The adaptive role of the aryl hydrocarbon receptor (Ah receptor or AHR) in protecting against disease-related conditions remains unclear in nonmammalian models, particularly teleosts. Therefore, this study focused on the potential role of AHR in response to biliary epithelial cell toxicity and hepatobiliary alteration in medaka. See-through medaka (STII strain) were exposed for 96 h using the biliary toxicant α-naphthylisothiocyanate (ANIT) as a reagent, and fish were evaluated daily using histological and ultrastructural analysis, and by imaging directly through the body wall of living fish. Brightfield and transmission electron microscopy showed that a single ANIT dose (40 mg/kg) specifically induced swelling and apoptosis of bile preductular epithelial cells (BPDECs) as early as 6 h after initial exposure. Following ANIT-induced BPDEC toxicity, in vivo imaging of STII medaka showed significant gallbladder discoloration from 48–72 h. Collectively, these pathologic data suggested that ANIT exposure resulted in acute hepatobiliary changes, lasting < 96 h following initial exposure. We then tested the potential role of AHR in response to ANIT-induced hepatobiliary alteration. Overall, we demonstrated that (1) transient AHR activation and cytochrome P450 1A (CYP1A) induction in livers occurred during ANIT-induced hepatobiliary impairment, (2) pretreatment with an AHR agonist partially protected against acute hepatobiliary alteration, and (3) using a luciferase-based reporter assay, the bile pigment bilirubin weakly activated mouse AHR and binding to medaka-specific CYP1A promoter, resulting in AHR element–driven transcription. Given that bile acids and pigments are present in mammalian and fish liver, these studies collectively suggest that bile-induced AHR activation may be conserved between teleosts and rodents.

Key Words: see-through medaka; AHR; liver; nuclear receptor; bile; ANIT.

Cholestasis may develop from extra- or intrahepatic physical obstruction of biliary flow or by abnormal secretion by hepatocytes. Stasis results in hepatic accumulation of bile (predominantly bile acids, bile pigments, and cholesterol) and, if chronic, can lead to hepatocellular necrosis and cirrhosis. In humans, intrahepatic cholestasis is commonly due to damage caused by infection or drug-induced toxicity within the liver, whereas extrahepatic cholestasis is frequently due to stones or tumors blocking the common bile duct outside the liver (Kumar et al., 2005). Although not widely reported in wildlife, cases of bile stasis with unknown etiology have been documented in birds (Ferrell et al., 2000; Ochiai et al., 2003), and cases resulting from biliary adenocarcinoma and inflammatory diseases (cholangiohepatitis, cholelithiasis, and cholecystitis) have been documented in domestic cats (Mayhew et al., 2002). To protect against severe and sustained liver injury, bile accumulation within hepatocytes initiates an adaptive response characterized by decreased bile acid and bilirubin uptake across sinusoidal membranes, as well as increased bile acid and bile pigment metabolism, detoxification, and transport across canicular membranes (Trauner et al., 1998). Consequently, the regulation of bile metabolism and transport is critical for maintaining biliary homeostasis and minimizing hepatocellular toxicity.

In the liver, a family of nuclear receptors directly regulates the transcription of genes involved in hepatic clearance of bile acids and pigments (Boyer, 2005). Currently known nuclear receptors for bile acids or bile pigments include farnesoid X receptor (FXR) (Makishima et al., 1999; Parks et al., 1999), vitamin D receptor (VDR) (Makishima et al., 2002), and pregnane X receptor (PXR) (Goodwin et al., 2003). During stasis, hepatocellular bile acid and bile pigment levels increase, approaching a critical, near-toxic concentration for activation of ligand-specific nuclear receptors. At the transcriptional level, these receptors then initiate a feed-forward system to induce or repress transcription of genes involved in regulating the intracellular levels of these ligands. For example, at physiologically relevant concentrations, the primary bile acid chenodeoxycholic acid activates FXR and, through induction of small heterodimer partner, decreases expression of cholesterol 7α-hydroxylase (CYP7A), the enzyme that synthesizes bile acids from cholesterol (Makishima et al., 1999). Normal receptor functions—ligand-binding, association with coregulators, and transactivation of promoter response elements or
enhancers—are critical for transducing signals at the level of gene expression, decreasing bile levels, and preventing massive bile-induced hepatocellular injury. In fact, dysfunction or loss of nuclear receptors in hepatocytes exacerbates bile-induced injury. For example, in mice, loss of PXR significantly increases susceptibility to bile acid-induced liver injury following bile duct ligation (Stedman et al., 2005).

Although recent nuclear receptor research has focused on FXR, VDR, and PXR as biological sensors for biliary compounds, little work has been done to determine whether bile acids or bile pigments activate the aryl hydrocarbon receptor (AHR), particularly in the context of in vivo experimental models of bile stasis. Although AHR is not by definition a nuclear receptor, nuclear receptors and AHR share the fundamental function of transducing endogenous ligand-initiated signal to the level of gene expression. AHR may play a major role as a receptor of intracellular biliary compounds because, in the non-ligand-binding state, AHR resides in the cytosol of hepatocytes where, during bile stasis, bile accumulation principally occurs. Similar to nuclear receptors, AHR may be critical for mediating an adaptive response that involves induction of genes responsible for the metabolism or transport of biliary compounds. Indeed, Sinal and Bend (1997) previously demonstrated that cytochrome P450 1A1 (CYP1A1) messenger RNA (mRNA) and ethoxyresorufin-O-deethylase activity was specifically induced by products of heme metabolism (heme, biliverdin [BV], and bilirubin) in wild-type mouse Hepa-1c1c7 cells but not mutant cells lacking AHR or aryl hydrocarbon receptor nuclear translocator (ARNT). In this study, bilirubin was the only compound to activate in vitro binding of AHR to Ah response element (AHRE) complexes, suggesting that bilirubin, the end product of heme metabolism in mammals, was the ultimate ligand for AHR. Phelan et al. (1998) then extended these observations to additional species by using guinea pig, rat, and human cell lines containing stably integrated AHRE-driven firefly luciferase reporter vectors.

To date, no studies have investigated the ability of bile acids or bile pigments to induce AHR activation in experimental models of bile stasis. Indirect evidence using the Gunn rat model suggests that an endogenous, bile-related ligand for AHR exists in vivo. Gunn rats are deficient in bilirubin uridine diphosphoglucuronosyltransferase (UDPGT) (Scragg et al., 1985)—the enzyme that catalyzes bilirubin conjugation and excretion—and, as a result, exhibit severe hyperbilirubinemia and jaundice (Billing, 1972). In order to eliminate toxic heme degradation products (ultimately bilirubin) in the absence of conjugation, these rats rely on an alternative mechanism of metabolism that involves bilirubin oxidation and biliary excretion of polar bilirubin metabolites. Compared to normal rats, these animals have significantly higher constitutive levels of hepatic CYP1A1 and CYP1A2 mRNA and protein, particularly in young (10-day-old) pups (Kapitulnik and Gonzalez, 1993), suggesting that CYP1A1 and CYP1A2 are involved in alternate pathways of bilirubin catabolism (Pons et al., 2003; Zaccaro et al., 2001).

Given the recent evidence demonstrating that nuclear receptors are bile acid receptors, in vitro work with AHR-driven luciferase reporter assays, and previous in vivo work with Gunn rat models, we hypothesized that biliary epithelial cell (BEC) toxicity in medaka liver may lead to AHR activation. The biliary toxicant 2-naphthylisothiocyanate (ANIT)—a chemical long used to induce cholestasis in rodents—was used as a reagent to induce acute hepatobiliary alteration in medaka. See-through medaka (STII strain) were utilized in order to monitor and image gallbladders (an indicator of biliary status) directly through the body wall of living fish. CYP1A mRNA induction was measured during ANIT-induced biliary impairment to initially determine the presence or absence of AHR activation. Additional experiments were then performed to determine whether pretreatment with an AHR agonist (dioxin) protected against ANIT-induced biliary impairment, and, using a luciferase-based reporter assay, whether bile-related compounds were potential AHR agonists.

**MATERIALS AND METHODS**

**Chemicals.** 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD or dioxin) (99% purity) was purchased from Cambridge Isotope Laboratories (Andover, MA). ANIT (95% purity), bilirubin (98% purity), cholic acid (98% purity), and taurocholic acid (95% purity) were purchased from Sigma-Aldrich (St Louis, MO). BV hydrochloride (98% purity) was purchased from Frontier Scientific, Inc. (Logan, UT) All chemicals were dissolved in 100% high-performance liquid chromatography (HPLC)-grade dimethyl sulfoxide (DMSO) and nominal chemical stocks were stored at room temperature in the dark.

**Animal models.** Adult male fish were collected from a STII medaka line (Wakamatsu et al., 2001) maintained since 2002 under standard recirculating aquaculture conditions in our colony at Duke University. STII medaka fish were used in order to visualize gross biliary changes (via inspection of the gall bladder under brightfield light) through the body wall of living fish (Fig. 1A). All fish were handled and treated according to protocols approved by the Duke Institutional Animal Care and Use Committee.

**ANIT exposures.** Adult male STII medaka approximately 4 months old and 270 ± 30 mg wet weight were used for all experiments described below. A 72-h range-finding study was initially performed to determine an effective single dose of ANIT with minimal mortality (LD10). Based on this preliminary study, the 72-h LD10 and LD50 in these fish were approximately 40 and 100 mg/kg, respectively. Therefore, an approximate LD10 ANIT dose of 40 mg/kg was used for the remainder of experiments in this study.

For all exposures, fish were ip-injected once with 1 μl of HPLC-grade DMSO (vehicle) or 1 μl of ANIT stock in the left lateral abdominal region with a sterile, glass 25-μl Hamilton syringe equipped with an ultralfine needle. Vehicle- and ANIT-injected fish were incubated at 25°C under 16:8 light:dark conditions in ethanol-rinsed 2-l glass beakers containing sterile embryo rearing medium (ERM) (17.1mM NaCl, 272μm CaCl2 2H2O, 402μm KCl, 661μm MgSO4 7H2O; pH 6.0 ± 0.2) (Kirchen and West, 1976). Fish were fed every 2 days starting 24 h following injection. ERM was −80%–renewed daily to ensure maximum water quality conditions throughout exposure periods. To assess and characterize the time-course of ANIT pathogenesis in medaka, 4-day exposures were performed to determine the onset of cellular toxicity.
Histological analysis. For 4-day ANIT exposures, vehicle- and ANIT-exposed fish were sampled at 6, 24, 48, and 72 h, anesthetized in ice-cold ERM, and caudal peduncle was transected distal to the anus using clean scissors and forceps. Clean scissors were then used to open the abdominal cavity, permitting fixative to infiltrate all internal viscera. Fish were fixed in 10% neutral-buffered formalin for 72 h at 4°C, and stored in 6% sucrose/phosphate-buffered saline (PBS) (pH 7.4) until processing, mounting, and sectioning. Fish were oriented in lateral recumbency, paraffin-embedded, and 6-μm thick-step sections through the whole body were mounted on glass slides and stained with hematoxylin and eosin (H&E). All animals were surveyed and imaged with a Nikon Eclipse E600 light microscope, a Nikon DXM 1200 digital camera, and EclipseNet imaging software (Nikon, Melville, NY).

Transmission electron microscopy analysis. For transmission electron microscopy (TEM), vehicle- and ANIT-exposed fish were sampled at 6, 24, 48, and 72 h, anesthetized in ice-cold ERM, and livers removed using clean scissors and forceps. Livers were minced into 1-mm pieces on paraffin dental wax byte pads using clean razor blades and stored in McDowell and Trump’s 4F:1G fixative at 4°C until processing for TEM (McDowell and Trump, 1976). After two rinses in 0.1M sodium phosphate buffer (pH 7.2), samples were postfixed in 1% osmium tetroxide/0.1M sodium phosphate buffer (pH 7.2) for 1 h at room temperature. Samples were then rinsed two times in distilled water, dehydrated in a series of ethanol solutions, and rinsed two times in 100% acetone. Tissues were then placed in a 1:1 mixture of Spurr’s resin (Spurr, 1969) and acetone for 30 min and transferred to 100% Spurr’s resin for 1 h. Samples were then 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.
Noninvasive serial in vivo imaging. Separate 4-day exposures for serial in vivo imaging were performed as above to enable continuous monitoring of live fish at 24, 48, 72, and 96 h. For pretreatment studies with TCDD, serial in vivo imaging of STII medaka was performed to enable continuous monitoring of gallbladder coloration in live fish at 24, 48, 72, and 96 h following the first injection. At each sampling time point, vehicle-, and ANIT-treated fish were anesthetized individually in ice-cold ERM and positioned on a piece of wet cotton secured within a plastic petri dish for no more than 1 min. Right lateral sides of each fish were viewed using a Leica MZ FLIII stereomicroscope. Using a Nikon CoolPix 990 digital camera, fish were imaged under bright light in right lateral recumbency in order to visualize changes in gall bladder coloration. After images were captured, fish were returned to ERM until the next sampling time point.

Gallbladder bile BV analysis. Relative concentrations of gallbladder bile BV in vehicle- and ANIT-exposed fish were estimated at 24, 48, 72, and 96 h. Fresh gallbladder bile samples collected from three replicate fish for each dose-time group, were centrifuged for 15 min at 13,200 rpm = 16,100 × g, and placed on ice for immediate analysis. Relative BV absorbance at 660 nm (Gray et al., 1961) was measured using a NanoDrop ND-1000 ultraviolet/visual (UV/Vi) Spectrophotometer in UV/Vi mode.

Pretreatment exposures with AHR agonist (TCDD). For AHR agonist (TCDD) pretreatment studies, individual fish were first ip-injected with 1 μl vehicle (DMSO) or TCDD stock (0.035 mg/ml) and treated for 24 h. Fish then received a 1 μl vehicle (DMSO) or ANIT dose (11 mg/ml) and were treated for 72 h. Fish were incubated under conditions as described above for ANIT-only exposures. The approximate TCDD dose based on the wet weight of these fish was approximately 0.1 μg/kg. This dose is minimally toxic to medaka liver and only results in reversible hepatocellular atrophy at 5 days postexposure (Vozl et al., 2006). The final dosing combinations were (first dose/second dose): vehicle/vehicle, vehicle/ANIT, TCDD/vehicle, and TCDD/ANIT.

Total RNA isolation. For liver RNA extractions, vehicle- and ANIT-exposed fish were sampled at 24, 48, 72, and 96 h. For each dose-time group, three replicate liver pools (three livers per pool) were collected and snap frozen in liquid nitrogen, yielding a total of 30 independent liver pools. Livers were homogenized with 1 ml of RNA Bee (TelTest, Friendswood, TX) using a stainless steel Polytron homogenizer (Kinematica, Newark, NJ) cleaned with RNaseZAP (Sigma), diethylpyrocarbonate-treated water, and sterile deionized water. Following homogenization, total RNA was isolated as described in Vozl et al. (2005). Prior to sample elution, each sample was on-column–digested with DNase to eliminate DNA contamination using an RNase-free DNase Set (Promega) according to manufacturer’s instructions (User Bulletin #2, ABI PRISM 7700 Sequence Detection System, Applied Biosystems).

Real-time PCR. Per manufacturer’s instructions, first-strand complementary DNAs were generated from a total of 30 RNA samples using SuperScript Reverse Transcriptase (Invitrogen). Relative levels of β-glutamyltranspeptidase 1 (GGT1), bile salt export pump (BSEP), CYP1A, uridine diphosphoglucuronosyl transferase (UDPGT), and β-actin transcripts in DMSO- and ANIT-treated livers were measured using real-time PCR. Sequences were identified using the Medaka Genome Browser (DNA Sequencing Center, National Institute of Genetics, Japan) available at http://dolphin.lab.nig.ac.jp/medaka, and the following medaka-specific real-time PCR primers were designed using PrimerQuest (Integrated DNA Technologies): GGT1, forward primer 5’-AGTGGTTGTTATTGGTCTGGTTGGCC-3’, reverse primer 5’-GGTGGTTCCTGACATTGCCAGAGA-3’; BSEP, forward primer 5’-TGGATTGGTTAGACATGACGACAGA-3’, reverse primer 5’-TTCTTACTCTGCTTAGGCCCC-3’, CYP1A, forward primer 5’-ACATGGCGCTTACCCCGAAGGATTTATATC-3’, reverse primer 5’-AGGTGGAGGTTGCTGCTGAAGGAAA-3’, reverse primer 5’-TTCTGATTGGTTTCTCCATAGTTA-3’, and β-actin, forward primer 5’-ACAAGCAACACCTTGCCAGAGA-3’, reverse primer 5’-AGGAGGAGTCTGCTGCTGCAAAG-3’. PCR products were amplified separately in duplicate using a Quantitect SYBR Green PCR Kit (Qiagen) and an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Relative quantitation of gene expression and

<table>
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<th>Primer name</th>
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*Primer direction (F = forward; R = reverse).

Primer melting temperature (°C).

*Sequencing was performed directly off the final expression construct for mAHRIα (in pSG5 vector) and reporter construct for mf1lp (in pGL3-Basic vector).

95% confidence intervals were calculated using the comparative cycle threshold (Ct) method provided in the manufacturer’s instructions (User Bulletin #2, ABI PRISM 7700 Sequence Detection System, Applied Biosystems).

Expression and reporter constructs. Per manufacturer’s instructions, genomic DNA was isolated from adult male STII medaka liver using a DNeasy Tissue Kit (Qiagen). All primers used for construction of expression and reporter plasmids are listed in Table 1.

The mAHRIα expression vector was constructed as follows. Primers for full-length STII medaka AHR1α were designed using a publicly available orange-red medaka AHR1α mRNA sequence (GenBank Accession No. AB065092). Full-length AHR1α cDNA was first amplified from 200 ng STII medaka liver cDNA using Advantage 2 Polymerase Mix (Clontech, Mountain View, CA) and primers without nested restriction sites (mfAHR1α-f and mfAHR1α-r). As medaka AHR1α contains a high G/C content (62%), the addition of DMSO (5% vol/vol) to the PCR reaction was necessary to ensure complete strand denaturation prior to primer annealing and extension. In addition, long-distance PCR (LD-PCR) was used to ensure complete amplification of full-length AHR1α (2.6 kb). LD-PCR reaction conditions were: 95°C for 1 min followed by 10 cycles of 95°C for 10 s and 72°C for 1 min, 25 cycles of 95°C for 10 s and 68°C for 3 min plus 10 s/cycle, and 1 cycle of 70°C for 10 min. PCR product was purified using a QIAquick PCR Purification Kit (Qiagen), and full-length AHR1α containing 5’-EcoRI and 3’-BglII restriction sites was reamplified using the same PCR reaction conditions as above. Gel-purified AHR1α containing restriction sites was ligated into pCR 2.1-TOPO vector (TOPO TA Cloning Kit, Invitrogen) and Subcloning Efficiency DH5α Competent Cells (Invitrogen) were transformed with the resulting plasmid. Plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen) and

TABLE 1

Primer Sequences for Cloning and Sequencing STII Medaka AHR1α (Full-Length) and CYP1A Promoter (Partial Sequence)
checked for AHR1x insert by digesting with EcoRI overnight at 37°C. Plasmids containing the AHR1x insert were renamed pCR 2.1-mfAHR1x. Empty pSG5 expression vector (Stratagene, Cedar Creek, TX) and pCR 2.1-mfAHR1x were double-digested with EcoRI and BglII for 5 h at 37°C. Gel-purified mfAHR1x was then directionally cloned into pSG5 vector using T4 DNA ligase (New England Biolabs, Ipswich, MA) and Subcloning Efficiency DH5α Competent Cells (Invitrogen) were transformed with the resulting plasmid. Plasmids containing the AHR1x insert were renamed pSG5-mfAHR1x.

The AHR-E driven luciferase reporter vector was constructed as follows. Primers were designed to amplify a partial fragment (1205 bp) of the STII medaka CYP1A promoter (936 bp upstream to 210 bp downstream of the transcriptional start site) (Table 1). This promoter sequence (named here as mf1Ap) was identified by mapping medaka CYP1A mRNA (GenBank Accession No. AJ297923) to version 200406 of the medaka genome (scaffold911: 25,996–29,142), and exported and searched manually for AHR-Es (5‘-CACGCA-T-3‘). Based on the medaka genome online, the promoter sequence used for cloning and construction of the reporter contained 6 AHR-Es upstream of the transcriptional start site. mf1Ap was amplified from 100 ng STII medaka liver genomic DNA using Advantage 2 Polymerase Mix (Clontech) and primers without nested restriction sites (mf1Ap-f and mf1Ap-r). PCR reaction conditions were: 95°C for 1 min followed by 35 cycles of 95°C for 10 s and 68°C for 1.5 min. PCR product was purified using a QIAquick PCR Purification Kit (Qiagen), and mf1Ap containing 5’-MluI and 3’-SmaI restriction sites was reamplified using the same PCR reaction conditions as above with a 10-min extension at 70°C. Gel-purified mf1Ap containing restriction sites was ligated into pCR 2.1-TOPO vector (TOPO TA Cloning Kit, Invitrogen) and Subcloning Efficiency DH5α Competent Cells (Invitrogen) were transformed with the resulting plasmid. Plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen) and checked for mf1Ap insert by digesting with SmaI for 1 h at 25°C and MluI overnight at 37°C. Empty pG3.Basic (Promega, Madison, WI) was also digested using the same restriction enzymes and incubation conditions. Gel-purified mf1Ap was directionally cloned into pGL3-Basic vector using T4 DNA ligase (New England Biolabs) and Subcloning Efficiency DH5α Competent Cells (Invitrogen) were transformed with the resulting plasmid. Plasmids containing mf1Ap insert were renamed mf1Ap-Luc.

Inserts were sequenced by MWG Biotech (High Point, NC) to verify correct sequence and orientation. To ensure complete sequence coverage of mfAHR1, T7 promoter primer and three primers nested within mfAHR1 were designed to amplify a partial fragment (1205 bp) of the STII medaka gene containing the AHR1 promoter (Stratagene, Cedar Creek, TX) and primers without nested restriction sites (mf1Ap-f and mf1Ap-r). PCR reaction conditions were: 95°C for 1 min followed by 35 cycles of 95°C for 10 s and 68°C for 1.5 min. PCR product was purified using a QIAquick PCR Purification Kit (Qiagen), and mf1Ap containing 5’-MluI and 3’-SmaI restriction sites was reamplified using the same PCR reaction conditions as above with a 10-min extension at 70°C. Gel-purified mf1Ap containing restriction sites was ligated into pCR 2.1-TOPO vector (TOPO TA Cloning Kit, Invitrogen) and Subcloning Efficiency DH5α Competent Cells (Invitrogen) were transformed with the resulting plasmid. Plasmids containing mf1Ap insert were renamed mf1Ap-Luc.

RESULTS

Control Liver Morphology

A description of the anatomy and biliary system of control adult STII medaka (Fig. 1A) was necessary before accurately interpreting hepatobiliary changes following acute ANIT exposure. For a detailed description of the intrahepatic biliary passageways in medaka, refer to Hardman et al. (2007). Hepatic tubules of medaka are two to three hepatocytes thick in longitudinal section and are individually separated by sinusoidal passageways (Fig. 1B). Similar to the lobular liver, canaliculi are filled by microvillar projections from plasma membranes of hepatocytes (Fig. 1C). However, in medaka liver, an interconnected network of canaliculi travels a short distance before draining into the tubule lumen (or bile preductule). Lining these tubule lumens are bile preductular epithelial cells (BPDECs) (Fig. 1B). BPDECs are small in size (approximately 30% the size of hepatocytes), have a high nuclear to cytoplasmic ratio, and share apical junctional complexes with hepatocytes (Fig. 1C). Because the lumens of hepatic tubules in medaka function as biliary channels, and these tubules anastamose throughout the liver, there is likewise a branching interconnected network of bile preductules (lined with BPDECs) and ductules (near the hilus, with cuboidal BECs) within the
parenchyma that ultimately drain into three large-diameter intrahepatic bile ducts. The latter merge to feed one or two bile ducts at the liver hilus (Fig. 1D). In medaka, cuboidal to low columnar biliary epithelia are present and line the intrahepatic ducts and common bile duct (Fig. 1D). The common extrahepatic bile duct is lined by columnar epithelium with well-developed luminal microvilli (Fig. 1D). As in mammals, the hepatic duct drains into the gallbladder via the cystic duct and intestine via the common bile duct, and the confluence of the efferent bile ducts, the afferent portal vein and the hepatic artery at the liver hilus, are surrounded by connective tissue (Fig. 1D).

**ANIT-Induced Pathogenesis**

Fish survival for all exposures performed in this study was approximately 100% for vehicle-treated fish and 90% for ANIT-treated fish. For 4-month-old male STII medaka weighing 270 ± 30 mg wet weight, an approximate effective dose of ANIT with 10% mortality (LD$_{10}$) was 40 mg/kg wet weight. All data henceforth is described based on the use of this ANIT dose. Following a single dose of ANIT, swelling of eosinophilic cells with small nuclei was observed by 6 h on routine histologic sections of the liver (Fig. 2A). These swollen features were present throughout the parenchyma of the liver in exposed fish. Because hepatocytes appeared healthy, these data suggested that BDPECs, and not hepatocytes, were injured. However, as the resolution of paraffin-embedded histologic sections (5–6 μm) was not adequate for unequivocal identification of the swollen cell type, high-resolution preparations were required for further examinations. At the same magnification as Figure 2A (100×), a semithin preparation (500 nm) readily revealed glycogen-containing normal hepatocytes but swollen and apoptotic BPDECs (Fig. 2B). Indeed, high magnification of ultrathin preparations (90 nm) revealed swollen BPDECs (Fig. 2C) and apoptotic BPDEC fragments within macrophages (Fig. 2D).

Based on mRNA-level data, the hepatobiliary effects due to a single ANIT dose appeared to persist for 72 h. Using real-time PCR, relative levels of GGT1 and BSEP mRNAs were measured at all time points in ANIT-exposed livers (Fig. 3). GGT1 is a specific marker for BECs (Okihiro and Hinton, 2000), whereas
BSEP is a specific marker for bile transporters localized to canalicular membranes (Green et al., 2000; Keitel et al., 2005). GGT1 transcript levels were significantly decreased by approximately 14-fold at the same time point (6 h) that showed significant BPDEC toxicity, and returned to control levels by 24 h (Fig. 3). BSEP transcript levels were significantly decreased by 2- and 3.5-fold at 48 and 72 h, respectively, and returned to control levels by 96 h (Fig. 3), suggesting that BSEP levels were affected following initial injury to BPDECs.

In vivo imaging of STII medaka showed pronounced discoloration of the gallbladder following ANIT exposure. Figure 4A shows representative brightfield images of the right lateral side of living STII medaka. Relative to vehicle-exposed fish, the gallbladders of ANIT-exposed fish first appeared darker green by 48 h, and progressively darkened in coloration by 72 h. However, by 96 h, the gallbladders of ANIT-exposed fish returned to a light green color similar to 24 h post-ANIT exposure. Because altered concentrations of bile pigments were probably responsible for these changes, these data suggested that bile flow and/or gallbladder bile composition was disrupted from 48- to 72-h post-ANIT exposure. Therefore, as an indicator of total bile concentration, relative levels of the dominant medaka gallbladder bile pigment BV were determined by measuring bile absorbance at 660 nm in vehicle- and ANIT-exposed gallbladders. The wavelength selected (660 nm) for analysis of gallbladder bile was the maximal absorption for BV (Gray et al., 1961). Using this approach, relative BV levels in fresh gallbladder bile from ANIT-exposed fish were significantly increased at 72 h, but approached levels similar to vehicle-exposed fish by 96 h (Fig. 4B).

**FIG. 3.** Fold change of GGT1 and BSEP mRNA in ANIT-treated liver for 96 h. Relative quantitation of gene expression (fold change) and 95% confidence intervals were calculated using the comparative cycle threshold ($C_t$) method. Negative fold changes were calculated by dividing $-1$ by the fractional change (between 0 and 1) determined using the comparative $C_t$ method. Fold changes above (+1) or below (−1) the horizontal solid lines indicate an increase or decrease, respectively, in gene expression relative to the time-matched control. Asterisk denotes significant difference between treatment and time-matched control ($p<0.05$).

**FIG. 4.** Biliary changes in STII medaka for 96 h after a single dose of ANIT. (A) Changes in gallbladder coloration for 96 h after a single dose exposure to ANIT. Live fish were anesthetized in chilled water, positioned in left lateral recumbency, and gallbladders imaged in right lateral view through the body wall under brightfield conditions. Note that ANIT-treated fish presented gallbladders with a darker green coloration from 48 to 72 h, with the darkest color occurring at 72 h. GB, gall bladder; L, liver. Scale bar = 0.5 cm. (B) Relative gallbladder biliverdin concentrations in STII medaka for 96 h after a single dose exposure to ANIT. Asterisk denotes significant difference between treatment and time-matched control ($p<0.05$).

**Transient AHR Activation during Hepatobiliary Alteration**

To assess in vivo AHR activation, hepatic transcript levels of UDPGT and CYP1A were measured at 6, 24, 48, 72, and 96 h following initial ANIT exposure. CYP1A transcripts were significantly induced in a time-dependent manner by 2.0, 3.5-, and 7-fold at 6, 24, and 48 h, respectively, and returned to control levels by 72 h (Fig. 5). In contrast, UDPGT transcript levels were not significantly induced at all time points sampled (Fig. 5). These data show that CYP1A is transiently induced following ANIT-induced biliary alteration, and endogenous bile-related compounds that accumulate in the liver may activate AHR and transcription of metabolism genes (e.g., CYP1A) involved in promoting bile clearance. To better address this hypothesis, in vivo studies were then performed to determine whether nontoxic levels of an AHR agonist (dioxin) could protect against or mitigate ANIT-induced biliary impairment.

A new cohort of fish were pretreated with either vehicle (DMSO) or dioxin (TCDD) for 24 h, then treated with vehicle
(DMSO) or ANIT for 72 h. Biliary impairment was evaluated by monitoring changes in gallbladder coloration every 24 h following initial pretreatment. As shown with ANIT-only exposures, fish pretreated with vehicle and then treated with ANIT resulted in the darkened gallbladder phenotype by 72 h post-ANIT exposure as described above (Fig. 6). The administered dioxin dose (0.1 μg/kg) did not appear to affect biliary status as fish pretreated with dioxin then vehicle did not show signs of discoloration (Fig. 6). Fish pretreated with dioxin and then treated with ANIT exhibited gallbladder coloration similar to ANIT-only–treated fish at 48 h, but then exhibited gallbladder coloration similar to controls (vehicle/vehicle and dioxin/vehicle) by 72 h post-ANIT exposure (Fig. 6). Therefore, although dioxin pretreatment did not protect ANIT-treated fish at 48 h, there was a clear protective effect of dioxin by 72 h post-ANIT exposure. At least with this injury model, these data suggest that AHR activation appears to attenuate biliary injury following ANIT-induced BPDEC toxicity at 6 h. However, these in vivo findings do not indicate what chemicals within bile may potentially activate AHR and initiate an adaptive response pathway.

Bilirubin-Induced AHR:AHRE-Mediated Transcription

A luciferase reporter-based system was then developed to determine if chemicals that are predominant in medaka bile initiate AHR-mediated transcription. The expression and reporter plasmids used were STII medaka AHR1α (pSG5-mfAHR1α), mouse AHR (pSPORT-mAHR), and a medaka CYP1A promoter-based firefly luciferase reporter vector (mf1Ap-Luc). Mutant mouse hepatoma cells (Hepa-1c1c7

![FIG. 5. Fold change of UDPGT and CYP1A mRNA in ANIT-treated liver for 96 h. Note that mRNA levels increase up to 48 h and return to near-control levels by 72 h. Relative quantitation of gene expression (fold change) and 95% confidence intervals were calculated using the comparative cycle threshold (Ct) method. Negative fold changes were calculated by dividing −1 by the fractional change (between 0 and 1) determined using the comparative Ct method. Fold changes above (+ 1) or below (−1) the horizontal solid lines indicate an increase or decrease, respectively, in gene expression relative to the time-matched control. Asterisk denotes significant difference between treatment and time-matched control (p<0.05).]

![FIG. 6. Changes in gallbladder coloration in STII medaka following a 24-h pretreatment with vehicle (DMSO) or 0.1 μg/kg TCDD and a 72-h treatment with vehicle (DMSO) or ANIT. Note that fish treated with vehicle then ANIT resulted in a darkened gallbladder by 72 h post-ANIT exposure (as shown in Fig. 4A), whereas fish pretreated with TCDD and then treated ANIT exhibit gallbladder coloration similar to controls (vehicle/ANIT and TCDD/vehicle) by 72-h post-ANIT exposure.]}
(c12 cells) with significantly reduced constitutive AHR activity were used in order to prevent high background levels of reporter activity and to definitively identify AHR-specific ligands. Dioxin (TCDD) was used as a positive control for reporter assay development and optimization. The chemical structures of all compounds screened are provided in Figure 7.

Prior to assay optimization, expression of STII medaka AHR1a mRNA following pSG5-mfAHR1a transfection into Hepa-1c1c7 c12 cells was determined using RT-PCR. Although this construct expressed well in these cells compared to cells transfected with empty vector (pSG5 only) (Fig. 8A), dioxin exposure (0.01–100nM) did not significantly induce luciferase reporter activity in cells expressing STII mfAHR1a (Fig. 8B). However, when cells were transfected with mouse AHR (pSPORT-mAHR), there was a significant dose-dependent response in reporter activity (Fig. 8B). Relative to vehicle-treated cells, significantly induced reporter activity was detected at 0.1, 1, 10, and 100nM (Fig. 8B). Although STII mfAHR1a did not respond to dioxin, STII mfAHR1a may be a receptor for other non-dioxin-like ligands. For this reason, the ability of the remaining chemicals in Fig. 7 to induce reporter activity were evaluated.

Observations in vivo indicated that hepatic CYP1A mRNA levels were increased following ANIT-induced biliary impairment (Fig. 5). This does not exclude the possibility that ANIT is simply an AHR agonist. Therefore, ANIT was screened to eliminate the possibility that AHR activation in vivo following ANIT-induced biliary impairment was due to ANIT binding and activation of AHR. The chemical structure for ANIT is provided in Figure 7. Using the in vitro reporter system, ANIT exposure (1–100μM) did not significantly induce luciferase reporter activity in cells transfected with empty vector, mouse AHR, or STII medaka AHR1a (Fig. 9A). Significantly decreased activity with each vector was detected at the highest dose (100μM) and was due to significant cytotoxicity at this dose. Thus, these data suggest that ANIT is not an agonist for mouse AHR or medaka AHR1a.

**FIG. 7.** Structures of chemicals screened for medaka AHR1α- and mouse AHR-dependent AHRE-driven firefly luciferase reporter activity in mutant Hepa-1c1c7 c12 cells. 2,3,7,8-TCDD (dioxin) was used as a positive control for testing reporter assay functionality. BV and bilirubin are bile pigments, and cholic acid and taurocholic acid are bile acids.
BILIARY TOXICITY AND AHR RESPONSE IN MEDAKA

Vertebrate bile is composed of a complex mixture of cholesterol, bile acids, and bile pigments. As bile acid and bile pigment composition varies widely across vertebrate taxa (Cornelius, 1991; Krasowski et al., 2005; Moschetta et al., 2005), it was imperative to estimate the predominant components of medaka bile (e.g., those that are relevant to this species) prior to screening biliary compounds for potential AHR agonist activity. Based on HPLC studies done in collaboration with Dr Lee Hagey (University of California, San Diego), medaka gallbladder bile is predominantly composed of C27 taurine-conjugated bile acids (~50%) and C24 taurine-conjugated bile acids (~50%). The dominant C24 taurine-conjugated cholic acid in medaka is the common bile salt taurocholic acid. Based on this composition, medaka probably has a bile salt pathway consisting of conversion of C27 alcohol sulfates to C27 taurine-conjugated acids to C24 taurine-conjugated acids (L. Hagey, personal communication). Analytical work has not yet been performed on the bile pigment composition; however, based on data presented in Figure 4 and previous research on bile pigments in fish (reviewed in Cornelius, 1991), medaka bile is probably composed of the bile pigment BV and BV conjugates with trace amounts of bilirubin and bilirubin conjugates. Therefore, bile-related chemicals screened using this reporter assay were BV, bilirubin, cholic acid, and taurocholic acid (Fig. 7).

Similar to ANIT, BV caused significant cytotoxicity at 100µM, resulting in decreased luciferase activity in cells transfected with each vector and did not induce luciferase reporter activity at the lower BV doses (Fig. 9B). In contrast, bilirubin exposure significantly increased reporter activity in cells transfected with mouse AHR (pSPORT-mAHR) and exposed to 50µM, but not 100µM (Fig. 9C). This effect was not observed in cells transfected with empty vector (pSG5) or STII medaka AHR1α (pSG5-mfAHR1α). Therefore, similar to TCDD, bilirubin is a specific agonist (albeit weak) for mouse AHR but not medaka AHR1α in this system. However, when cells were exposed to equimolar mixtures of BV and bilirubin, significant toxicity was observed at a total concentration of 50 and 100µM (Fig. 9D). Because cytotoxicity was only observed at 100µM BV exposure, and toxicity was not observed with all bilirubin doses tested, these data demonstrate that coexposure to BV and bilirubin results in the potentiation of BV cytotoxicity by bilirubin. Moreover, luciferase reporter induction by bilirubin-only exposure is prevented when cells are also exposed to BV. Lastly, cholic acid or taurocholic acid exposure did not induce luciferase reporter activity with all plasmids and doses tested (Figs. 9E and 9F). As these bile acids are predominant in medaka bile, these data suggest that bile acids are not agonists for medaka AHR1α.

DISCUSSION

In this study, ANIT was selected on the basis of its well-established biliary toxicity including cholestasis in mammalian animal models. In rodents, ANIT diffuses from the sinusoidal lumen into hepatocytes and forms a reversible conjugate with glutathione (GSH) (Carpenter-Deyo et al., 1991). Following active transport across canalicular membranes into bile, ANIT:GSH dissociation results in high-dose exposure of BECs to ANIT (Carpenter-Deyo et al., 1991). Consequently, within 24–48 h postexposure, BEC necrosis is extensive, bile flow and bile acid excretion are significantly reduced, and serum bile acids and total bilirubin are significantly increased (Kossor et al., 1993, 1995). By 72 h, debris from necrotic BECs is cleared and, by 96 h, normal bile flow is restored, serum bile acids and bilirubin levels return to control levels, and bile acid and bilirubin excretion is increased (Kossor et al., 1993). In addition, BEC hyperplasia occurs within 48–72 h following restoration of biliary function (Kossor et al., 1995, 1998).

In medaka, ANIT specifically targeted BDPECs localized within hepatic tubules by 6 h following exposure (Fig. 2). Significantly decreased levels of GGT1 mRNA—a marker

![Figure 8](image-url)
specific to medaka BDPECs (Okihiro, 1996)—in ANIT-exposed fish also suggested toxicity to BPDECs as early as 6-h postexposure (Fig. 3). Interestingly, BPDEC-specific toxicity appears to be a function of the unique architecture of fish liver. In medaka liver, canicular passageways are short in length and traverse the width of one hepatocyte before abutting with the tubule lumen wall (Hardman et al., 2007). BPDECs are located within tubule lumens and, because tubules

FIG. 9. AHR:AHRE-driven firefly luciferase activity following transfection with empty expression vector (pSG5), STII medaka AHR1α expression vector (pSG5-mfAHR1α), or mouse AHR expression vector (pSPORT-mAHR). All cells were transfected with AHRE-driven reporter vector (mf1Ap-Luc) and transfection control (pRL-CMV) to assess firefly and Renilla luciferase activity respectively. Immediately following a 5-h transfection, cells were exposed for 18 h to (A) α-naphthylisothiocyanate (ANIT), (B) BV, (C) bilirubin, (D) BV and bilirubin, (E) cholic acid, or (F) taurocholic acid. Crosses (†) denote significant treatment effect (p<0.05) relative to vehicle controls (N= 4 wells per treatment).
anastamose throughout the entire liver, BPDECs are abundant within the hepatic parenchyma (Hardman et al., 2007). Consequently, BPDECs were exposed to ANIT once ANIT was transported into the hepatic tubule lumen (or bile preductule) and acute BPDEC damage resulted in swelling and obstruction of biliary passageways.

Evidence for biliary toxicity from high-resolution light microscopy and TEM showed BPDEC swelling, necrosis, and apoptotic fragmentation. Whether cholestasis was produced was difficult to assess in these small organisms. Repeated attempts of serum enzymatic analysis—a hallmark feature of was difficult to assess in these small organisms. Repeated attempts of serum enzymatic analysis—a hallmark feature of

Consequently, BPDEC swelling resulted in swelling from 24 to 48 h, direct, clinical evidence of bile stasis (e.g., elevated serum bilirubin) was not conclusively demonstrated. Using conventional colorimetric assays, elevated serum total bilirubin, alanine aminotransferase, alkaline phosphatase, and GGT was measured in vehicle- and ANIT-treated fish. Based on these assays, treatment-level differences were not detected at any of the time points sampled. Although these assays were scaled down to 96-well format, the lack of effect was probably due to assay sensitivity because microliter serum quantities were only attainable from the caudal vein of each fish. Therefore, at this time, definitive clinical data corroborating widely published clinical data in rodent models of ANIT-induced cholestasis does not exist for medaka. However, the data presented herein collectively suggest that ANIT-induced hepatobiliary injury in medaka resulted in a bile stasis-like phenotype similar to rodents. In rats, maximal cholestasis (24–48 h) following ANIT exposure is accompanied by significantly reduced canalicular bile flow and bile acid excretion (Kossor et al., 1993). In ANIT-exposed medaka liver, relative levels of BSEP mRNA progressively decreased up to 72 h and returned to control levels by 96 h (Fig. 3), suggesting that alterations in bile transport occurred during ANIT-induced biliary injury.

ANIT-induced biliary obstruction resulted in marked alteration of gallbladder bile color (light green to dark green) from 48–72 h that was visible through the body wall of STII medaka (Fig. 4A). Suspecting that the coloration change was due in part to altered bile pigment concentrations, relative levels of BV in vehicle- and ANIT-exposed gallbladder bile were measured over 96 h. As shown in Figure 4B, significantly increased BV levels were strongly correlated with the dark green color of the gallbladder at 72 h. Similar changes in the color of gallbladders have also been observed in fasted fish (Love, 1980). Heme degradation is catalyzed by oxidation of heme to BV by heme oxygenase, and reduction of BV to bilirubin by BV reductase (Kapitulnik, 2004). In mammals, bilirubin conjugation is mediated by UDPGT prior to excretion into bile, and, during oxidative stress and increased cytochrome P450 activity, bilirubin may be oxidized to BV (De Matteis et al., 2006). However, in fish, BV reductase and UDPGT levels are significantly lower than mammals and, consequently, bile tends to be dominated by BV rather than conjugated bilirubin (Cornelius, 1991). Therefore, a significant increase in gallbladder BV and its derivatives (rather than conjugated or unconjugated bilirubin) in ANIT-exposed fish at 72 h probably contributed to the dark green color observed through the body wall of STII medaka.

A significant time-dependent increase in CYP1A transcription preceded the dark green gallbladder phenotype (an indicator of bile clearance), suggesting that CYP1A may be involved in bile clearance following ANIT-induced biliary injury. Therefore, we assessed whether enhanced AHR activation prior to ANIT exposure could mitigate the bile stasis-like phenotype observed in medaka. By 72-h post-ANIT exposure, fish pretreated with dioxin (0.1 μg/kg) for 24 h exhibited gallbladders that were similar to controls (vehicle/vehicle and dioxin/vehicle) and were significantly different than fish pretreated with vehicle and then treated with ANIT (Fig. 5). Although not conclusive, these data suggest that AHR activation partially protected against ANIT-induced bile accumulation. Similar studies in rodents have demonstrated that PXR or FXR activation by agonist pretreatment protects against bile acid-induced liver injury (Liu et al., 2003; Staudinger et al., 2001). However, only one published study has demonstrated that AHR activation may ameliorate liver injury due to bile accumulation. In this study, Gunn rats were treated with vehicle or dioxin (10 μg/kg), and plasma bilirubin concentrations, bilirubin kinetics, and bile pigment excretion were measured (Kapitulnik and Ostrow, 1977). Rats treated with dioxin exhibited a 60% decrease in plasma bilirubin with a significant increase in bile flow. However, bile acid concentration and bile acid output were not affected by pretreatment. The authors concluded that dioxin stimulated increased bilirubin (and not bile acid) elimination in the absence of UDPGT-meditated glucuronidation. Similarly, dioxin may have provided partial protection from ANIT-induced biliary changes in medaka, a mechanism in part due to sustained bile pigment metabolism by AHR target genes such as CYP1A.

Because CYP1A transcription was significantly increased during ANIT-induced biliary injury, an AHRE-driven firefly luciferase-based reporter assay was developed to directly determine whether bile-related chemicals were potential AHR agonists in medaka. For reasons currently unknown, medaka AHR1α was not responsive to dioxin in this system even though mRNA expression levels of this construct were high compared to cells transfected with empty vector (Fig. 8B). However, cells transfected with mouse AHR were strongly responsive to dioxin with maximal reporter induction at 10 and 100nM (Fig. 8B). This response was specifically a function of AHR transfection, as cells transfected with empty expression vector did not respond to dioxin exposure (Fig. 8B). Thus, although medaka AHR1α was not responsive, dioxin did activate mouse AHR and binding to medaka-specific CYP1A promoter. Despite the interspecies variation in the primary
AHR protein sequence (Fig. 10), these data demonstrate that, when activated, mouse AHR binds to the medaka-specific CYP1A promoter, resulting in AHRE-driven transcription. Medaka AHR1α may not have been responsive to dioxin for several reasons. First, and most probable, AHR1α may not be the dioxin receptor ortholog in medaka. Additional explanations for negative dioxin responsiveness are (1) lack of sufficient medaka AHR1α mRNA expression in mouse Hepa-1c1c7 cells (albeit, expression did occur as shown in Fig. 8A), (2) lack of medaka AHR1α translation in mouse Hepa-1c1c7 cells, (3) lack of medaka AHR1α heterodimerization with mouse ARNT, and/or (4) lack of signals or coregulators in mouse Hepa-1c1c7 cells that are required for normal medaka AHR1α signaling. AHR1α was originally chosen because of high sequence similarity to killifish AHR1, the functional dioxin receptor in this teleost. As AHR1β is a splice variant of AHR1α containing a minimally truncated C-terminal end (99 amino acid truncation), and there are otherwise no sequence differences in the 879-aa protein, AHR1β is probably not the dioxin receptor in medaka. However, functional tests are needed to confirm or refute this hypothesis. In zebrafish, AHR2 is the dioxin receptor, whereas AHR1α is nonresponsive to TCDD exposure (Andreasen et al., 2002; Karchner et al., 2005). Likewise, Prasch et al. (2003) demonstrated that zebrafish AHR2 mediates TCDD-induced developmental toxicity in zebrafish. Therefore, AHR2 may be the functional dioxin receptor in medaka. Medaka have an AHR2 gene, as a predicted AHR2 mRNA sequence was mapped to scaffold4360, position: 32,879–42,180 (genome assembly revision 200406), and phylogenetic and sequence analysis indicates that it clusters with zebrafish and killifish AHR2 (Fig. 10). During the course of this work, attempts to amplify STII medaka AHR2 from hepatic cDNA were unsuccessful. Future functional studies using medaka-specific AHR2 and ARNT expression plasmids are needed to test whether AHR2 is the dioxin receptor, and whether medaka ARNT is specifically required for transactivation in mouse hepatoma cells.

Reporter assay data for all chemicals are summarized in Table 2. Of the five compounds screened (ANIT, BV, bilirubin, cholic acid, and taurocholic acid) (Fig. 7), bilirubin (50 μM) was the only chemical to induce luciferase activity following transfection of mouse AHR (but not medaka AHR1α) (Fig. 9C). On the one hand, these data simply confirm previous findings demonstrating that bilirubin weakly activates in vitro binding of AHR to AHRE complexes in mouse Hepa-1c1c7 cells (Sinal and Bend, 1997). On the other hand, similar to
dioxin, these data uniquely demonstrate that bilirubin can activate mouse AHR and binding to medaka-specific CYP1A promoter, resulting in AHR-driven transcription.

Exposure to the remaining chemicals exhibited no significant treatment differences in luciferase activity, or induced cytotoxicity at the highest dose (100 μM) tested. Coexposure of BV and bilirubin resulted in elimination of bilirubin-induced reporter activity in mouse AHR-transfected cells; in fact, the potentiation of BV cytotoxicity by bilirubin was observed at combined doses of 50 and 100 μM (Fig. 9D). These data show that BV, cholic acid, and taurocholic acid are not ligands for mouse AHR, and BV, bilirubin, cholic acid, and taurocholic acid are not ligands for STII medaka AHR1α. However, future tests are needed to assess the possibility of upstream, indirect activation of AHR by bile acids or bile pigments. Similar indirect mechanisms of activation have been shown with constitutive androstane receptor–mediated protective responses to toxic bilirubin levels in mammals (Huang et al., 2003).

In summary, ANIT exposure in medaka resulted in BPDEC toxicity and hepatobiliary alteration, leading to the potential involvement of AHR in the protective response to ANIT-induced toxicity. A single ANIT dose specifically induced swelling and apoptosis of BPDECs as early as 6 h following exposure. Following ANIT-induced BPDEC toxicity, in vivo imaging of STII medaka showed significant gallbladder discoloration from 48–72 h, possibly due to elevated BV concentrations within the gallbladder. We also demonstrated that (1) transient AHR activation and CYP1A induction occurs during hepatobiliary impairment up to 48 h following a single dose of ANIT, (2) pretreatment with an AHR agonist partially protects against ANIT-induced biliary effects, and (3) the bile pigment bilirubin weakly activates mouse AHR (but not medaka AHR1α) and AHR-driven transcription in mouse hepatoma cells. These findings contribute to the body of AHR research by showing that cholestatic-like liver disease in medaka results in AHR activation, a response that is, similar to nuclear receptors, partially required for protection against bile-induced hepatocellular injury.

### TABLE 2
Summary of AHR Reporter Assay Data

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Vector control</th>
<th>Medaka AHR1α</th>
<th>Mouse AHR</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCDD</td>
<td>No</td>
<td>No</td>
<td>Yes (0.1, 1, 10, 100nM)</td>
</tr>
<tr>
<td>ANIT</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>BV</td>
<td>No</td>
<td>No</td>
<td>No</td>
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<tr>
<td>Bilirubin (BR)</td>
<td>No</td>
<td>No</td>
<td>Yes (50μM)</td>
</tr>
<tr>
<td>BV + BR</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Cholic acid</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Taurocholic acid</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

*Note. A significant increase (p < 0.05) in AHR:AHRE-driven firefly luciferase reporter activity is denoted by “Yes” with statistically significant dose(s) in parentheses.*

### SUPPLEMENTARY DATA

Color version of Figures 8 and 9 are available at http://toxsci.oxfordjournals.org/.

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### REFERENCES


