in medaka (Toussaint et al., 2001a, 2001b; Wolf and Wolfe 2005; Hardman et al., 2008; Volz et al., 2008). The role of BPDECs in the hepatobiliary toxicity after exposure to GW4064 required use of a tiered histopathological approach. Toluidine blue stained semithin sections provided higher resolution and valuable information on lipid (aquamarine droplets) in hepatic parenchyma and identification as well as alteration in biliary epithelial cells (position between rows of hepatocytes in hepatic tubules, lipid vesicles in cytoplasm) and hepatocyte response (enlarged mitochondria, lipid vesicles). Analysis of TEM extended and confirmed these observations. Through our tiered approach, our observations suggest that not all circular vesicles, thought to

be due to hepatocyte lipid, are in fact fatty change but may be swollen BPDECs (see discussion below). The application of morphometry provided quantitative support to our subjective findings and illustrated that hepatocyte injury including altered nuclei, nucleoli, mitochondrial swelling and lipid accumulation occurred in a dose responsive manner.

The oil red O whole mount staining of intact medaka EEs allowed for an initial, rapid assessment of lipid accumulation in the liver following exposure to GW4064. Lipid inclusions, completely absent from livers of control EEs, were readily visible in exposed individuals where they increased in a dose-responsive fashion. This hallmark feature of liver toxicity suggests that medaka as well as their zebrafish (*Danio rerio*) counterparts may be useful in rapid screening and preclinical safety decision making in relation to development of pharmaceuticals (Redfern et al., 2008).

While found throughout the liver parenchyma, the small size of BPDECs makes observing them in paraffin sections difficult (Fig. 5), if not impossible. Some swelling and expansion of biliary passageways may also be present but it is difficult to see these in paraffin sections. Most importantly, BPDECs are indistinguishable from the hepatocytes due to the low resolving power of paraffin preparations. Vacuolation resulting from toxicant exposure could not be definitively ascribed to the alcohol dehydration of tissues during paraffin processing and the subsequent removal of lipid. However, due to our findings with oil red O whole mount staining and TEM analysis as discussed above, it is likely that some vacuoles in paraffin-embedded material originally contained lipid.

Since paraffin sections did not allow for the visualization of BPDECs, high resolution light microscopy using glycol methacrylate (GMA) sections was used. BPDECs were clearly visible in GMA with the inherent increase in resolution. In addition, alterations were prominent. Vacuolation in hepatocytes was apparent after GW4064 exposure. In these sections however, as in paraffin, the nature of the vacuoles was uncertain since alcohol dehydration is used in processing. Glycogen deposition and depletion in hepatocytes were also noted. However, even higher resolution was needed to determine the nature of BPDEC alteration. The secondary fixative used for processing of these specimens for TEM, osmium tetroxide, preserves lipids (Dykstra, 1992) and provides information not seen in paraffin and GMA sections. Clear vacuoles, free of lipid, but occasionally containing membranous debris were also seen in hepatocytes and BPDECs (Fig. 7). Clear vacuoles in hepatocytes may be caused by depletion of glycogen from depot stores; or, they may be hydropic vacuoles as observed previously in STII medaka EEs after exposure to ANIT (Hardman et al., 2008). Damaged, fragmented hepatic nucleoli were also visible in these high-resolution sections. The small size of the EEs makes the use of semithin sections and toluidine blue stains attractive. When individuals were placed in a horizontal longitudinal orientation and sections taken beginning at the ventral body wall, only three to five sections were required before liver was present. Skip sections in this orientation provide significant liver area for analysis.

Alterations of individual organelles were noted only through TEM. Apoptotic vesicles, phagocytosed by neighboring BPDECs, were observed and were likely the main cause of altered BPDEC morphology in GMA and toluidine blue-stained sections. Phagocytosis of these fragments resulted in increased BPDEC cytoplasmic area and altered position and shape of BPDEC nuclei. Damaged and ruptured hepatocellular mitochondria, disrupted endoplasmic reticulum, glycogen depletion and loss of hepatocellular polarity were noted upon exposure of medaka EEs to 10 μ M GW4064. Inflammation was absent in all of our sections providing further evidence that the cellular degeneration was apoptosis.

The alterations in mitochondria may be a major factor underlying lipid accumulation within hepatocytes and BPDECs of GW4064 exposed individuals. Mitochondria are the site for

breakdown of triglycerides by β -oxidation, and impairment of this process is observed in drug-induced liver injury and non-alcoholic steatohepatitis (NASH) (Pessayre, 2007; Vickers, 2009). Use of a mitochondrial DNA damage assay such as the long-amplicon quantitative real-time PCR method could be performed to characterize the alterations to mitochondria by GW4064 in more detail (Jung et al., 2009). As presented above, nuclear conformational change, from circular toward ellipsoids occurred and in a dose responsive manner. In addition, nucleoli were condensed and often located at the nuclear periphery rather than the central nuclear region as seen in controls. It is possible that the mitochondrial swelling and the nuclear conformational changes could be explained on the basis of apoptosis (Green and Reed, 1998; Morishima, 1999) however additional research will be needed to confirm this possible explanation.

Toxicity of GW4064 has not been reported to date, but concerns about potential toxicity have been raised due to the presence of a stilbene functional group (Akwabi-Ameyaw et al., 2008). This study clearly revealed sublethal toxicity of GW4064 to medaka EEs and suggests that the changes in expression of genes crucial to biliary homeostasis may also lead, under certain conditions, to a toxic phenotype.

Hepatic injury in medaka liver as a result of GW4064 exposure may indicate the potential for toxicity in mammalian liver as well; but, to date this has not been described. Rather, ANIT toxicity was reduced in rodents upon co-administration of GW4064 (Liu et al., 2003; Cui et al., 2009). Toxic injury in mammalian liver could be anticipated despite differences in overall liver architecture between mammals and teleosts because of similarities in response at the molecular level; both medaka and mammalian FXR α are strongly activated by GW4064. It would be interesting to see if GW4064 causes hepatotoxicity in a species with a non-GW4064 responsive FXR α such as the little skate (*Raja erinacea*) (Cai et al., 2007). In this manner, the mechanism by which GW4064 causes sublethal hepatotoxicity could be further elucidated.

Future studies should employ morpholino knockdown technologies to determine if the liver toxicity seen following GW4064 exposure was the result of overactivation of Fxr α in this model organism, or from off-target responses. Similarly, since our qPCR data showed expression of Fxr α in heart, gut, kidney, and spleen, future morphological analyses should be performed on these organs.

Finally, the results of this study argue that the efforts under REACH legislation (Registration, Evaluation, Authorization and Restriction of Chemicals) to replace, reduce and refine the use of animals in toxicity testing might consider a more robust use of embryos (Lammer et al., 2009) and EEs in future testing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FXR α	farnesoid X receptor alpha
Fxr α	medaka FXR α
dpf	days post fertilization
ERM	embryo-rearing medium
GMA	glycol methacrylate
TEM	transmission electron microscopy
PBS	phosphate-buffered saline
BSEP/Bsep	bile salt export pump
SHP/Shp	small heterodimer partner
CYP7A1/Cyp7a1	cytochrome P450 7A1
NTCP/Ntcp	sodium taurocholate cotransporting peptide
MRP2/Mrp2	multidrug resistant protein 2
BPDEC	bile preductular epithelial cell
ANIT	α -naphthylisothiocyanate

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.taap.2009.11.022.

Fig. 1.

(A) Quantitative, real-time PCR data showing expression of two medaka *Fxrα* isoforms in tissues of 6 month old OR males (black bars) and females (gray bars). Relative mRNA levels were measured in brain (B), gill (Gi), gut (Gu), heart (H), kidney (K), liver (L), skeletal muscle (M), spleen (S), and gonad (testis: T; ovary: O). Data was normalized to 18S rRNA levels and is represented as the mean relative mRNA level $\pm$ SEM ($n = 5$). (B) Relative mRNA level of *Fxrα1* (black bars) and *Fxrα2* (gray bars) within medaka embryos and EEs as measured by qPCR. Data was normalized to 18S rRNA levels and is represented as the mean relative mRNA level $\pm$ SEM ($n = 3$ pooled samples).

Fig. 2. Quantitative, real-time PCR data showing alteration in Bsep (A), Shp (B), and Cyp7a1 (C) expression in medaka EEs 10–12 dpf following exposure to GW4064 at 0.01, 0.1, 1, 5, or 10 μM. Data was normalized to β-actin and is represented as the mean mRNA level ± SEM ($n = 6$). A significant change in Bsep, Shp and Cyp7a1 was noted compared to DMSO controls (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$). Expression of Mrp2 (D) and Ntcp (E) were not significantly altered as a result of GW4064 exposure.

Fig. 3.

Quantitative, real-time PCR data showing the alteration in Bsep (A) and Shp (B) expression (in medaka EEs 10–12 dpf) as a result of exposure to 5 μ M GW4064 over time. Data was normalized to β -actin levels and is represented as the mean mRNA level $\pm$ SEM ($N = 3$). A significant difference in Bsep and Shp expression is noted after 24, 48, and 72 h of exposure to GW4064 (gray bars) but not at 6 h compared to time-matched controls (black bars). Significant differences between expression among dosed individuals at different time points is noted by brackets (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$).

Fig. 4.

Oil red O whole mount staining of EEs exposed to control (DMSO) and GW4064 for 48 h. Bar = 100 μm. Inset image in the left panel shows positive staining oil droplet (od) in control, Retained in larval development, this structure serves as an internal control and was located in the rostral peritoneal cavity between liver (L) and septum transversum. Y = yolk sac. Larger panels show liver fields from individuals exposed to DMSO, 1, 5, and 10 μM GW4064, demonstrating a dose-dependent increase in lipid accumulation (red stained vacuoles) as a result of GW4064 exposure. Morphometric analyses confirmed the apparent increase in lipid vacuoles (see Results section).

Fig. 5. Representative images from paraffin-embedded liver sections (5 μm) of control and treated (10 μM GW4064) medaka EEs stained with H&E. Scale bar = 10 μm . (A) Control liver at 48 h showing tubules of hepatocytes with darkly staining nuclei and unstained regions of the cytoplasm. Gu: gut; Bw: body wall. (B) Liver from fish treated with 10 μM GW4064 for 48 h. Disruption of the hepatic tubules and pronounced vacuolation (arrowheads) of parenchymal cells are seen. Bv, blood vessel.

Fig. 6.

Representative higher-resolution images (3 μm) from control (DMSO, vehicle) and treated (10 μM GW4064) medaka EEs embedded in GMA and stained with H&E. Scale bar = 10 μm . (A) Control (DMSO) liver at 48 h shows normal morphology for hepatocytes and bile preductular epithelial cells (BPDECs) (black arrowheads) which intervene between longitudinal arrays of hepatocytes in a single hepatic tubule. Canaliculi are not resolved with this technique.

Representative elements of a transitional biliary passageway (i.e.: hepatic tubule lumen, or bile preductule) are shown in the rectangle at right of field. In upper right hand corner is an endothelial cell and its associated sinusoid. White arrowhead at left hand margin of rectangle denotes nucleated erythrocyte in sinusoid. Between the two sinusoids (Bv) are hepatocytes in

a double row configuration. A blue-gray transitional biliary passageway (bile preductule) is denoted by Bp. Note biliary epithelial cell at bottom right corner (black arrowhead) illustrating that these passageways are lined by both hepatocytes and transitional biliary epithelial cells (bile preductular epithelial cells). Hepatocyte cytoplasm contains meandering basophilic structures corresponding to rough endoplasmic reticulum of hepatocytes—see Fig. 8. Basophilic areas contrast with pink stained areas of hepatocyte cytoplasm, denoting glycogen depots. (B) Liver from EE exposed to 10 μ M GW4064 for 48 h. Lower right hand corner of micrograph shows interface of liver surface with intestinal wall (dashed line). The remainder of the field is hepatic parenchyma with occasional microcirculatory elements (sinusoids). Hepatocytes (round nuclei with prominent nucleoli) and surrounded by lightly stained cytoplasm containing variously sized clear vacuoles (white arrowheads). Biliary epithelial cells (BPDECs) with elongated nuclei and dark and beaded cytoplasm (black arrowheads).

Fig. 7. Representative images from semithin (500 nm) liver sections of control or 10 μ M GW4064 treated EEs, stained with toluidine blue. Scale bar = 10 μ m. (A) Control section. Left upper corner is rostral surface of liver and adjacent sinus venosus. Exocrine pancreas is at right lower corner of field. Remainder is hepatic parenchyma and microvasculature. Hepatocytes are free of vacuolation, stain gray-blue, and contain single nucleus with prominent nucleolus. Three biliary epithelial cells (BPDECs) are shown (arrow points to center cell). By comparison to hepatocytes, BPDECs are lightly stained. (B) Liver section from individual exposed to 10 μ M GW4064 for 48 h. S = sinusoid. Black arrowheads denote BPDECs surrounding lumen or biliary passageway and containing numerous vacuolar alterations. Hepatocytes contain single

prominent nucleus with altered (elongated) nucleolus. Note numerous aquamarine lipid vacuoles in hepatocytes. Two of these are denoted (white arrowheads). Black lines on these vacuoles are due to section artifact (chatter). M = enlarged mitochondria. (C) Liver section from medaka larva exposed to 10 μ M GW4064 for 72 h. Large lipid inclusions (Li) are visible throughout hepatic parenchyma. Small clear vacuoles (white arrowheads) are present, some containing membranous debris. Nucleolar fragmentation (Nu) and BPDEC alteration (black arrowheads) are also observed. Bv: blood vessel.

Fig. 8.

Transmission electron micrographs of EE liver. (A) Control. This micrograph (11.2k magnification) shows fine structure of hepatocytes and two BPDECs. Together, these form a bile passageway (transitional passageway, or bile preductule). Adjacent hepatocytes are joined by junctional complexes (white arrowheads). Note numerous plasma membranes processes from hepatocytes into the biliary lumen. In addition, junctions are seen between BPDECs and hepatocytes (white arrowheads). Control hepatocyte morphology includes parallel arrays of rough endoplasmic reticulum, extensive glycogen depots (G), numerous mitochondria (black arrowheads) and occasional residual bodies (Rb). Note the large hepatocyte nuclei with rounded electron dense nucleoli. Bar = 1 μ m. (B) Electron micrograph (5600 $\times$ magnification)

of EE liver from individual exposed to 10 μ M GW4064 for 48 h. Hepatocytes show altered contour and presence of clear and lipid-containing vacuoles, (white arrowheads and Li, respectively). Endoplasmic reticulum shows marked cisternal swelling. Nuclear change is apparent in altered morphology of hepatocellular nucleoli. Hepatocellular mitochondria (black arrowheads) are enlarged and abnormally shaped. Triangular shaped nucleus at the center of the field is a BPDEC. BPDECs were targets for GW4064 as shown by the numerous lipid vesicles and phagocytosed apoptotic vesicles (Av). Bar = 3 μ m.

Table 1

Sequences for primers used in quantitative, real-time PCR.

Primer name	Sequence (5'→3')
Fxr α 1 F	CATCAGAGTACTTGAAGAAGATGTTGTCAGAGGACGG
Fxr α 1 R	CTCCTTCCGACATGGAGAAGTCATCGC
Fxr α 2 F	GAAGGGGAGATGGTTTTGTTGTAAAGGCTCAAAG
Fxr α 2 R	TCTTGAAGACTGCATCTGTACCAGGGCCAT
18S F	CCTGCGGCTTAATTTGACTC
18S R	GACAAATCGCTCCACCAACT
β -actin F	ACAACGGATCTGGCATGTGCAAAG
β -actin R	AGGGCTGTGATCTCCTTCTGCATT
Bsep F	TGGATGGTTGACAGTCGATGAGCA
Bsep R	ATCCTATTCTGTCAGCGTGCCCTT
Shp F	CCCTCCACAAACAATCCAGCCTTT
Shp R	CATTAAGAGCCTGCCTGCGTTCAA
Cyp7a1 F	TGTCAGTAAAGCCAGGACCCATGT
Cyp7a1 R	TCCAGAGCCAAAGGGCATGTAGAA
Mrp2 F	ACGGTGAACCTCATGTAGCAGAT
Mrp2 R	ATGCCGCACAATGTCAGGTTCAAG
Ntcp F	ATGACAACCTTGCTCCACCTTTGCC
Ntcp R	CAGTGTGAAGCCAATCAACGGCAT