A Novel Plant Isoflavonoid that Causes Biliary Atresia

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Supplementary Materials

Eight figures (Fig. S1, Fig. S2, Fig. S3, Fig. S4, Fig. S5, Fig. S6, Fig. S7, Fig. S8) and two movies (Movie S1, Movie S2).

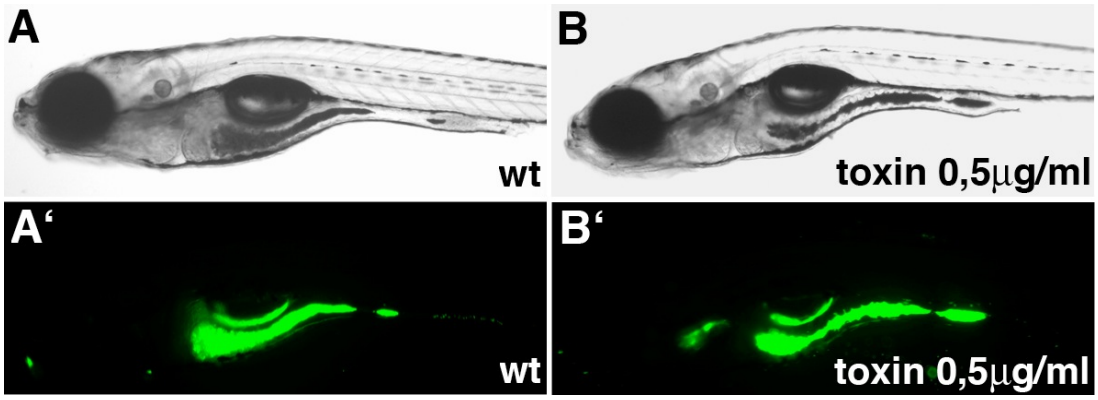

Fig. S1. Biliatresone does not alter swallowing function. Top panels (A, B) are lateral images of live wild type control (A) and biliatresone-treated larvae (72 hr treatment) that ingested fluorescent microspheres during treatment. Bottom panels (A′, B′) show corresponding fluorescent images of these larvae indicating that the control and toxin-treated larvae have ingested comparable amounts of the fluorescent microspheres. These images are representative of 20 larvae in each group.

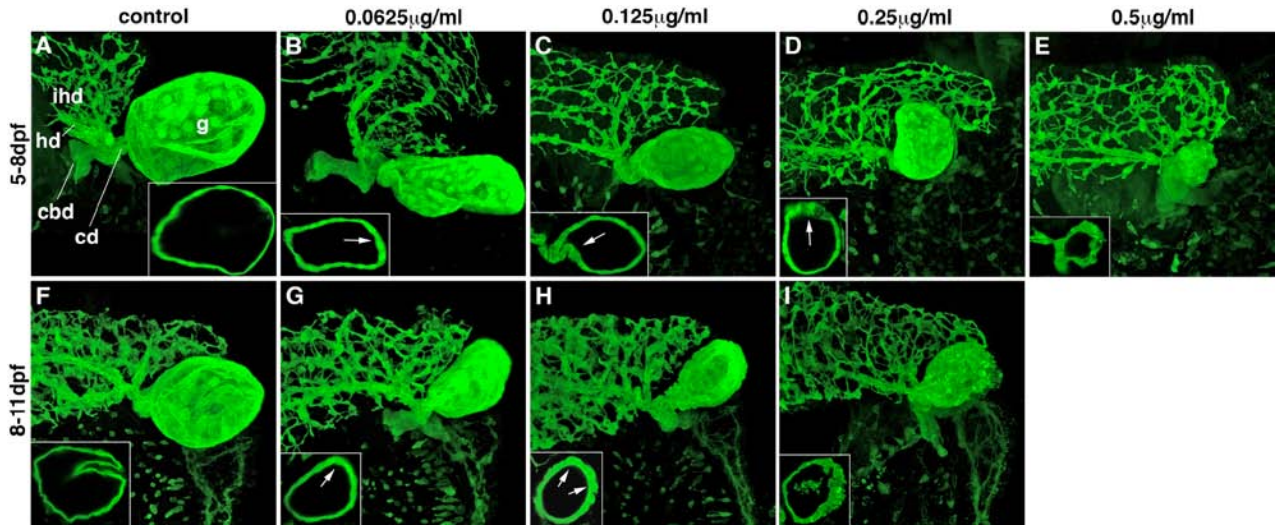

Fig. S2. Biliatresone dose response in zebrafish. (**A-D**) Confocal projections of the gallbladder and extrahepatic bile ducts of an 8 dpf immunostained control larva (untreated) and larvae treated with 4 different concentrations of biliatresone. The biliatresone dose is indicated above each panel. Insets show thin (1 μm) sections through the gallbladder that demonstrate progressive abnormalities of the gallbladder epithelium. Gallbladder epithelial defects (arrows) were first detected in larvae treated with 0.0625 μg per ml, and were more pronounced at higher doses. The earliest changes are manifest as asymmetric thickening of the epithelial layer. (**F-I**) Confocal projections of the gallbladder and extrahepatic bile ducts of an 11 dpf immunostained control larva (untreated) and 4 biliatresone-treated larvae (doses indicated). Gallbladder epithelial defects are first detected in larvae treated with 0.0625 μg per ml (thickened gallbladder wall seen in inset), and are more pronounced at higher doses.

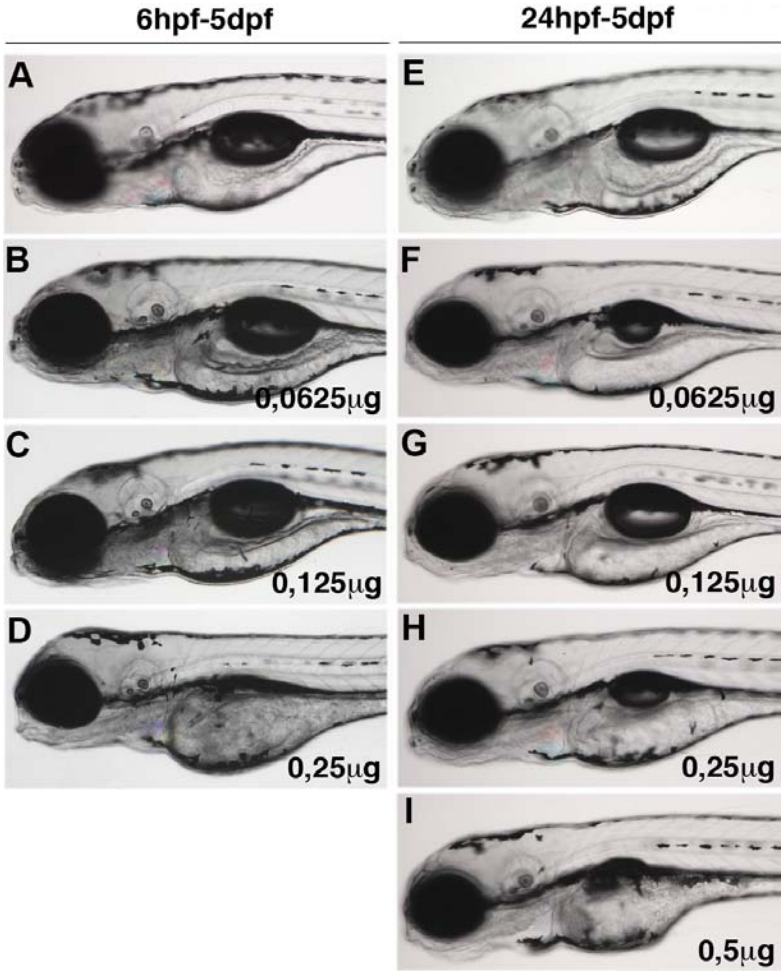

Fig. S3. Biliatresone treatment during embryogenesis. Lateral images of live larvae. (**A-D**) Larvae treated with biliatresone (0.0625 µg/ml and 0.125 µg/ml) beginning at 6 hpf through 5 dpf. The larvae treated with 0.0625 µg/ml and 0.125 µg/ml appear normal, whereas the larva treated with a higher dose (0.25 µg/ml) shows developmental delay but overall normal morphology. (**E-I**) Similar responses are seen with fish treated beginning at 24 hpf through 5 dpf.

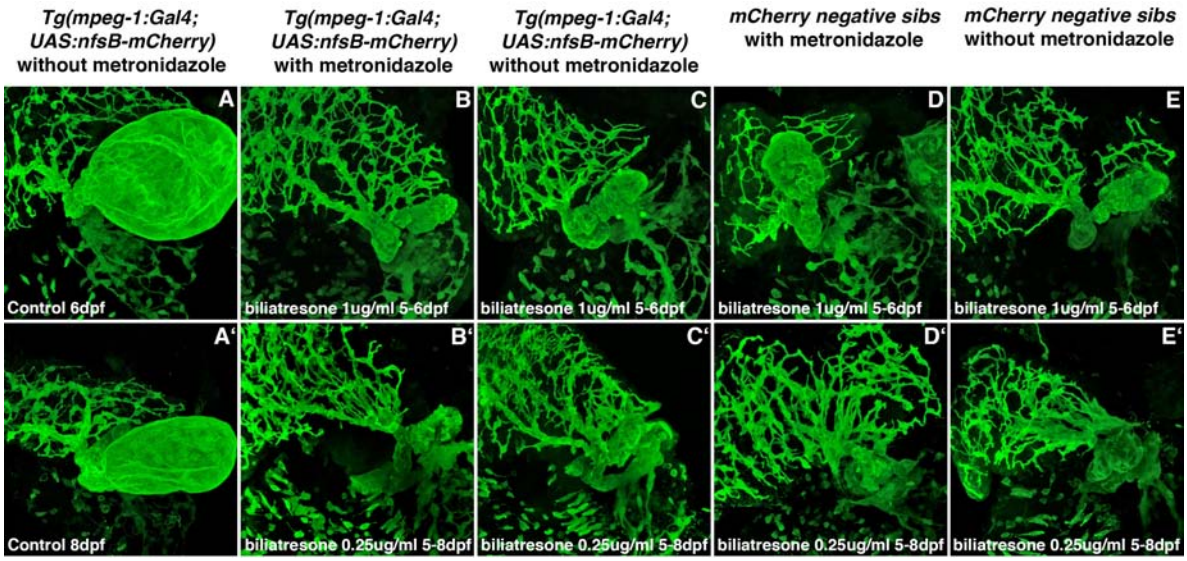

Fig. S4. Macrophages are not required for biliatresone-induced biliary injury. Confocal projections through the liver of *Tg(mpeg-1:Gal4; UAS:nfsB-mCherry)* larvae (A,A'-C,C'), and sibling larvae that lack mCherry expression (D,D'-E,E'). Larvae were sorted for mCherry expression at 24 hpf and immunostained with the 2F11 monoclonal antibody following the various treatments. Bigenic larvae express the mCherry fusion protein in macrophages. Bigenic larvae treated with metronidazole have few, if any macrophages, as determined by fluorescent microscopy. (A-E) Control bigenic larva that did not receive biliatresone (A) and larvae treated with biliatresone 1 ug/ml for 24 hrs beginning at 5 dpf (B-E). (A'-E') Control bigenic larva that did not receive biliatresone (A'), and larvae treated with biliatresone 0.25 ug/ml for 72 hrs beginning at 5 dpf (B'-E'). Metronidazole treatment induced macrophage ablation as evidenced by loss of mCherry cells (not shown). Biliatresone treatment causes profound changes in gallbladder morphology regardless of whether macrophages have been ablated (B,B') or not (C,C'-E,E'). Images representative of 6 larvae from each group.

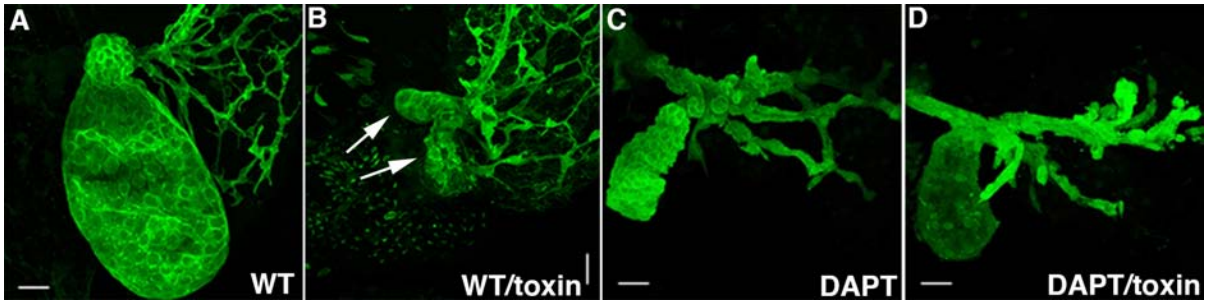

Fig. S5. Biliatresone toxicity is dependent on bile secretion. Confocal projections through the gallbladder and liver of 6 dpf larvae immunostained with the 2F11 antibody. (A) Wild type (WT) control larva. (B) Wild type larva following 24 hr treatment with biliatresone (1.0 ug/ml; 120 hpf to 144 hpf). There is nearly complete atresia of the gallbladder (arrows). (C) Wild type larva treated with DAPT from 48 hpf to 144 hpf. Gallbladder size is reduced by DAPT treatment and there are only a small number of enlarged, underdeveloped intrahepatic ducts that do not communicate with hepatocytes *(44)*. (D) Wild type larva treated with DAPT from 48 hpf to 144 hpf and biliatresone (1.0 ug/ml) from 120 hpf to 144 hpf. Gallbladder size is unchanged by biliatresone treatment in the DAPT-treated larva. These images are representative of 10 biliatresone treated larvae (Table S1).

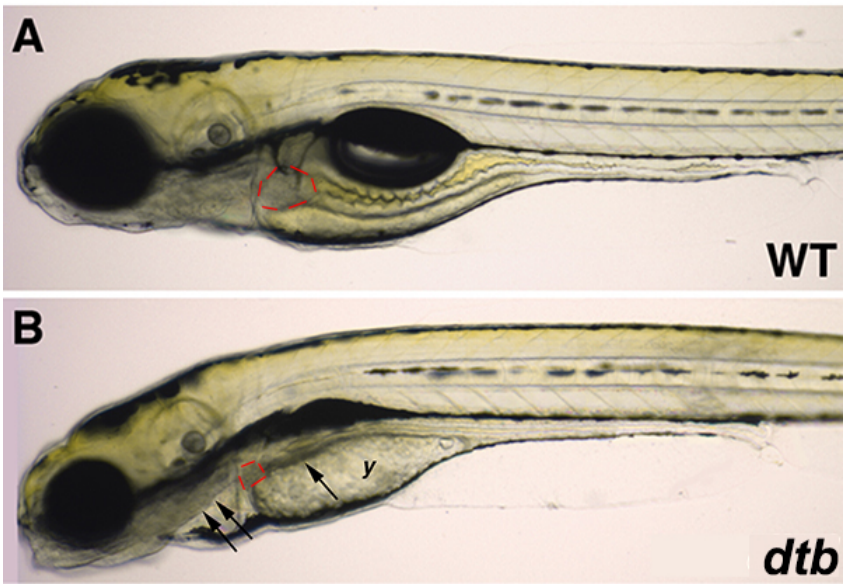

Fig. S6. Zebrafish *ductbend* mutants. Lateral images of live 5 dpf wild type (WT) and *ductbend* (*dbd*) larvae. Compared to the WT larva, the *dbd* larva has intestinal (arrow) and liver hypoplasia (red lines) and delayed branchial arch development (double arrows). The head and eye of the mutants are both smaller than in WT. *y* – residual yolk.

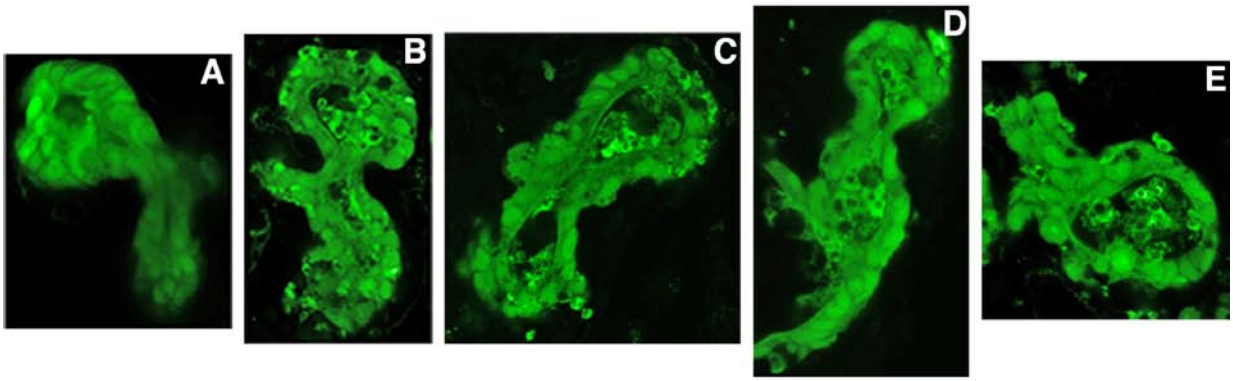

Fig. S7. *ductbend* sensitivity to biliatresone. Confocal images (5 uM) through the gallbladder and extrahepatic ducts of 4 *ductbend* mutants immunostained with the 2F11 monoclonal antibody. (A, B) Identical mutant larvae presented in Fig. 4. Panels show tissue morphology in an untreated (A) and biliatresone treated (B) larva. Note cellular debris in the gallbladder and duct lumen as well as evidence of cholangiocyte degeneration. (C-E) Similar findings in 3 additional biliatresone treated *ductbend* mutants.

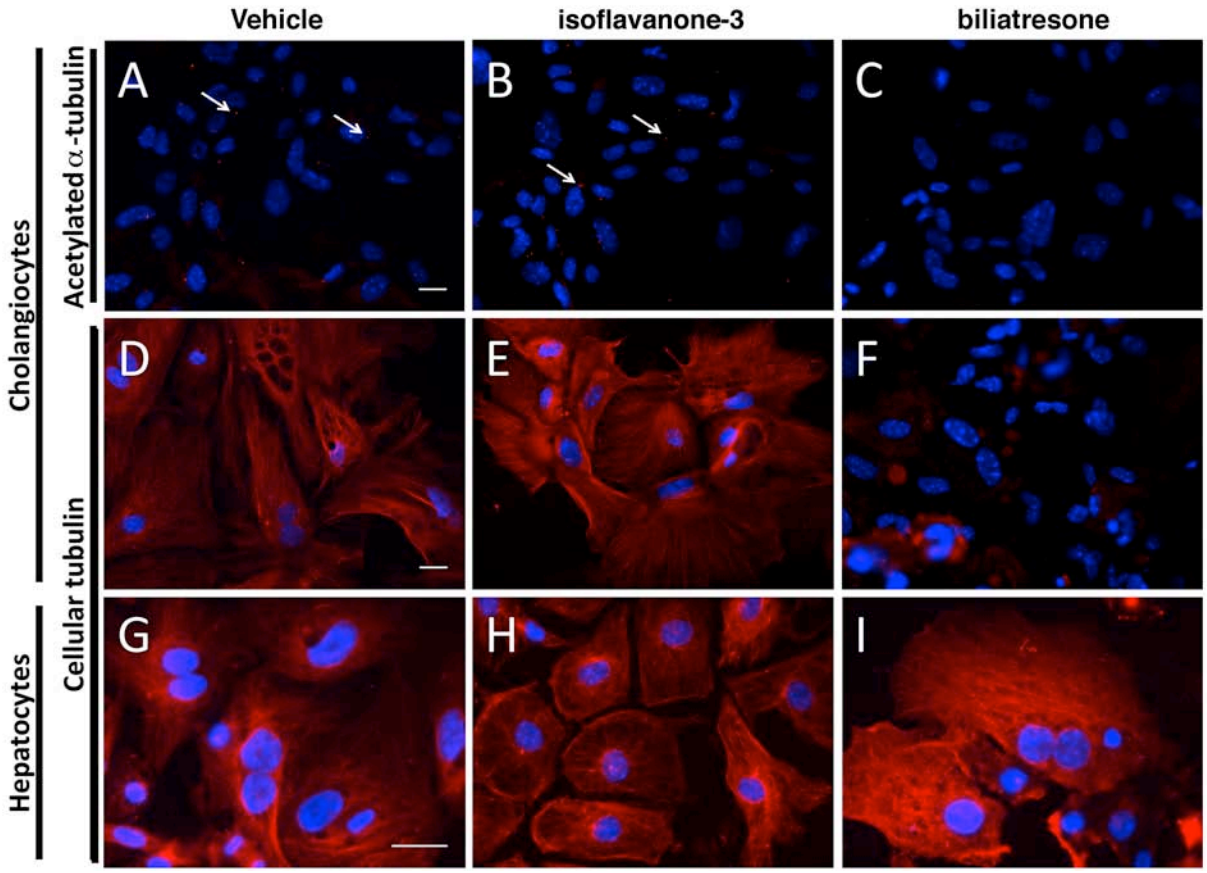

Fig. S8. Biliatresone-induced cholangiocyte cilia and microtubule defects. (**A-F**) Primary neonatal cholangiocytes were treated for 24 h with vehicle (**A**, **D**), 2 μg/ml isoflavanone-3 (**B**, **E**), or 2 μg/ml biliatresone (**C**, **F**), then stained with antibodies against acetylated α-tubulin to detect primary cilia or cellular tubulin to detect microtubules. Arrows show representative primary cilia. (**G-I**) Primary neonatal hepatocytes were treated for 24 h with vehicle (**G**), 3 μg/ml isoflavanone-3 (**H**), or 3 μg/ml biliatresone (**I**), then stained with antibodies against cellular tubulin. Antibody staining, red; DAPI nuclear stain, blue. Scale bar, 25 μm. Representative images from 3-5 independent experiments each, from independent primary cell isolations.

Table S1: Biliatresone toxicity requires active bile secretion.

	DAPT Treatment	Biliatresone Treatment	Mortality	Gallbladder Morphology	Intrahepatic ducts	Comment / Conclusion
1	None	None	0/10	Normal	Normal	Control for gallbladder morphology in wild type larvae (126 hpf)
2	None	None	0/10	Normal	Normal	Control for gallbladder morphology in wild type larvae (144 hpf)
3	None	1.0 µg/ml; 120 - 126 hpf	0/10	Small in all	Normal in all	Control for biliatresone activity in wild type larvae (6 hr treatment)
4	None	0.5 µg/ml; 120 - 144 hpf	1/15	3/14 small, 11/14 absent	Normal in all	Control for biliatresone activity in wild type larvae (24 hr treatment)
5	120 - 126 hpf	None	0/10	Normal in all	Normal in all	No effect of 6 hr DAPT treatment on gallbladder or intrahepatic ducts
6	120 - 126 hpf	1.0 µg/ml; 120 - 126 hpf	0/12	Small in all	Normal in all	Biliatresone activity not disrupted by 6 hr co-incubation with DAPT
7	120 - 144 hpf	None	0/15	Normal in all	Normal in all	No effect of 24 hr DAPT treatment on gallbladder or intrahepatic ducts
8	120 - 144 hpf	0.5 µg/ml; 120 - 144 hpf	0/15	3/15 Small, 12/15 absent	Normal in all	Biliatresone activity not disrupted by 24 hr co-incubation with DAPT
9	48 - 126 hpf	None	0/10	Normal* in all	Severely reduced to absent	Control to confirm DAPT activity
10	48 - 126 hpf	1.0 µg/ml; 120 - 126 hpf	0/14	Unchanged^ in all	Severely reduced to absent	Biliatresone is not active in DAPT-treated larvae
11	48 - 144 hpf	None	0/8	Normal in 7; absent in 1	Severely reduced to absent	Control to confirm DAPT activity
12	48 - 144 hpf	0.5 µg/ml; 120 - 144 hpf	5/15	Unchanged in 9; absent in 1.	Severely reduced to absent	Biliatresone is not active in DAPT-treated larvae. There is absence of biliary defects despite increased mortality from DAPT.

Results of 2F11 immunostaining depicted in Fig. S5. **Control experiments, rows 1-4.** Biliatresone preparation used for these experiments was more active than previous preparations, perhaps due to higher purity. Thus, there is a profound effect on gallbladder morphology in the absence of DAPT as early as 24 hrs in larvae exposed to 0.5 μg/ml (row 4) and a more modest effect as early as 6 hrs in larvae exposed to 1.0 μg/ml (row 3). **DAPT exposure experiments, rows 5-12.** Biliatresone is inactive in larvae that lack or have very few intrahepatic bile ducts. * Gallbladders are small in DAPT-treated larvae compared to untreated controls. ^ Biliatresone does not alter gallbladder size or morphology in DAPT-treated larvae.

Movie S1. Biliary anatomy of a wild type larva. Confocal projection through the liver and biliary system of a 8 dpf wild type larva immunostained with the 2F11 monoclonal antibody showing the gallbladder, extrahepatic ducts and a portion of the intrahepatic biliary ductal network.

Movie S2. Biliary anatomy of a toxin-treated wild type larva. Confocal projection through the liver and biliary system of a toxin-treated 8 dpf wild type larva(biliatresone 0.5 μg/ ml for 72 hrs) immunostained with the 2F11 monoclonal antibody. The gallbladder is markedly dysmorphic and the extrahepatic ducts are not identifiable. The structure of the intrahepatic biliary ductal network is preserved. Intestinal and pancreatic cells that express the 2F11 epitope are visible surrounding the liver. These cells are out of the plane of focus in the corresponding wild type larva (Movie S1).