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Dual Farnesoid X Receptor/TGR5 Agonist INT-767 Reduces Liver Injury in the *Mdr2*$^{-/-}$ (*Abcb4*$^{-/-}$) Mouse Cholangiopathy Model by Promoting Biliary HCO_3^- Output

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Abstract

Chronic cholangiopathies have limited therapeutic options and represent an important indication for liver transplantation. The nuclear farnesoid X receptor (FXR) and the membrane G protein-coupled receptor, TGR5, regulate bile acid (BA) homeostasis and inflammation. Therefore, we hypothesized that activation of FXR and/or TGR5 could ameliorate liver injury in *Mdr2*$^{-/-}$ (*Abcb4*$^{-/-}$) mice, a model of chronic cholangiopathy. Hepatic inflammation, fibrosis, as well as bile secretion and key genes of BA homeostasis were addressed in *Mdr2*$^{-/-}$ mice fed either a chow diet or a diet supplemented with the FXR agonist, INT-747, the TGR5 agonist, INT-777, or the dual FXR/TGR5 agonist, INT-767 (0.03% w/w). Only the dual FXR/TGR5 agonist, INT-767, significantly improved serum liver enzymes, hepatic inflammation, and biliary fibrosis in *Mdr2*$^{-/-}$ mice, whereas INT-747 and INT-777 had no hepatoprotective effects. In line with this, INT-767 significantly induced bile flow and biliary HCO_3^- output, as well as gene expression of carbonic anhy-drase 14, an important enzyme able to enhance HCO_3^- transport, in an Fxr-dependent manner. In addition, INT-767 dramatically reduced bile acid synthesis via the induction of ileal *Fgf15* and hepatic *Shp* gene expression, thus resulting in significantly reduced biliary bile acid output in *Mdr2*$^{-/-}$ mice.

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Additional Supporting Information may be found in the onbline version of this article.

Conclusion—This study shows that FXR activation improves liver injury in a mouse model of chronic cholangiopathy by reduction of biliary BA output and promotion of HCO_3^--rich bile secretion.

Current pharmacological strategies for chronic cholangiopathies, such as primary sclerosing cholangitis (PSC), have limited efficacy,[1,2] and novel therapies are eagerly awaited. Bile acids (BAs) are potent signaling molecules that, through activation of the nuclear receptor, farnesoid X receptor (FXR; NR1H4),[3–5] and the membrane G protein-coupled receptor, TGR5 (also called GPBAR1 or M-BAR/ BG37),[6,7] modulate BA homeostasis, inflammation, and lipid and glucose metabolism.[8] In the liver, FXR is highly expressed in hepatocytes, whereas cholangiocytes show a weak expression.[9] In contrast, TGR5 is highly expressed in the biliary epithelium, sinusoidal endothelial cells, and Kupffer cells.[10–13] FXR activation inhibits BA synthesis[14,15] and has anti-inflammatory effects in atherosclerosis,[16] inflammatory bowel disease,[17] and experimental cholestasis,[18] whereas TGR5 activation, via cAMP-mediated pathways, reduces proinflammatory cytokine production in macrophages[6] and Kupffer cells.[11] In addition, FXR and TGR5 mutations have been identified in intrahepatic cholestasis of pregnancy[19] and PSC,[20] respectively, emphasizing that these receptors are attractive novel therapeutic targets.

We, therefore, hypothesized that selective FXR activation by INT-747,[21] selective TGR5 stimulation by INT-777,[22] and/or dual FXR/TGR5 activation by INT-76[23] could exert beneficial therapeutic mechanisms on liver inflammation and fibrosis in mice lacking the phospholipid (PL) flippase multidrug resistance protein 2 (*Mdr2*) (*Mdr2*$^{-/-}$ or *Abcb4*$^{-/-}$) with sclerosing cholangitis.[24,25] In this study, we have identified the dual FXR/TGR5 agonist, INT-767, as a novel promising treatment in a mouse model of chronic cholangiopathy and characterized the underlying molecular and cellular mechanisms.

Materials and Methods

Animal Experiments

Mdr2$^{-/-}$ mice (FVB/N background) were obtained from Jackson Laboratory (Bar Harbor, ME) and housed in a 12-hour light-dark house facility with water and mouse chow diet (SSNIFF, Soest, Germany) *ad libitum*. *Fxr*$^{+/+}$ (C57BL6/J background) mice were from Himberg, Austria. *Fxr*$^{-/-}$ mice were originally obtained from a colony at the National Institutes of Health (NIH; Bethesda, MD).[26] Heterozygous *Tgr5-Tg* mice were described previously.[27] Common bile duct ligation (CBDL) was performed on C57BL6/J mice, as previously described.[28] Experimental protocols were approved by the local animal care and use committee, according to criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by U.S. National Academy of Sciences (NIH publication 86-23, revised 1985).

Feeding Protocols

INT-747 (selective FXR agonist), INT-777 (selective TGR5 agonist), and INT-767 (dual FXR/TGR5 agonist) (see Supporting Information) were synthesized by Intercept Pharmaceuticals (New York, NY) (Fig. 1A). Eight-week-old male *Mdr2*$^{-/-}$ mice received either a chow diet or a diet supplemented with INT-747, INT-777, and INT-767 (0.03% w/ w, equaling 30 mg/kg) for 4 weeks. CBDL was performed after 5 days of prefeeding either a chow diet or a diet supplemented with INT-747, INT-777, or INT-767. Experimental feeding was continued for an additional 3 days, and mice were sacrificed on day 4 after CBDL.

Methods for further biochemical, molecular, and histological analysis and *in vitro* cell-culture experiments are described in the Supporting Information.

Statistical Analysis

Results were evaluated using SPSS software (Release 14.0, 2005; SPSS Inc., Chicago, IL). Statistical analysis was performed using the one-way analysis of variance test, followed by the Mann-Whitney U test. Data are reported as means of 5 animals per group (unless otherwise noted) ± standard deviation (SD). A P value ≤ 0.05 was considered significant.

Results

Dual FXR/TGR5 Agonist INT-767 Decreases Liver Injury in Mdr2$^{-/-}$ Mice

Treatment with INT-767 significantly reduced, whereas INT-747 and INT-777 increased serum alanine aminotransferase (ALT) levels in *Mdr2*$^{-/-}$ mice (Fig. 1B). However, all treatments increased serum alkaline phosphatase (ALP) levels (Fig. 1B) (modest increase by INT-767) and liver weight/body weight (LW/BW) ratio (Supporting Fig. 1A). Histological examination (i.e., hematoxylin and eosin [H&E] staining) of INT-767-treated *Mdr2*$^{-/-}$ mouse livers showed less portal inflammation and bile duct proliferation (Fig. 1C), compared with untreated mice. In contrast, INT-747 aggravated liver damage in *Mdr2*$^{-/-}$ mice, as reflected by increased bile duct proliferation, portal tract expansion (Fig. 1C), and single-cell necrosis with lobular inflammation (Supporting Fig. 1B), whereas no significant changes were detected after treatment with INT-777.

INT-767 Suppresses Hepatic Inflammation, Reactive Cholangiocyte Activation, and Fibrosis in Mdr2$^{-/-}$ Mice

INT-767 treatment reduced *F4/80*, tumor necrosis factor alpha (*Tnf*-α), and interleukin (*Il*)-*1*β messenger RNA (mRNA) levels (Fig. 2A–C) as well as the number of cluster of differentiation (CD)-11b- and F4/80-positive cells (Supporting Fig. 2A,B). In contrast, INT-747 increased *Il-1*β mRNA levels (Fig. 2C) and portal CD-11b-positive cell accumulation in *Mdr2*$^{-/-}$ mice (Supporting Fig. 2A). The reactive cholangiocyte phenotype was also reduced by INT-767, as reflected by significantly lowered *K19* and vascular cell adhesion molecule-1 (*Vcam-1*) mRNA levels and by immunohistochemical staining (Supporting Fig. 3). INT-747 increased *Vcam-1* and monocyte chemotactic protein 1 (*Mcp-1*) mRNA levels and induced Vcam-1 staining in cholangiocytes, inflammatory cell infiltrates, and periportal hepatocytes, whereas INT-777 increased only *Mcp-1* mRNA levels (Supporting Fig. 3). Liver fibrosis was reduced in INT-767-treated *Mdr2*$^{-/-}$ mice, as reflected by hepatic hydroxyproline (HP) content, inhibition of collagen type 1 alpha 1 (*Col1a1*) gene expression, and reduced spleen weight (SW)/BW ratio (Fig. 2D–F). In contrast, HP, *Col1a1* mRNA, as well as SW/BW ratio increased in INT-747-fed mice, but remained unchanged in INT-777-fed mice. These findings were also confirmed by Sirius red staining (Supporting Fig. 4). Ki-67 staining revealed increased hepatocyte proliferation by INT-767 and INT-747 in *Mdr2*$^{-/-}$ (data not shown) and *Fxr*$^{+/+}$ mice, but not in *Fxr*$^{-/-}$ mice (Supporting Fig. 5).

Potential direct anti-inflammatory and antifibrotic effects of INT-767 were addressed in macrophage, cholangiocyte, and hepatocyte cell lines and isolated primary myofibroblasts (MFBs). Notably, despite the potent *in vivo* effects, INT-767 had only a modest or not statistically significant effect on lipopolysaccharide-induced *Il-6* expression in RAW264.7 macrophages, Tnf-α-induced *Vcam-1* gene expression in biliary epithelial cells (BEC), and TNF-α–induced *TNF*-α gene expression in HepG2 cells, despite pronounced inhibition of cholesterol 7 alpha-hydroxylase (*CYP7A1*) as a positive control (Supporting Fig. 6).

Furthermore, INT-767 had no significant effect on 10% fetal calf serum (FCS)-induced *Col1a1* gene expression in portal MFBs (Supporting Fig. 7).

INT-767 Inhibits BA Synthesis by Induction of Ileal Fgf15 and Hepatic Shp Gene Expression

Importantly, both INT-747 and INT-767 dramatically inhibited *Cyp7a1* (Fig. 3A) and *Cyp8b1* (Supporting Fig. 8A) and stimulated *Fgf15* gene expression (Fig. 3B). However, only INT-767 increased hepatic *Shp* gene expression (Supporting Fig. 8B). *Ntcp* was repressed by INT-747 and INT-767 at mRNA and protein levels, whereas only INT-767 increased bile salt export pump (Bsep) protein levels (Supporting Fig. 8C–E) and reduced serum BA levels in *Mdr2*$^{-/-}$ mice (Fig. 3C). No significant alterations of multidrug resistance-associated protein 2 (*Mrp2*), multidrug resistance-associated protein 3 (*Mrp3*), and multidrug resistance-associated protein 4 (*Mrp4*) were observed (Supporting Fig. 8E).

INT-767 Stimulates HCO_3^--rich Bile Flow and Inhibits BA Output via Fxr Activation

INT-767 significantly increased bile flow and HCO_3^- output in *Mdr2*$^{-/-}$ mice, whereas biliary BA output was reduced (Fig. 4). In contrast, bile flow and bile composition remained unchanged in response to INT-747 and INT-777 feeding in *Mdr2*$^{-/-}$ mice. Because INT-767 represents a potent FXR, as well as TGR5 agonist, we next aimed to further discriminate the specific impact of each receptor in INT-767-induced choleresis with the aid of *Fxr*$^{-/-}$ mice. Bile flow and biliary HCO_3^- output, increased by INT-767, were abolished in *Fxr*$^{-/-}$ mice (Fig. 5A,B), whereas INT-747 and INT-777 had no impact on bile flow or biliary HCO_3^- output. By using a genetic model of Tgr5 overexpression (*Tgr5-Tg* mice), we further confirmed that bile flow and biliary HCO_3^- secretion was independent of Tgr5 *in vivo* (Fig. 5C,D). In line with BA synthesis inhibition, INT-767 decreased biliary BA and, consequently, cholesterol and PL output (Fig. 6A–C) in an Fxr-dependent manner. INT-747 showed only modest reduction of BA output. Intriguingly, INT-777 decreased biliary PL and cholesterol output in *Fxr*$^{+/+}$ mice (Fig. 6B,C), whereas glutathione output remained unchanged by all three compounds in both genotypes (Supporting Fig. 9). Biliary concentration of INT-767 was higher in *Fxr*$^{-/-}$, compared with *Fxr*$^{+/+}$ mice, whereas INT-747 and INT-777 concentrations did not differ between genotypes (Fig. 6D). However, INT-777 showed the lowest biliary enrichment.

INT-767 Induces Gene Expression of Hepatic Carbonic Anhydrase 14

In human gallbladder epithelium, FXR was shown to induce HCO_3^--rich secretion[30] via vasoactive intestinal peptide receptor (VPAC-1) induction. However, INT-767 even decreased hepatic *Vpac-1* mRNA levels in *Mdr2*$^{-/-}$ as well as *Fxr*$^{+/+}$ mice, (Supporting Fig. 10), indicating that Vpac-1 is unlikely to be responsible for HCO_3^--rich secretion in INT-767-treated mice. Gene expression of hepatocellular and cholangiocellular HCO_3^- output transporter *Ae2*[31–33] as well as *Slc4a4*, an additional transporter in mouse cholangiocytes,[34] remained unchanged in *Mdr2*$^{-/-}$, *Fxr*$^{+/+}$, and *Fxr*$^{-/-}$ mice (Supporting Fig. 11). Because none of the INT compounds altered gene expression of HCO_3^- input transporter *Slc4a5* in *Mdr2*$^{-/-}$ mice (data not shown), we studied the regulation of different carbonic anhydrases (Cas) by INT-767. INT-767 significantly increased the expression of hepatocellular membrane-bound *Ca14*[35] in *Mdr2*$^{-/-}$ and *Fxr*$^{+/+}$ mice and in human HepG2 hepatocytes, but not in *Fxr*$^{-/-}$ mice (Fig. 7A–C).

INT-767 Shows Moderate Protection Even in a Model of Total Biliary Obstruction

INT-747 and INT-767 increased the size and amount of bile infarcts, as well as LW/BW ratio, in CBDL mice (Supporting Fig. 12A,B), whereas only INT-767 significantly

decreased SW/BW ratio (Supporting Fig. 12C) and showed a trend to reduction of serum ALT (Supporting Fig. 13A). Although histological examination of H&E-stained livers revealed bile infarcts in all the groups, only INT-747 increased infiltration of inflammatory cells within the portal fields (Supporting Fig. 13B). In line with serum ALT levels, INT-767-fed CBDL mice had reduced expression of proinflammatory genes *Tnf*-α and *Il-1*β and less CD-11b- and F4/80-positive cells around bile infarcts (Supporting Fig. 14A,B). However, keratin 19 (*K19*) and *Vcam-1* gene expression remained unchanged in CBDL mice after INT-747, INT-777, and INT-767 feeding (Supporting Fig. 15).

Discussion

In this study, we have addressed the therapeutic mechanisms of BA receptor signaling through the nuclear BA receptor, FXR, and the G-protein-coupled membrane BA receptor, TGR5, in the *Mdr2*$^{-/-}$ mouse cholangiopathy model. We report herein that, in this model, the novel FXR/TGR5 agonist, INT-767, reduces bile toxicity by decreasing biliary BA output and inducing HCO_3^--rich choleresis in an FXR-dependent manner.

BAs are important signaling molecules with hormonal actions through dedicated nuclear and G-protein-coupled receptors, such as FXR and TGR5, respectively.[8] *TGR5* and *FXR* polymorphisms [19,20] further support the importance of BA signaling in human cholestatic diseases, such as PSC. Liver injury in *Mdr2*$^{-/-}$ mice is considered to evolve because of detergent properties of nonmicellar-bound free biliary BAs,[29] leaving many open questions for the potential role of BA signaling in modulating biliary pathophysiology Only the dual FXR/TGR5 agonist, INT-767, was hepatoprotective in the *Mdr2*$^{-/-}$ model, as reflected by reduced serum ALT, decreased hepatic inflammation, improved reactive cholangiocyte phenotype, and reduced fibrosis. We could neither observe significant direct anti-inflammatory effects of INT-767 in RAW264.7 macrophages (with very low endogenous *Fxr* and *Tgr5* expression), BEC cholangiocytes, or HepG2 hepatocytes (both with high levels of *Fxr* and very low *Tgr5*; data not shown) nor direct antifibrotic effects in primary MFBs (with very low endogenous *Fxr* and *Tgr5* expression) as major fibrogenic cells in the *Mdr2*$^{-/-}$ model. Absent expression of FXR and TGR5[9,11] in hepatic stellate cells further indicates that FXR and TGR5 signaling may have no direct antifibrotic effects. These findings led us hypothesize that INT-767 might improve liver injury by directly impacting on bile formation and composition. Indeed, via Fxr activation, INT-767 inhibited BA synthesis (by ileal *Fgf15* and hepatic *Shp* induction), thus resulting in decreased biliary BA output while significantly increasing bile flow and-unexpectedly-HCO_3^- output.

HCO_3^- formation and secretion into bile is considered to be protective by regulating intercellular pH, or bile alkalinization and sustaining BA-independent bile flow.[36] Because INT-767 increased HCO_3^--rich choleresis via Fxr, we focused on the regulation of genes involved in HCO_3^- transport and production. FXR was shown to increase biliary HCO_3^- secretion in human gallbladder epithelium via VPAC-1 induction.[30] However, in our experiments, *Vpac-1* expression was even decreased by INT-767 in *Mdr2*$^{-/-}$ and *Fxr*$^{+/+}$ mice, indicating that other mechanisms may contribute to the INT-767-stimulated HCO_3^- secretion. Biliary HCO_3^- export is mediated by anion exchanger 2 (Ae2) in hepatocytes[31–33] and Ae2 and Slc4a4 in cholangiocytes.[34] Impaired expression of Ae2 has been characterized in the pathogenesis of cholangiopathies,[37] and induction of AE2 expression was found to be an important mechanism for the beneficial effects of combined therapy with UDCA and corticosteroids.[38] Neither *Ae2* nor *Slc4a4* gene expression were altered by INT-767 in *Mdr2*$^{-/-}$ mice, showing that an alternative mechanism may be responsible. HCO_3^- secretion can be facilitated by the induction of Cas which, via formation of functional complexes with Aes, form so-called ‘‘HCO_3^- transport metabolon’’[39] to maximize HCO_3^- flux.[40,41] More

specifically, the subgroup of membrane-bound or extracellular Cas facilitate Aes and HCO_3^- transport to buffer the extracellular fluids.[40,42–44] In addition, the role of membranebound Cas was suggested to propagate the HCO_3^- umbrella at the apical surface of cholangiocytes.[36] Expression of *Ca4*, an isoform expressed in apical membrane of cholangiocytes,[45] was undetectable and remained unchanged *in vivo* and *in vitro* (BECs) by INT-767, whereas expression of cholangiocellular basolateral *Ca9*[46,47] increased by INT-767 in $Fxr^{+/+}$, but not in $Mdr2^{-/-}$, mice and remained unchanged in BECs (data not shown). However, INT-767 induced the gene expression of *Ca14*, a membrane-bound enzyme expressed in hepatocytes,[35] in $Mdr2^{-/-}$ mice. The Fxr dependence of this finding was confirmed by showing an induction in $Fxr^{+/+}$ and no increase in $Fxr^{-/-}$ mice after INT-767 administration and is further supported by the presence of inverted repeat 1, an FXR-responsive element,[48] on the CA14/Ca14 promoter (identified *in silico* by Nuscan and Matinspector). Finally, we could show that INT-767 significantly induced *CA14* mRNA levels in HepG2 cells, which show high FXR and undetectable TGR5 gene expression. The functional and physical interaction of Ca14 with Cl^-/HCO_3^- exchanger anion exchanger 3 (Ae3) was proven to be an efficient mechanism to facilitate HCO_3^- transport in the mouse brain.[42] Therefore, it is tempting to speculate that INT-767 via Fxr-dependent induction of *Ca14* expression in hepatocytes promotes the formation of HCO_3^- transport metabolon involving Ca14 and Ae2. Whether INT-767 is also able to influence the insertion of Ae into plasma membrane as well as direct regulation of the *Ca14* gene by Fxr remain to be established. Future studies will also have to address whether these compounds have direct effects on cholangiocellular bile secretion resulting from their BA-signaling properties.

In contrast to INT-767, the selective FXR agonist, INT-747, enhanced liver injury and fibrosis in the $Mdr2^{-/-}$ model. Although low-dose INT-1–747 had no impact on liver damage in $Mdr2^{-/-}$ mice (data not shown), a high dose (0.03% w/w) aggravated it, despite the induction of *Fgf15* and inhibition of BA synthesis. Interestingly, INT-747 did not induce hepatic *Shp* gene expression, suggesting that in contrast to INT-767, which efficiently activates Fxr in the intestine and liver, INT-747 is less likely to have hepatic activity in $Mdr2^{-/-}$ mice. However, *Ntcp* gene expression was inhibited by INT-747, which may reflect direct repression by BAs or by proinflammatory cytokines caused by induced liver injury.[49] In addition, INT-747 had no impact on *Ca14* gene expression *in vivo* and *in vitro* (data not shown). Together, these findings point out to a differential regulation of Fxr-dependent genes by INT-747 and INT-767. Ligand binding to FXR can favor receptor conformations that, in turn, allow only specific cofactor recruitment, depending on the DNA-binding sequence, therefore resulting in selective modulation of gene expression.[50] Our findings suggest that INT-767 acts as a specific FXR modulator in a way similar to other natural or synthetic FXR modulators.[51–53]

BA hydrophobicity is another important factor directly linked to BA detergent properties.[54,55] Hydrophobic BAs are toxic to hepatocytes at micromolar concentrations.[56] Endogenous BAs and INT-747 are hydrophobic BAs, whereas INT-767 is hydrophilic.[23] INT-767 reduces bile toxicity and prevents further progression of liver injury via strong inhibition of endogenous BA synthesis, replacing hydrophobic BAs with the hydrophilic INT-767 and inducing HCO_3^--rich bile secretion. In contrast, accumulation of the hydrophobic INT-747 in the liver without stimulation of hepatoprotective mechanisms may act as an additional important factor for the promotion of liver damage in $Mdr2^{-/-}$ mice. Nevertheless, preliminary data from clinical phase II trials reported a beneficial effect of INT-747 on serum liver enzymes in patients with primary biliary cirrhosis.[57] However, Fxr-mediated stimulation of bile flow may be deleterious in obstructive cholestasis.[58] Importantly, despite promoting bile infarcts (because of increased bile flow) in the CBDL model, INT-767 even moderately reduced serum ALT levels and ameliorated

proinflammatory cytokine expression, possibly because of low concentrations of endogenous hydrophobic BA and high HCO_3^- content.

Because TGR5 signals through cAMP, an important regulator of chloride channel CFTR,[10] we expected increased bile flow and HCO_3^- output by the selective TGR5 agonist, INT-777. Surprisingly, INT-777 did not influence bile secretory function, as reflected by unchanged bile flow and biliary HCO_3^- output in *Mdr2*$^{-/-}$, *Fxr*$^{+/+}$ and *Fxr*$^{-/-}$ mice, despite its ability to activate TGR5 *in vitro* and *in vivo*.[22,27] Interestingly INT-777 showed the lowest biliary enrichment, indicating limited bioavailability and, subsequently, the lack of choleretic effect of this compound in mice. In addition to pharmacological TGR5 activation, by using *Tgr5-Tg* mice, we could confirm that Tgr5 over-expression also had no impact on bile secretion. However, INT-777 decreased biliary PL and cholesterol output in *Fxr*$^{+/+}$ mice in the presence of unchanged BA concentrations. These findings are consistent with previous report showing that *Tgr5*$^{-/-}$ mice had higher biliary PL, compared with *Tgr5*$^{+/+}$ mice, and were protected from gallstone development upon lithogenic diet feeding.[59] Altogether, these data suggest that despite a beneficial effect of TGR5 activation in diabesity,[8,27] TGR5 is unlikely to be benecial in cholangiopathies and diseases with impaired bile composition as well as gallbladder function. However, the failure of INT-777 to improve disease progression in *Mdr2*$^{-/-}$ does not rule out the possibility that other TGR5 activators might help to delay or cure cholestatic liver injury in humans.

In conclusion, our study demonstrates that FXR activation by INT-767, a novel, highly potent FXR/ TGR5 agonist, modifies bile flow and reduces bile toxicity by decreasing endogenous BA output and increasing HCO_3^- output, resulting in the repression of hepatic inflammation as well as biliary fibrosis in *Mdr2*$^{-/-}$ mice.

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Abbreviations

Ae2	anion exchanger 2
Ae3	anion exchanger 3
ALT	alanine aminotransferase
ALP	alkaline phosphatase
BA	bile acid
Bsep (Abcb11)	bile salt export pump
BW	body weight
Ca4	carbonic anhydrase 4
Ca9	carbonic anhydrase 9
CA14 (Ca14)	carbonic anhydrase 14
CBDL	common bile duct ligation

CD	cluster of differentiation
Col1a1	collagen type I alpha 1
Cyp7a1	cholesterol 7 alpha-hydroxylase
FCS	fetal calf serum
FXR (Fxr)	farnesoid X receptor
H&E	hematoxylin and eosin staining
HP	hydroxyproline
Il	interleukin
K19	keratin 19
LW	liver weight
Mdr2 (Abcb4)	multidrug resistance protein 2
Mcp-1	monocyte chemotactic protein 1
MFB	myofibroblast
mRNA	messenger RNA
Mrp2 (Abcc2)	multidrug resistance-associated protein 2
Mrp3 (Abcc3)	multidrug resistance-associated protein 3
Mrp 4 (Abcc4)	multidrug resistance-associated protein 4
PL	phospholipid
PSC	primary sclerosing cholangitis
SD	standard deviation
SW	spleen weight
TNF-α (Tnf-α)	tumor necrosis factor alpha
VPAC-1 (Vpac-1)	vasoactive intestinal peptide receptor-1
Vcam-1	vascular cell adhesion molecule-1

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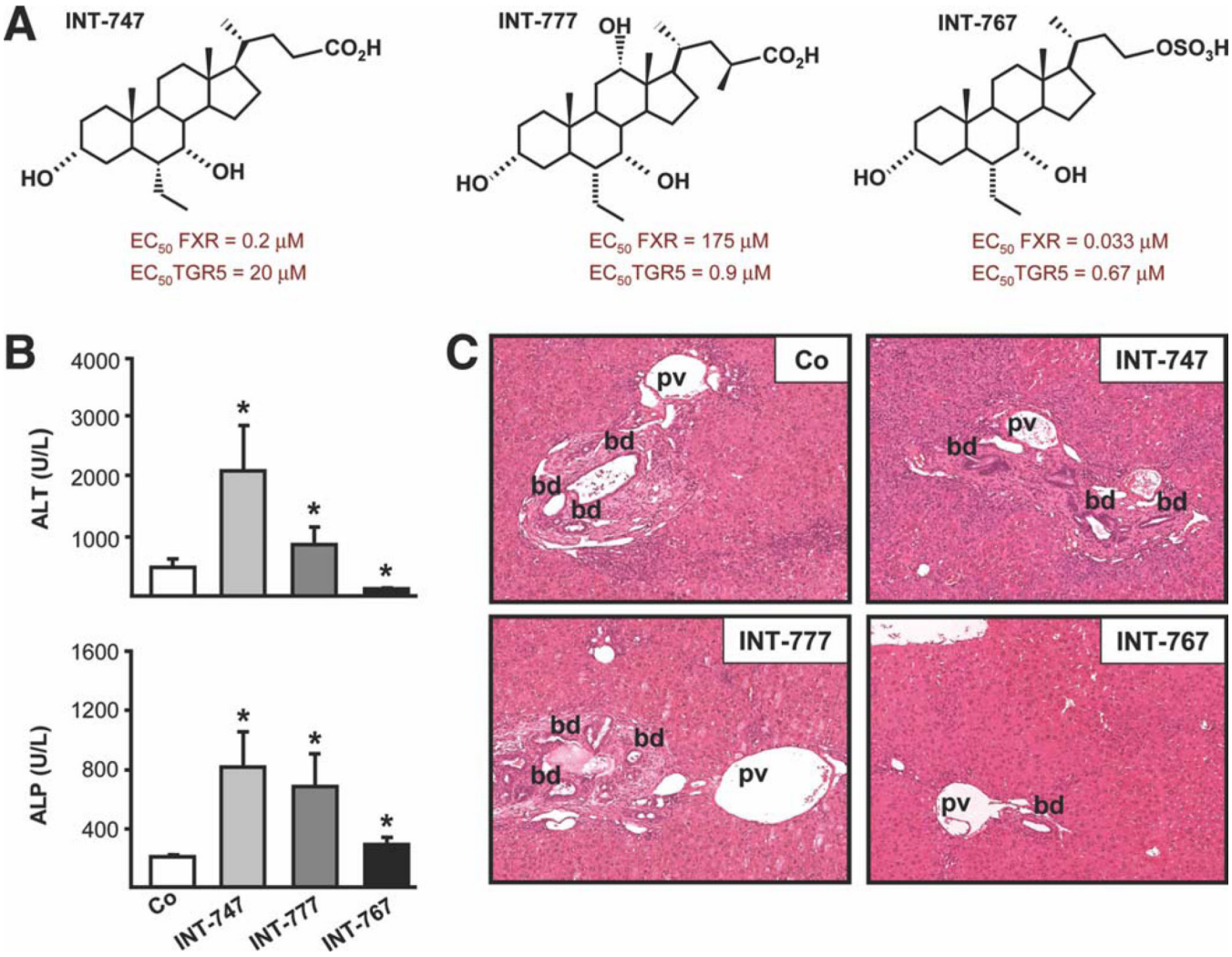

Fig. 1.

Dual FXR/TGR5 agonist INT-767 improves liver injury in *Mdr2*$^{-/-}$ mice. *Mdr2*$^{-/-}$ mice were either fed a chow diet or a diet supplemented with the FXR agonist, INT-747, the TGR5 agonist, INT-777, or the dual FXR/TGR5 agonist, INT-767 for 4 weeks. (A) Chemical structures of INT-747, INT-777, and INT-767 compounds with respective FXR and TGR5 EC_{50}. (B) INT-747 and INT-777 increased serum ALT and ALP, whereas INT-767 significantly reduced serum ALT, but not ALP, levels. Means of 6 mice/group ± SD. *$P < 0.05$ INT-747, INT-767, and INT-777 versus controls. (C) Representative histological pictures of H&E-stained livers. Bile duct proliferation and portal infiltration of inflammatory cells was reduced by INT-767. The INT-747-fed *Mdr2*$^{-/-}$ mouse showed increased bile duct proliferation, expansion of biliary tract, and accumulation of inflammatory cells. No obvious alterations were detected in the INT-777-fed *Mdr2*$^{-/-}$ mouse liver. Co, chow-fed littermates; pv, portal vein; bd, bile duct.

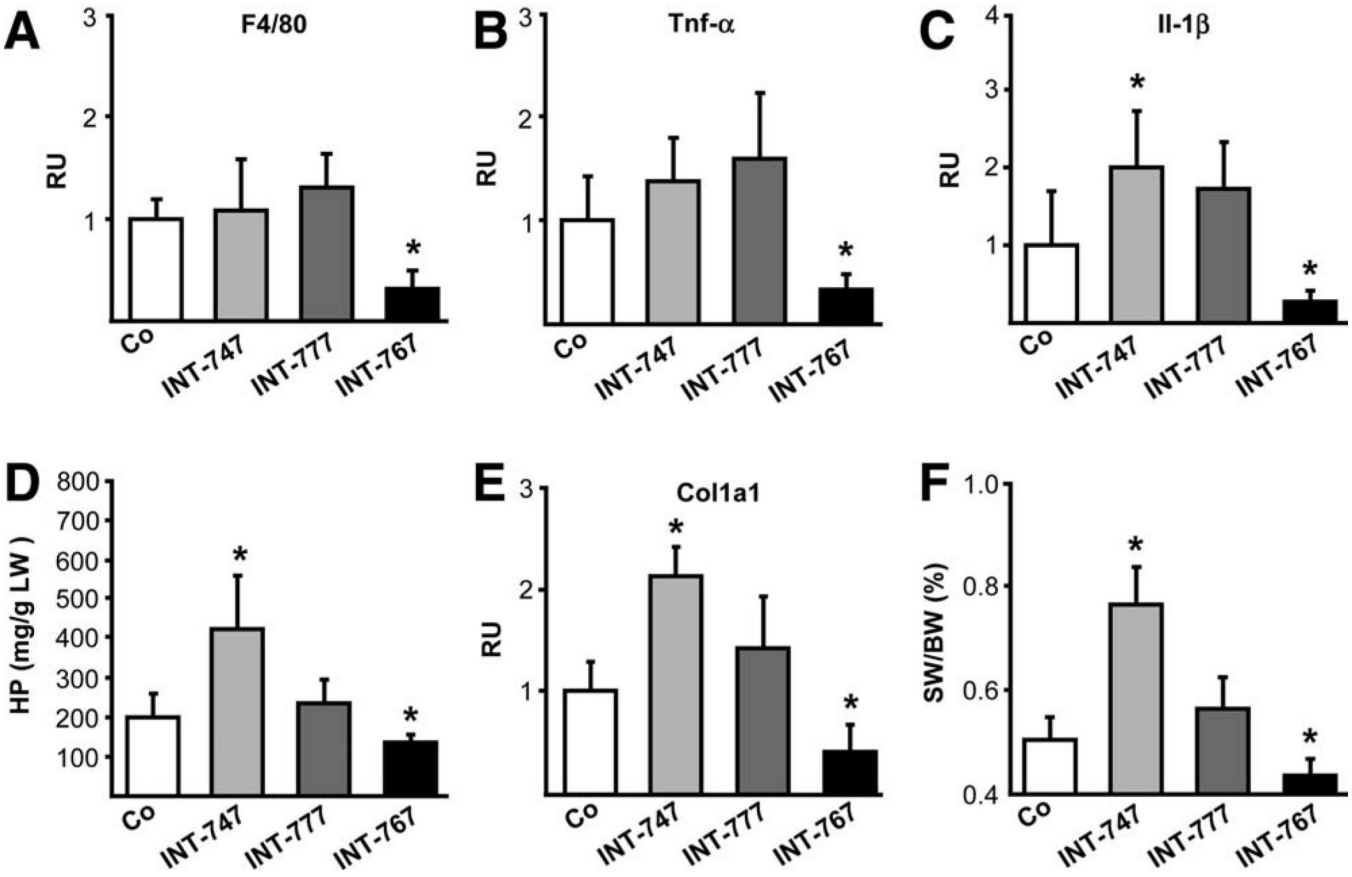

Fig. 2.

INT-767 reduces hepatic inflammation and fibrosis in *Mdr2*$^{-/-}$ mice. INT-767 inhibited the gene expression of macrophage marker *F4/80* (A) and proinflammatory cytokines *Tnf*-α (B) and *Il-1*β (C) in *Mdr2*$^{-/-}$ mice, whereas INT-747 induced *Il-1*β gene expression. INT-767 lowered hepatic HP levels (D) as well as *Col1a1* gene expression (E) in *Mdr2*$^{-/-}$ mice, whereas INT-747 increased and INT-777 did not modify HP or *Col1a1* gene expression. (F) Spleen weight was normalized to body weight, and percent ratio is presented (SW/BW). Means of 5–6 mice/ group ± SD are presented. Gene-expression levels are normalized to the *36b4* housekeeping gene and the mean expression value of untreated *Mdr2*$^{-/-}$ mice (Co) is accepted as 1. **P* < 0.05 INT-747 and INT-767 versus Co.

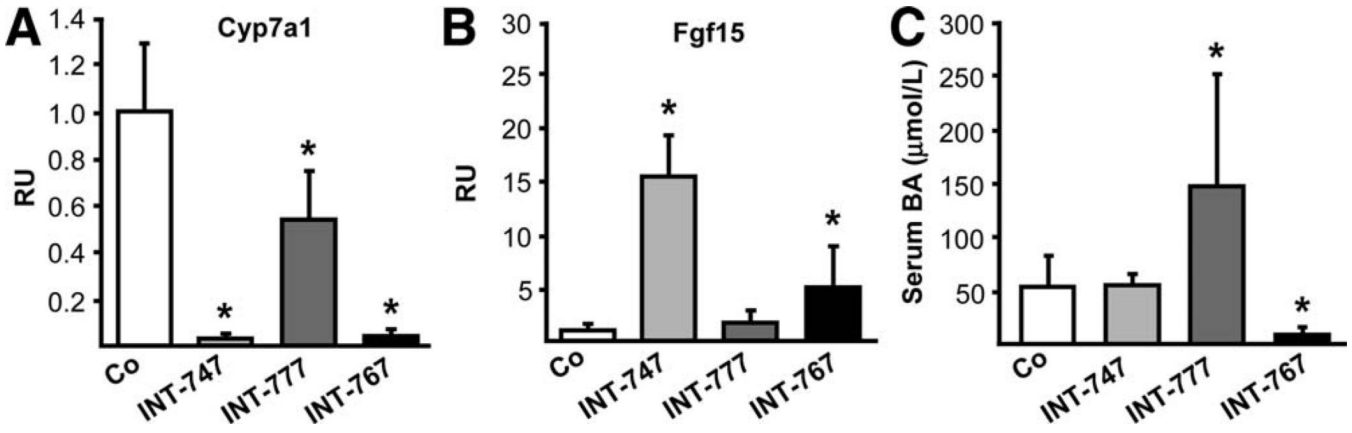

Fig. 3.

INT-747 and INT-767 inhibit BA synthesis in *Mdr2*$^{-/-}$ mice. Both INT-747 and INT-767 dramatically inhibited *Cyp7a1* (A) while inducing ileal *Fgf15* (B) gene expression in *Mdr2*$^{-/-}$ mice. (C) Only INT-767 significantly decreased, whereas INT-777 increased serum BA levels in *Mdr2*$^{-/-}$ mice. Means of 6 animals/group ± SD are presented. Gene-expression levels are normalized to the *36b4* housekeeping gene, and the mean expression value of untreated *Mdr2*$^{-/-}$ mice (Co) is accepted as 1. **P* < 0.05 INT-747, INT-767, and INT-777 versus Co.

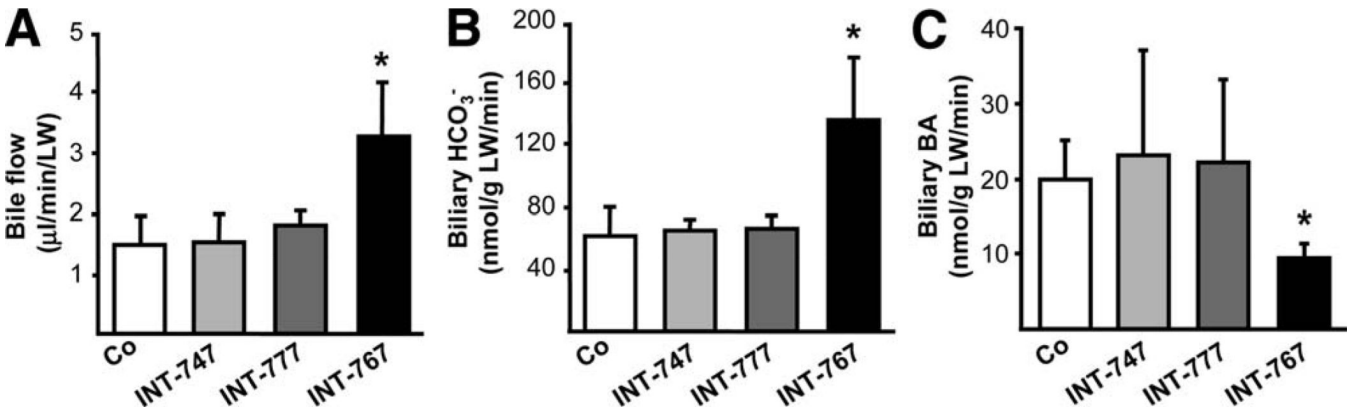

Fig. 4.

INT-767 stimulates HCO_3^--rich bile flow in *Mdr2*$^{-/-}$ mice. Bile flow and composition were measured in *Mdr2*$^{-/-}$ mice after 7 days of feeding either a chow or a diet supplemented with INT-747, INT-777, or INT-767. (A) INT-767 increased bile flow and (B) biliary HCO_3^- output. (C) Biliary BA output was significantly decreased by INT-767. Neither INT-747 nor INT-777 modified bile flow, biliary HCO_3^-, or BA output in *Mdr2*$^{-/-}$ mice. Means of 4–5 mice/ group ± SD are presented. *$P < 0.05$ INT-767 versus chow-fed *Mdr2*$^{-/-}$ mice (Co).

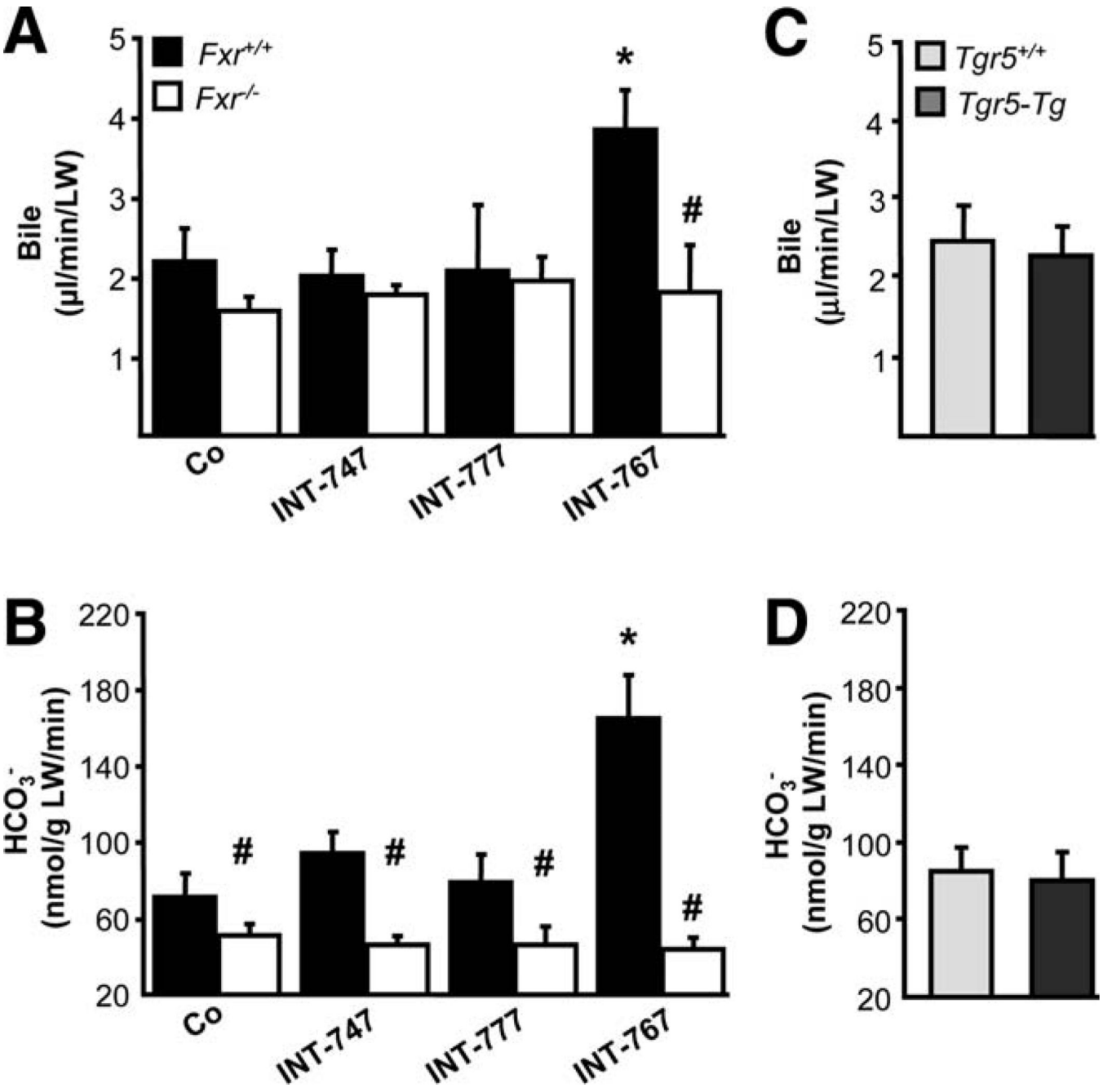

Fig. 5.

Induction of HCO_3^--rich choleresis by INT-767 depends on Fxr, but not Tgr5. Bile flow and biliary HCO_3^- output were measured in *Fxr*$^{+/+}$ and *Fxr*$^{-/-}$ mice after 7 days of feeding on a chow or INT-747-, INT-777-, or INT-767-supplemented diet. (A) INT-767 induced bile flow only in *Fxr*$^{+/+}$ mice, whereas INT-747 and INT-777 did not change bile flow in both *Fxr*$^{+/+}$ and *Fxr*$^{-/-}$ mice. (B) Only INT-767 induced biliary HCO_3^- output in *Fxr*$^{+/+}$, but not in *Fxr*$^{-/-}$, mice. Means of 4–5 mice/group ± SD are presented. *$P < 0.05$ INT-767-fed versus chow-fed *Fxr*$^{+/+}$ mice (Co). $^{\#}P < 0.05$ *Fxr*$^{-/-}$ versus *Fxr*$^{+/+}$ mice. Bile flow (C) and biliary HCO_3^- (D) output were measured in age-matched *Tgr5-Tg* and *Tgr5*$^{+/+}$ female mice. Bile

flow, as well as biliary HCO_3^- output, was undistinguishable between *Tgr5-Tg* and *Tgr5*$^{+/+}$ mice. Means of 4–5 mice/group ± SD. are presented.

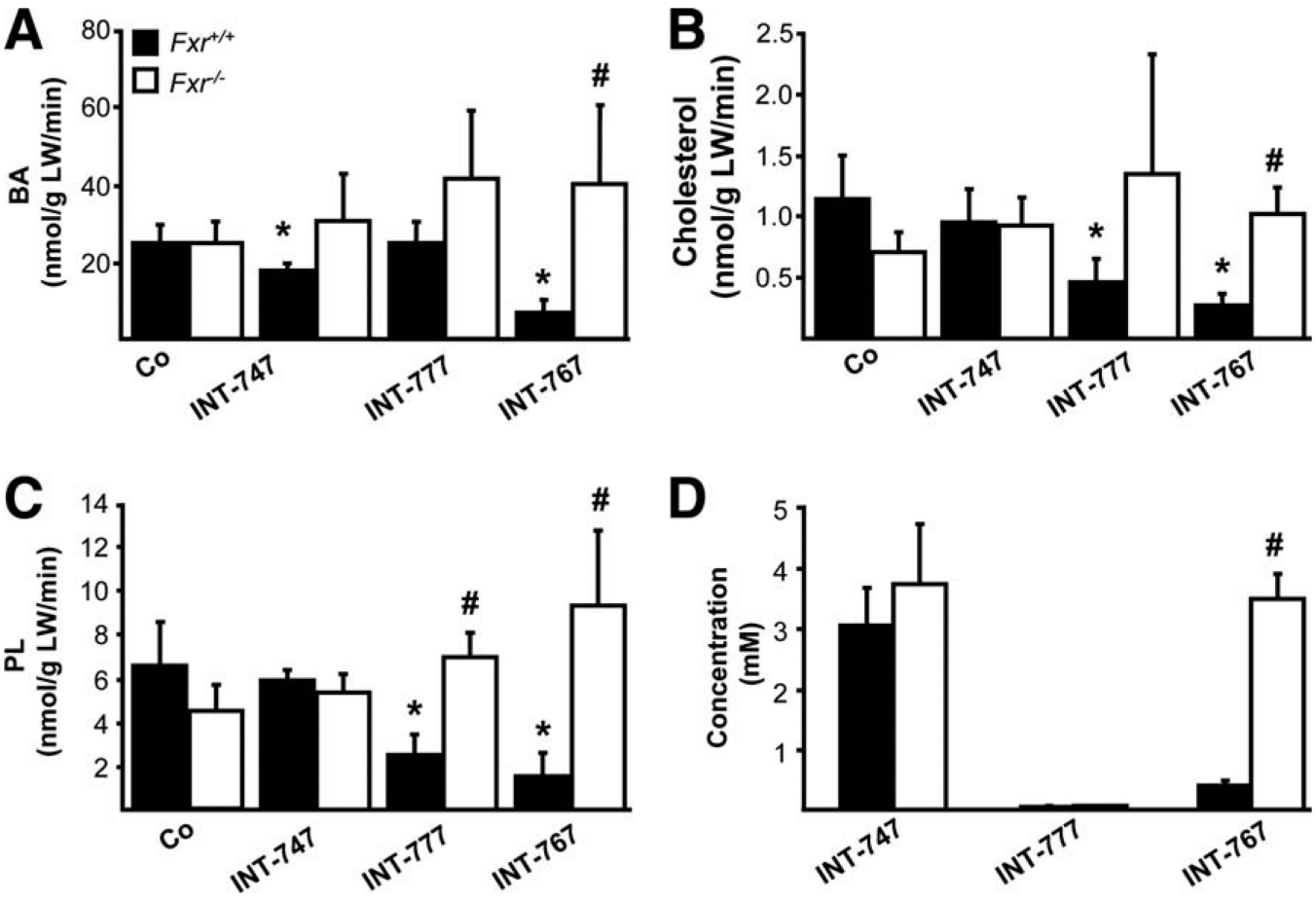

Fig. 6.

INT-767 reduces biliary output of endogenous BAs in an Fxr-dependent manner. Output of biliary BAs (A), cholesterol (B), and PLs (C) was reduced in $Fxr^{+/+}$ mice by INT-767 while remaining unchanged in $Fxr^{-/-}$ mice. INT-747 modestly reduced biliary BA output (A), whereas INT-777 reduced PL (B) and Cholesterol (C) output in $Fxr^{+/+}$ mice. (D) Biliary concentrations of respective INT compounds in bile samples of $Fxr^{+/+}$ and $Fxr^{-/-}$ mice. Means of 4–5 animals/group ± SD are presented. $^{*}P < 0.05$ INT-747, INT-767 and INT-777-fed versus chow-fed $Fxr^{+/+}$ mice (Co). $^{\#}P < 0.05$ $Fxr^{-/-}$ versus $Fxr^{+/+}$ mice.

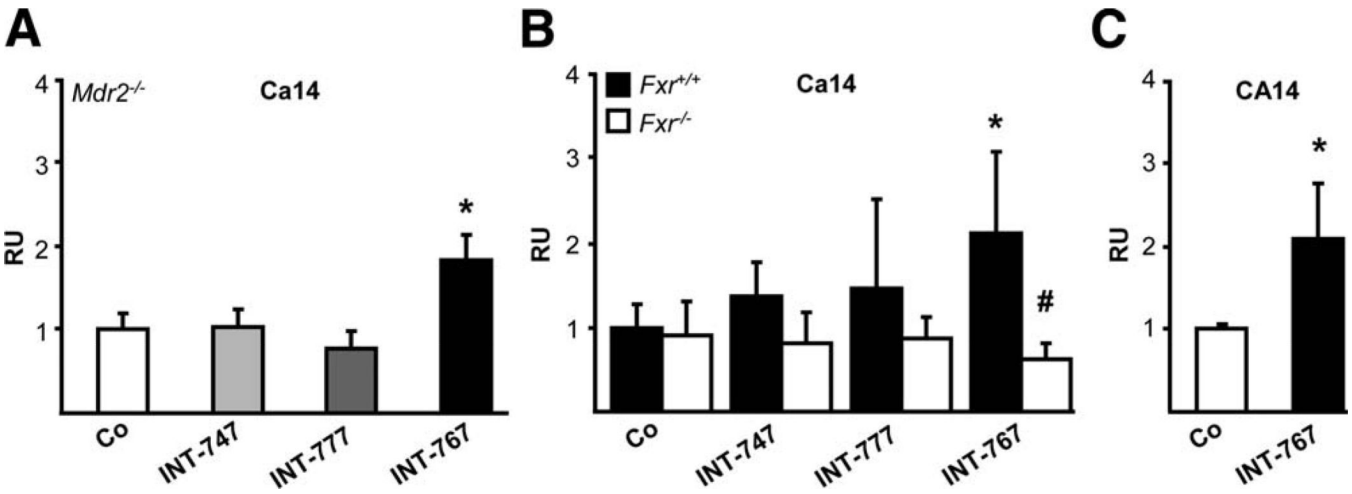

Fig. 7.

INT-767 significantly induces carbonic anhydrase 14 (Ca14) gene expression *via* Fxr. INT-767 significantly stimulated hepatic *Ca14* gene expression in $Mdr2^{-/-}$ (A) and $Fxr^{+/+}$ mice (B), whereas no alterations were detected in $Fxr^{-/-}$ mice. Means of 5–6 animals/group ± SD are presented. Gene-expression levels are normalized to the *36b4* housekeeping gene, and the mean expression value of chow-fed $Mdr2^{-/-}$, $Fxr^{+/+}$, and $Fxr^{-/-}$ mice is accepted as 1 (Co). $^{*}P < 0.05$ INT-767-fed $Fxr^{+/+}$ and $Mdr2^{-/-}$ mice versus respective controls. $^{\#}P < 0.05$ $Fxr^{-/-}$ versus $Fxr^{+/+}$ mice. (C) INT-767 (10 μM) significantly induced *CA14* gene expression in HepG2 cells. Values are presented as means of 3 samples per group ± SD. Expression levels are normalized to *36B4*. $^{*}P < 0.05$ INT-767- versus control medium-incubated (Co) cells. RU, relative units.

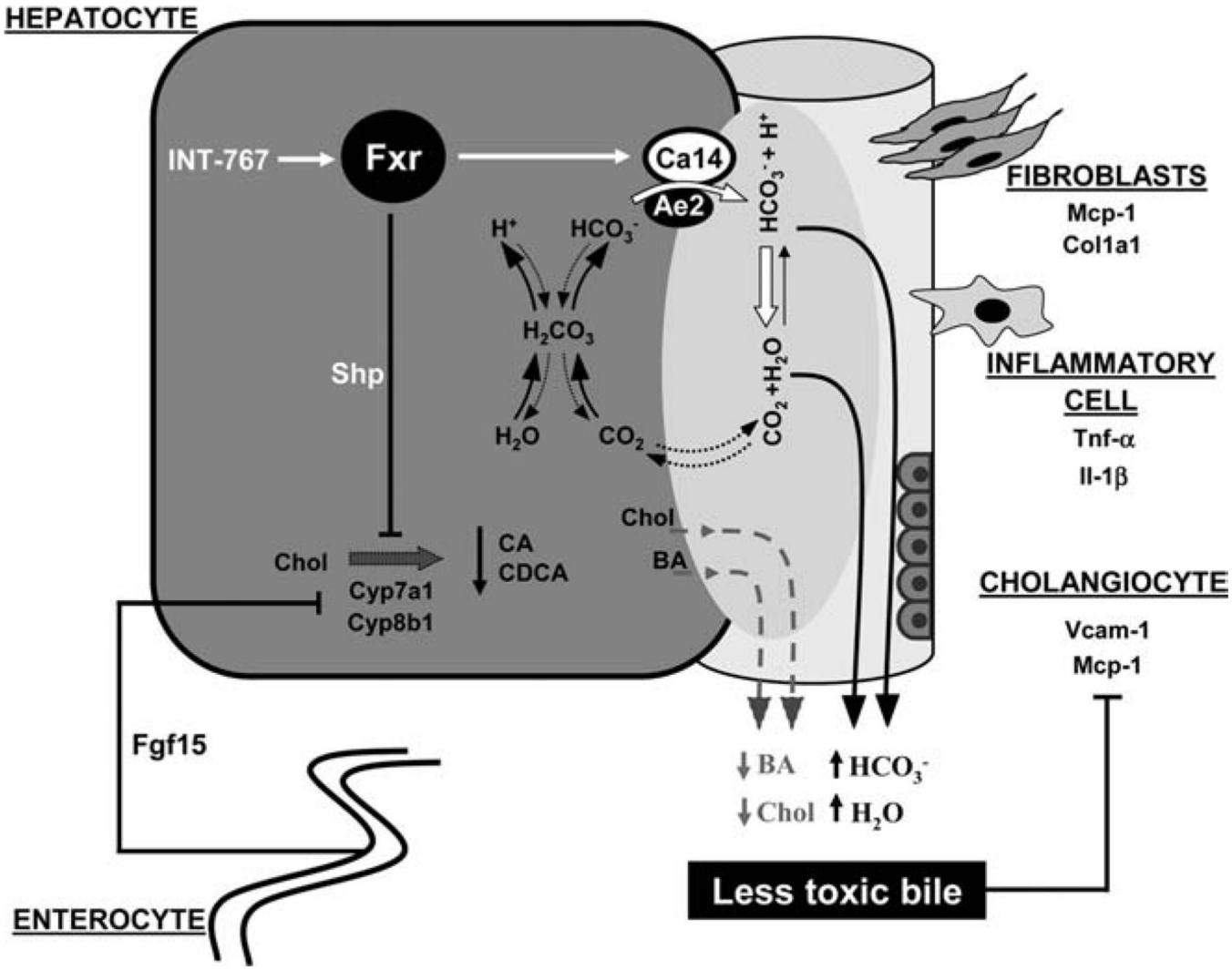

Fig. 8.

Proposed model of INT-767-mediated beneficial effects in *Mdr2*$^{-/-}$ mice. INT-767 activates Fxr in the ileum and liver. By the induction of ileal *Fgf15* and hepatic *Shp*, INT-767 profoundly inhibits endogenous BA synthesis, resulting in a significant reduction of biliary output of hydrophobic BAs and cholesterol. In addition, INT-767 via Fxr activation induces the expression of hepatocellular membrane-bound *Ca14*, which, in turn, promotes HCO_3^- output and HCO_3^--rich choleresis because of (1) complex formation with HCO_3^- transporter Ae2 and (2) hydration of HCO_3^- with H^+ into CO_2 and H_2O, thus increasing the local transmembrane HCO_3^- gradient to further facilitate HCO_3^- export. CO_2, being easily permeable across the cell membrane, can enter cells where it is rehydrated to H^+ and HCO_3^-, thus contributing to HCO_3^- recycling. Collectively, INT-767, by reducing the concentration of detergent BAs and increasing HCO_3^--rich choleresis, contributes to less reactive alterations of bile duct epithelium and results in decreased portal inflammation and fibrosis.