Published in final edited form as:

Toxicology. 2016 July 15; 365: 9–16. doi:10.1016/j.tox.2016.07.021.

Inhibition of PAR-4 and P2Y₁₂ receptor-mediated platelet activation produces distinct hepatic pathologies in experimental xenobiotic-induced cholestatic liver disease

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Abstract

Emerging evidence supports a protective effect of platelets in experimental cholestatic liver injury and cholangiofibrosis. Coagulation-mediated platelet activation has been shown to inhibit experimental chronic cholestatic liver necrosis and biliary fibrosis. This occurs through thrombin-mediated activation of protease activated receptor-4 (PAR-4) in mice. However, it is not known whether other pathways of platelet activation, such as adenosine diphosphate (ADP)-mediated receptor P2Y₁₂ activation is also protective. We tested the hypothesis that inhibition of P2Y₁₂-mediated platelet activation exacerbates hepatic injury and cholangiofibrosis, and examined the impact of P2Y₁₂ inhibition in both the presence and absence of PAR-4. Treatment of wild-type mice with the P2Y₁₂ receptor antagonist clopidogrel increased biliary hyperplasia and cholangiofibrosis in wild-type mice exposed to the xenobiotic alpha-naphthylisothiocyanate (ANIT) for 4 weeks compared to vehicle-treated mice exposed to ANIT. Interestingly, this effect of clopidogrel occurred without a corresponding increase in hepatocellular necrosis. Whereas biliary hyperplasia and cholangiofibrosis were increased in PAR-4^{-/-} mice, clopidogrel treatment failed to further increase these pathologies in PAR-4^{-/-} mice. The results indicate that inhibition of receptor P2Y₁₂-mediated platelet activation exacerbates bile duct fibrosis in ANIT-exposed mice, independent of hepatocellular necrosis. Moreover, the lack of an added effect of clopidogrel

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Disclosures of Conflicts of Interest: The authors have no conflicts to disclose

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Final approval of the version to be published: Nikita Joshi, Anna K. Kopec, Jessica Ray, James P. Luyendyk

administration on the exaggerated pathology in ANIT-exposed PAR-4^{-/-} mice reinforces the prevailing importance of coagulation-mediated platelet activation in limiting this unique liver pathology.

Graphical abstract

Role and importance of specific platelet activation pathways in control of experimental xenobiotic-induced cholestatic liver injury and fibrosis

Normally quiescent platelets circulate in the blood until activated by a range of stimuli. Thrombin is among the most powerful activators of platelets through activation of G-protein coupled protease activated receptors [(PAR)-3/4 complex in mice (PAR-1 in humans)]. Thrombin activation of platelets induces a conformational change in the $\alpha_{IIb}\beta_3$ integrin (expressed on platelets) required to engage ligands such as fibrin(ogen), an interaction involved in processes such as platelet aggregation. Activated platelets release intracellular granules containing stored mediators such as ADP and serotonin. Released ADP amplifies platelet activation via activation of the G-protein coupled receptor P2Y₁₂. Diminished platelet activation in PAR-4^{-/-} mice was coupled to exaggerated hepatocellular necrosis and biliary fibrosis in mice with xenobiotic (i.e., ANIT)-induced cholestatic liver injury (Joshi et al. 2015). Moreover, fibrin(ogen) engagement of the platelet integrin $\alpha_{IIb}\beta_3$ inhibits hepatocellular necrosis in ANIT-exposed mice (Joshi et al. 2015). The present studies reveal that inhibition of the ADP receptor P2Y₁₂ with clopidogrel increases xenobiotic-mediated cholangiofibrosis in wild-type mice. However, clopidogrel did not increase hepatocellular necrosis in ANIT-exposed mice, suggesting that platelet activation by ADP is not a component of the mechanism whereby platelets inhibit liver injury in this experimental setting. Clopidogrel also failed to exacerbate cholangiofibrosis in PAR-4^{-/-} mice, suggesting thrombin-mediated PAR-4 activation is central to the protective consequences of platelet activation in ANIT-exposed mice.

Keywords

Platelets; liver disease; fibrosis; bile ducts

1. Introduction

Blood platelet count is often affected in patients with chronic liver disease (Afdhal et al. 2008; Giannini 2006; Wang et al. 2004), and an increasing number of clinical and experimental studies suggest that platelet function contributes to the progression of liver disease (Kodama et al. 2010; Lang et al. 2008; Laschke et al. 2008; Murata et al. 2008; Sullivan et al. 2010a; Watanabe et al. 2009). However, the precise role of platelets in experimental liver injury appears to depend on the cause of the injury and nature of the liver pathology. Several studies have examined the role of platelets in models of cholestatic liver injury, where intrahepatic bile ducts are injured by various pathologic or toxic stimuli. Platelets exaggerate acute hepatocyte necrosis in models of obstructive cholestasis (bile duct ligation) and acute chemical-induced bile duct damage (Bailie et al. 1994; Laschke et al. 2008; Sullivan et al. 2010a). However, fibrosis of the bile ducts (i.e., cholangiofibrosis) increased significantly in thrombocytopenic mice subjected to long-term bile duct ligation (Watanabe et al. 2009). Likewise, in another setting of xenobiotic-induced bile duct fibrosis, mice with defects in coagulation-mediated platelet activation had increased cholangiofibrosis (Joshi et al. 2015). However, in another model of cholestasis-associated biliary fibrosis driven by Mdr2 deficiency, administration of aspirin, a relatively weak anti-platelet drug, significantly reduced cholangiofibrosis (Yoshida et al. 2014). Collectively, there is experimental evidence supporting both damaging and protective effects of platelets in experimental cholestatic liver disease.

Platelets circulate in a quiescent state and their activation can be systematically driven by a variety of stimuli including collagen, von Willebrand Factor, adenosine diphosphate (ADP), and the coagulation protease thrombin (Siess 1989). Thrombin is among the most potent physiological activators of platelets (Gear and Burke 1982; Haslam 1964; Kinlough-Rathbone et al. 1977; Packham et al. 1973; Siess 1989), and does so by direct activation of G-protein coupled, protease activated receptors (PARs) on platelets (Hamilton et al. 2004; Kahn et al. 1999; Sambrano et al. 2001; Stalker et al. 2012; Vu et al. 1991). Thrombin stimulates activation of human platelets via PAR-1 and PAR-4, whereas mouse platelets are activated through a complex of PAR-3/4 (Hamilton et al. 2004; Kahn et al. 1999; Kahn et al. 1998). Thrombin activated platelets release additional mediators, such as ADP (Haslam 1964; Packham et al. 1973; Siess 1989; Stalker et al. 2012). The G-protein coupled receptor P2Y₁₂ serves as a chemoreceptor for ADP and its interaction promotes additional platelet activation (Murugappa and Kunapuli 2006). P2Y₁₂ is the target of antiplatelet drugs such as clopidogrel (Savi et al. 2006). Previous studies have shown that PAR-4 is an important activator of platelets in experimental cholestasis, and PAR-4 deficiency worsened biliary fibrosis and liver necrosis in mice with chemical-induced cholestatic liver disease (Joshi et al. 2015). However, the role of P2Y₁₂ receptor activation in cholestatic liver injury is not well understood. Moreover, it is currently not known whether combined defects in multiple platelet activation pathways exacerbate cholestatic liver disease.

We sought to discern the role of different platelet activation pathways in chronic experimental cholestatic liver injury and fibrosis. Utilizing an established model of chemical-induced cholestatic liver injury and cholangiofibrosis, we examined the effect of

the irreversible P2Y₁₂ receptor antagonist clopidogrel on liver injury and fibrosis in both wild-type and PAR-4^{-/-} mice.

2. Materials and Methods

2.1 Mice

PAR-4^{-/-} mice and wild-type mice backcrossed at least 8 generations on the same C57BL/6J background, were maintained by homozygous breeding (Sambrano et al. 2001). Age-matched male mice between the ages of 8–14 weeks were used for these studies. Mice were housed at an ambient temperature of approximately 22°C with alternating 12 hour light/dark cycles and provided water and rodent chow *ad libitum* prior to study initiation. Mice were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility at Michigan State University. All animal procedures were approved by Michigan State University Institutional Animal Care and Use Committee.

2.2 ANIT diet model and pharmacological intervention

Custom diets were prepared by Dyets, Inc. (Bethlehem, PA). The ANIT diet was an AIN-93M purified diet containing 0.025% ANIT (Sigma-Aldrich, St. Louis, MO), as we have described previously (Joshi et al. 2015). Groups of mice were fed ANIT diet for a total of 4 weeks, *ad libitum*. For platelet inhibition studies, mice were allowed *ad libitum* access to purified drinking water, or purified drinking water containing the P2Y₁₂ receptor antagonist clopidogrel bisulfate (Matrix Scientific, Columbia, SC) (0.15 mg/mL) for the duration of ANIT exposure (4 weeks). Based on typical daily consumption of water by the mice (4–5 ml per day), this produced an approximate daily dose of 30 mg/kg. Previous studies have shown that this dose prolongs time to occlusion in a model of thrombosis (Lorrain et al. 2004). Mice were anesthetized with isoflurane, and blood was collected from the caudal vena cava into sodium citrate (final, 0.38%) or an empty syringe for the collection of plasma and serum, respectively. The liver was removed and washed with saline. Sections of the left lateral lobe were fixed in 10% neutral buffered formalin for 48 hours prior to routine processing. The remaining liver was cut into approximately 100 mg sections and flash-frozen in liquid nitrogen.

2.3 Histopathology and clinical chemistry

For analysis of liver histopathology by light microscopy, formalin-fixed liver sections were cut at 5 microns and stained with hematoxylin and eosin (H&E), sirius red, and cytokeratin-19 (CK-19) antigen by the Michigan State University Investigative Histopathology Laboratory as described previously (Joshi et al. 2015; Joshi et al. 2016). At least 2 sections of liver from the left lateral lobe of each animal were qualitatively and quantitatively evaluated in their entirety to calculate area of necrosis. Quantitative assessment of necrotic area in H&E-stained sections were performed in a masked fashion. For quantification of sirius red (collagen deposits) and CK-19 staining (bile duct epithelial cells), images of stained liver sections were captured using a Virtual Slide System VS110 (Olympus, Hicksville, NY) with a 20× objective. The area of positive sirius red and CK-19 staining in at least 200 images per tissue was determined in an automated and unbiased fashion using a batch macro and the color de-convolution tool in ImageJ. Serum activity of

alanine aminotransferase (ALT) was determined using a commercial reagent (Thermo Scientific, Waltham, MA). Plasma serotonin levels were determined using a commercial enzyme-linked immunosorbent assay kit (Eagle Biosciences, Nashua, NH).

2.4 RNA isolation, cDNA synthesis, and real-time PCR

Total RNA was isolated from approximately 15 mg of snap-frozen liver or adherent macrophages using TRI Reagent (Molecular Research Center, Cincinnati, OH). 1 µg of total RNA was utilized for the synthesis of cDNA, accomplished using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) and a C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). SYBR Green quantitative real-time PCR (qPCR) amplification was performed using a CFX Connect thermal cycler (Bio-Rad) with primers purchased from IDT (Coralville, IA). Primers were purchased from IDT (Coralville, IA). The expression of each gene was normalized to the housekeeper gene GAPDH and the relative levels of each gene were evaluated using the $\Delta\Delta C_t$ method. Mouse GAPDH primer sequences were 5'-GTGGACCTCATGGCCTACAT-3' (forward primer), 5'-TGTGAGGGAGATGCTCAGTG-3' (reverse primer). Mouse COL1A1 primer sequences were 5'-GAGCGGAGAGTACTGGATCG-3' (forward primer), 5'-GCTTCTTTTCCTTGGGGTTC-3' (reverse primer). Mouse ITGβ6 primer sequences were 5'-CTCACGGGTACAGTAACGCA-3' (forward primer), 5'-AAATGAGCTCTCAGGCAGGC-3' (reverse primer). Mouse TGFβ2 primer sequences were 5'-CCCCGGAGGTGATTCCATC-3' (forward primer), 5'-GATGGCATTTCGGAGGGGA-3' (reverse primer). Mouse TIMP1 primer sequences were 5'-GAGACACACCAGAGCAGATACC-3' (forward primer), 5'-CCAGGTCCGAGTTGCAGAAG-3' (reverse primer). Mouse TGFβ1 primer sequences were 5'-CTCCCGTGGCTTCTAGTGC-3' (forward primer), 5'-GCCTTAGTTTGGACAGGATCTG-3' (reverse primer).

2.5 Statistics

Two-way analysis of variance was used and Student-Newman-Keuls test was used for post-hoc comparisons. Data were log-transformed as required to achieve a normal distribution. The criterion for statistical significance was $p < 0.05$.

Results

3.1 Impact of clopidogrel on ANIT-induced liver injury in PAR-4^{-/-} mice

Platelets are the primary source of plasma serotonin, and plasma serotonin levels increase in wild-type mice exposed to ANIT, reflecting platelet activation (Joshi et al. 2015). In agreement with our previous studies, plasma serotonin levels were significantly reduced in ANIT-exposed PAR-4^{-/-} mice (Figure 1A). Moreover, clopidogrel significantly reduced plasma serotonin levels (Figure 1A), indicative of inhibition of platelet activation after ANIT exposure.

PAR-4 deficiency is not associated with overt changes in liver histopathology (Joshi et al. 2015). Likewise, liver histopathology was unremarkable in wild-type mice treated with clopidogrel alone (data not shown). Mice exposed chronically to ANIT in the diet develop

biliary hyperplasia and cholangiofibrosis in the absence of marked hepatocyte necrosis (Joshi et al. 2015; Joshi et al. 2016; Joshi et al. 2014). In agreement with our previous study (Joshi et al. 2015), PAR-4 deficiency resulted in an atypical increase in hepatocellular injury in ANIT-exposed vehicle-treated mice, indicated by elevated serum ALT activity and hepatocellular necrosis compared to wild-type mice (Figure 1B–C). Interestingly, clopidogrel administration did not affect serum ALT activity or necrosis in wild-type mice exposed to ANIT (Figure 1B–C). However, clopidogrel administration significantly reduced serum ALT activity and necrosis in PAR-4^{-/-} mice (Figure 1B–C). The results suggest that inhibition of P2Y₁₂ receptor-mediated platelet activation is insufficient to worsen ANIT-induced hepatocellular necrosis in wild-type mice, and that P2Y₁₂ receptor activation may exaggerate hepatocyte necrosis in the absence of PAR-4.

2.2 Clopidogrel and PAR-4 deficiency independently increase ANIT-induced bile duct hyperplasia

ANIT-induced bile duct hyperplasia was evaluated by immunohistochemical staining for cytokeratin-19 (CK-19), a biomarker of bile ducts in mouse liver. In agreement with our previous study (Joshi et al. 2015), biliary hyperplasia was markedly increased in ANIT-exposed PAR-4^{-/-} mice (Figure 2A–B). Despite having no effect on hepatocellular necrosis, clopidogrel administration exacerbated biliary hyperplasia in ANIT-exposed wild-type mice compared to vehicle-treated mice exposed to ANIT (Figure 2A–B). However, clopidogrel administration did not increase bile duct hyperplasia in PAR-4^{-/-} mice (Figure 2A–B). The results indicate that clopidogrel increases ANIT-induced bile duct hyperplasia in wild-type mice, but lacks this effect in PAR-4^{-/-} mice.

3.3 Clopidogrel and PAR-4 deficiency independently increase ANIT-induced cholangiofibrosis

Compared to ANIT-exposed wild-type mice treated with vehicle, hepatic expression of profibrogenic COL1A1, ITGβ6 and TIMP1 mRNAs increased significantly in livers of ANIT-exposed wild-type mice that were given clopidogrel (Figure 3A–C). Hepatic expression of TGFβ1 and TGFβ2 mRNA was not significantly affected by genotype or treatment (Figure 3D–E). In agreement with these indicators of profibrogenic response, hepatic collagen protein deposition, indicated by sirius red staining and quantification, significantly increased in ANIT-exposed wild-type mice administered clopidogrel compared to vehicle-treated mice (Figure 4A–B). These results indicate that clopidogrel treatment worsens cholangiofibrosis in ANIT-exposed wild-type mice. We have shown previously that cholangiofibrosis is increased in PAR-4^{-/-} mice exposed chronically to ANIT (Joshi et al. 2015). Notably, administration of clopidogrel did not further exacerbate either hepatic profibrogenic gene induction or cholangiofibrosis in ANIT-exposed PAR-4^{-/-} mice (Figure 3A–E, 4A–B). Collectively these studies indicate clopidogrel administration significantly increases cholangiofibrosis in ANIT-exposed wild-type mice, but does not exacerbate the fibrosis elicited by PAR-4 deficiency in this experimental setting.

3. Discussion

Building on previous studies identifying PAR-4-mediated platelet activation as protective in chronic ANIT-mediated liver fibrosis, we found that administration of the P2Y₁₂ antagonist clopidogrel also significantly increased biliary hyperplasia and fibrosis in ANIT-exposed mice. This result provides further evidence in support of platelets exerting antifibrotic activity in this experimental setting. However, clopidogrel had no effect on liver fibrosis in PAR-4^{-/-} mice, suggesting PAR-4 is potentially a stronger determinant of anti-fibrotic activity in this model. Interestingly, unlike inhibition of PAR-4, clopidogrel did not increase liver necrosis in ANIT-exposed mice. Rather, and to our surprise, clopidogrel administration reduced liver necrosis in ANIT-exposed PAR-4^{-/-} mice. The basis for this unexpected protection conferred by clopidogrel is not clear, although clopidogrel did significantly reduce necrosis in mice given a single acute dose of ANIT, most likely by blocking neutrophil activation (Sullivan et al. 2010a).

Available evidence on the role of platelets in experimental systems of cholestatic liver injury and fibrosis is complex and often puzzling, making it very difficult to assign a single function to platelets in this disease process (Kopec et al. 2016; Lisman 2015). Despite concurrent protection from hepatocellular damage, thrombocytopenic mice subjected to chronic bile duct ligation develop markedly increased cholangiofibrosis (Kodama et al. 2010). Likewise, loss of thrombin-mediated platelet activation increased liver fibrosis induced by chronic exposure to ANIT (Joshi et al. 2015). Here, we extend these studies to note that clopidogrel administration increases cholangiofibrosis in mice exposed chronically to ANIT. However, another inhibitor of platelets, aspirin, had the opposite effect in another model of cholangiofibrosis, Mdr2^{-/-} mice (Yoshida et al. 2014). The basis for the discrepant effects of these various anti-platelet interventions on fibrosis is not known, and these studies highlight the challenge in interpreting studies examining the role of platelets in cholestatic liver injury and fibrosis.

One challenge is the diversity of strategies utilized to evaluate the role of platelets in experimental cholestatic liver disease. In part, the variation in efficacy and target of the anti-platelet intervention could underpin the observed differences across studies. For example, near complete thrombocytopenia caused severe hepatic injury in mice given a single large dose of ANIT (Sullivan et al. 2010a). However, in similar studies where platelet depletion was incomplete (10% of normal), ANIT-induced hepatocellular injury was reduced (Sullivan et al. 2010a). Thus, the extent of thrombocytopenia has the potential to affect experimental outcome. There are also some differences in the antibodies utilized to deplete platelets, and off-target effects such as hypothermia and lung injury have been reported with some antibodies (Nieswandt et al. 2000; Nieswandt et al. 1999).

The impact of platelet inhibitors and thrombocytopenia on cholangiofibrosis is also varied. Mice with megakaryocyte-specific deletion of Bcl-xL developed severe thrombocytopenia, and had greatly increased liver fibrosis after long-term bile duct ligation, demonstrating a protective role of platelets in chronic cholestatic liver fibrosis (Kodama et al. 2010). Similarly, lack of thrombin-mediated platelet activation via PARs, a strong platelet activation signal, or lack of fibrin(ogen) engagement of the platelet integrin $\alpha_{IIb}\beta_3$, significantly

increased liver fibrosis (Joshi et al. 2015). In contrast, another study found that treatment with aspirin, a relatively weak anti-platelet drug, significantly reduced cholangiofibrosis in *Mdr2*^{-/-} mice (Yoshida et al. 2014). The basis for these different observations is not completely understood, but like the distinction between complete and partial thrombocytopenia, it is plausible that the efficacy of platelet inhibition may determine the effect on liver pathology.

Cholestatic liver injury is associated with two distinct lesions. Focal hepatocellular necrosis, often termed bile-infarcts, occur after bile duct ligation or ANIT and are driven primarily by excessive neutrophil activation (Gujral et al. 2003; Laschke et al. 2008; Sullivan et al. 2010a). In contrast, neutrophils and neutrophil chemokines are not required for cholangiofibrosis in these models (Saito et al. 2003; Xu et al. 2004). Mechanisms mediating cholangiofibrosis, such as $\alpha_v\beta_6$ -integrin mediated TGF β 1 activation, do not affect the development of hepatocellular injury (Patsenker et al. 2008; Popov et al. 2008; Sullivan et al. 2010b; Wang et al. 2007). It is conceivable that platelets could have differential impact on each of these pathologies. Indeed, this could explain how after bile duct ligation, thrombocytopenia can concurrently protect mice from hepatocellular injury, yet exacerbate cholangiofibrosis (Kodama et al. 2010). In other experimental settings where hepatocyte injury is intrinsically linked to the profibrotic response, the role of platelets in liver injury and fibrosis may be more easily aligned, as is observed in viral hepatitis-associated liver disease (Lang et al. 2008).

4. Conclusion

The present studies provide clues as to the relative importance of specific platelet activation pathways in experimental cholestatic liver injury. Complementing our previous studies (Joshi et al. 2015), we demonstrate that both PAR-4 and P2Y₁₂ contribute to platelet activation in ANIT-exposed mice. It seems plausible that thrombin-mediated PAR-4 activation promotes further ADP-driven platelet activation in ANIT-exposed mice. This would explain the impact of clopidogrel on biliary hyperplasia and cholangiofibrosis in ANIT-exposed wild-type mice, but not in PAR-4^{-/-} mice. Collectively, these results suggest that thrombin-mediated PAR-4 activation inhibits hepatocellular necrosis and cholangiofibrosis in ANIT-exposed mice, and suggest that P2Y₁₂ receptor activation also plays a central role in the mechanism whereby platelets inhibit cholangiofibrosis. Development of *in vitro* systems to assess the impact of platelet-derived mediators on fibroblast activation, particularly in co-culture with cholangiocytes, could reveal novel mechanisms whereby the hemostatic system controls biliary fibrosis. Overall, these studies highlight a continued need to define the impact of different platelet activation pathways on experimental liver disease, particularly given emerging evidence that anti-platelet therapies may have beneficial effects on fibrosis driven by some human liver diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding information

This work was supported by the National Institutes of Health National Institute of Environmental Health Sciences [R01 ES017537]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health..

Abbreviations

ANIT	alpha-naphthylisothiocyanate
ADP	adenosine diphosphate
PAR	protease activated receptor
H&E	hematoxylin and eosin
CK-19	cytokeratin-19
ALT	alanine aminotransferase
TGFβ	transforming growth factor beta
COL1A1	type I collagen
ITGβ6	integrin beta 6
TIMP1	tissue inhibitor of matrix metalloproteinase 1
α_{IIb}β₃	alphaIIbbetaIII integrin
α_vβ₆	alphaVbeta6 integrin

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Figure 1. Impact of clopidogrel administration on ANIT-induced liver injury in wild-type and PAR-4^{-/-} mice

Wild-type and PAR-4^{-/-} mice were fed diet containing 0.025% ANIT for 4 weeks. Clopidogrel bisulfate dissolved in drinking water (Clopi, 0.15 mg/mL) or vehicle (Veh, drinking water) was given for the duration of ANIT exposure at a dose of 30 mg/kg. (A) Plasma serotonin levels were determined by ELISA. (B) Serum ALT activity and (C) area of necrosis were determined as described in Materials and Methods. Data are expressed as mean±SEM; n=4–7 mice per group. *p<0.05 vs. wild-type mice receiving the same treatment. #p<0.05 vs. vehicle-treated mice of the same genotype.

Figure 2. Clopidogrel administration and PAR-4 deficiency independently increase ANIT-induced bile duct hyperplasia

Wild-type (WT) and PAR-4^{-/-} mice were fed diet containing 0.025% ANIT for 4 weeks. Clopidogrel bisulfate dissolved in drinking water (Clopi, 0.15 mg/mL) or vehicle (Veh, drinking water) was given for the duration of ANIT exposure at a dose of 30 mg/kg. (A) Representative photomicrographs (100×) show liver sections stained for cytokeratin-19 (CK-19, brown) in ANIT-exposed wild-type and PAR-4^{-/-} mice. (B) CK-19 staining was quantified as described in Materials and Methods. Data are expressed as mean+SEM; n=4–7

mice per group. * $p < 0.05$ vs. wild-type mice receiving the same treatment. # $p < 0.05$ vs. vehicle-treated mice of the same genotype.

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Figure 3. Clopidogrel and PAR-4 deficiency independently increase ANIT-induced profibrogenic gene induction

Wild-type (WT) and PAR-4^{-/-} mice were fed diet containing 0.025% ANIT for 4 weeks. Clopidogrel bisulfate dissolved in drinking water (Clopi, 0.15 mg/mL) or vehicle (Veh, drinking water) was given for the duration of ANIT exposure at a dose of 30 mg/kg. Hepatic expression of mRNAs encoding the profibrogenic genes (A) COL1A1, (B) ITGβ6, (C) TIMP1, (D) TGFβ2, and (E) TGFβ1 was determined by real-time qPCR. Data are expressed as mean±SEM; n=6–7 mice per group. *p<0.05 vs. wild-type mice receiving the same treatment. #p<0.05 vs. vehicle-treated mice of the same genotype.

Figure 4. Clopidogrel and PAR-4 deficiency independently increase ANIT-induced cholangiofibrosis

Wild-type (WT) and PAR-4^{-/-} mice were fed diet containing 0.025% ANIT for 4 weeks. Clopidogrel bisulfate dissolved in drinking water (Clopi, 0.15 mg/mL) or vehicle (Veh, drinking water) was given for the duration of ANIT exposure at a dose of 30 mg/kg. (A) Representative photomicrographs show liver sections stained for sirius red (100×). (B) Sirius red staining was quantified as described in Materials and Methods. Data are expressed as

mean+SEM; n=4–7 mice per group. *p<0.05 vs. wild-type mice receiving the same treatment. #p<0.05 vs. vehicle-treated mice of the same genotype.

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