PGE2-regulated wnt signaling and N-acetylcysteine are synergistically hepatoprotective in zebrafish acetaminophen injury

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Acetaminophen (APAP) toxicity is the most common drug-induced cause of acute liver failure in the United States. The only available treatment, *N*-acetylcysteine (NAC), has a limited time window of efficacy, indicating a need for additional therapeutic options. Zebrafish have emerged as a powerful tool for drug discovery. Here, we developed a clinically relevant zebrafish model of APAP toxicity. APAP depleted glutathione stores, elevated aminotransferase levels, increased apoptosis, and caused dose-dependent hepatocyte necrosis. These outcomes were limited by NAC and conserved in zebrafish embryos. In a targeted embryonic chemical screen, prostaglandin E2 (PGE2) was identified as a potential therapeutic agent; in the adult, PGE2 similarly decreased APAP-associated toxicity. Significantly, when combined with NAC, PGE2 extended the time window for a successful intervention, synergistically reducing apoptosis, improving liver enzymes, and preventing death. Use of a wnt reporter zebrafish line and chemical genetic epistasis showed that the effects of PGE2 are mediated through the wnt signaling pathway. Zebrafish can be used as a clinically relevant toxicological model amenable to the identification of additional therapeutics and biomarkers of APAP injury; our data suggest combinatorial PGE2 and NAC treatment would be beneficial for patients with APAP-induced liver damage.

acetaminophen liver toxicity | chemical screen

Acetaminophen (*N*-acetyl-p-aminophenol; APAP) is a commonly used analgesic and antipyretic. Although safe at therapeutic doses, accidental or suicidal drug overdose can cause dose-dependent liver damage. APAP is the most common cause for liver transplantation for toxin-induced fulminant hepatic failure and results in more than 300 deaths annually in the United States (1). The only antidote in clinical use is *N*-acetylcysteine (NAC), which reduces mortality by ~20–28% (2); however, the time interval of effective intervention after ingestion is typically <12 h, and delayed or prolonged treatment can negatively impact clinical outcome and survival (3). Therapeutic options for hepatic failure are limited to best supportive care and liver transplantation.

APAP toxicity results from a hepatotoxic metabolite, *N*-acetyl-p-benzoquinone imine (NAPQI), produced by the cytochrome P450 enzymes CYP1A2, 2E1, and 3A4 (4). At therapeutic doses, NAPQI is efficiently inactivated in the liver by glutathione (GSH) conjugation (5). At toxic doses, excess production of NAPQI depletes hepatic GSH. Unconjugated NAPQI causes dysfunction of critical liver proteins, oxidative stress, and mitochondrial damage (6–8). NAC is a true antidote and limits damage by repleting GSH. Clinical efficacy of NAC treatment was shown for patients presenting after APAP ingestion; however, no conclusive clinical trials were conducted to elucidate its efficacy and optimal treatment window. Therapeutic benefits have been shown in mammalian models for other antioxidants (9, 10) that function to restore GSH levels. Compounds that support liver recovery from APAP injury rather than antagonizing the mechanism of toxicity are lacking. Chemical inhibitors of mitochondrial damage have been tested in mouse models (11); however, in vivo screens for novel compounds to act synergistically with NAC have not been performed.

Fish have been used to assess water quality and detect toxins. Genetic conservation between zebrafish and mammals was documented in liver development, regeneration, and carcinogenesis (12); however, the functional conservation of toxic–metabolic effects on the liver has not been evaluated. Proteomic analysis of the zebrafish liver has revealed potential indicators of xenobiotic response (13), implying the suitability for translational toxicological studies. Zebrafish embryos are uniquely amenable to chemical screening approaches for the identification of novel compounds. Recently, we have shown that findings in embryonic screens can be extended to adult zebrafish physiology and translated into therapeutic applications (14).

Here, we characterize a zebrafish model for APAP liver toxicity and identify therapeutics that work cooperatively with NAC. APAP was found to decrease zebrafish liver GSH content and elevate serum alanine aminotransferase (ALT) levels. Survival and histological evidence of liver injury were dose-dependent. Progressive APAP injury was inhibited by NAC, making this an excellent preclinical model. Liver-specific APAP toxicity was conserved in zebrafish embryos, enabling a targeted chemical genetic evaluation for hepatoprotectants, which identified prostaglandin E2 (PGE2) as a potential therapeutic; in the adult, it worked synergistically with NAC to limit hepatic APAP injury and enhance survival, extending the therapeutic window for chemical intervention. PGE2 increased wnt pathway activity, a known regulator of liver regeneration, after APAP injury to improve outcome in embryos and adults. These results show the therapeutic relevance of modeling organ-specific toxicity in zebrafish and point to clinical strategies to reduce APAP-mediated liver damage.

Results

APAP Causes Liver Toxicity in Adult Zebrafish. APAP exposure in mammals leads to the rapid depletion of GSH stores (15). Similarly, in the adult zebrafish liver, GSH was significantly reduced

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Conflict of interest statement: T.E.N and W.G. have filed a patent for the combined use of PGE2 and NAC in acetaminophen liver injury. T.E.N, L.I.Z., and W.G. receive patent royalties and consulting fees from FATE Therapeutics for the use of PGE2 in hematopoietic stem cell formation.

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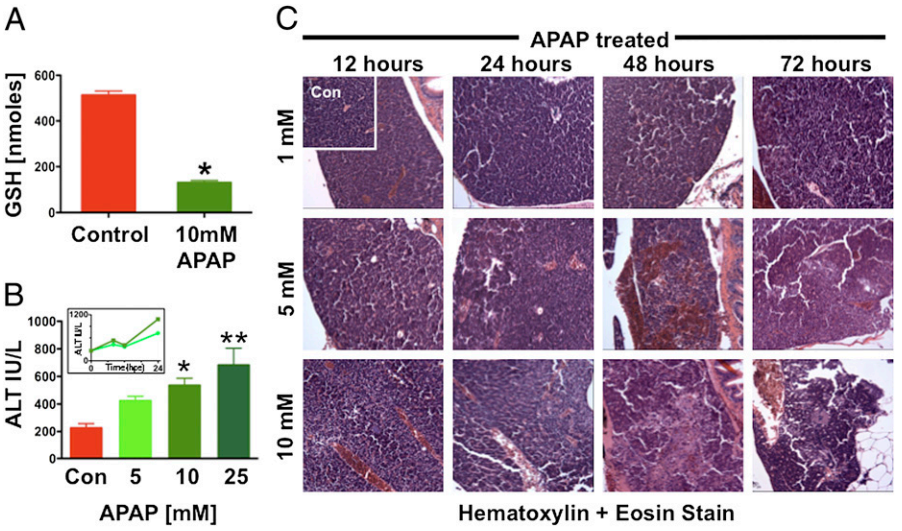


Fig. 1. Acetaminophen causes liver toxicity in adult zebrafish. Adult zebrafish were exposed to APAP for 24 h. (*A*) Total liver GSH was significantly diminished after exposure to 10 mM APAP. **t* test, $P \leq 0.001$, $n \geq 4$. (*B*) ALT levels were measured 6 hpe to 5, 10, and 25 mM APAP. *Significant vs. control. **Significant vs. 5 mM, ANOVA, $P < 0.01$, $n = 20$/sample for three replicates. (*Inset*) ALT levels measured over time after exposure to 5 and 10 mM APAP rose over the first 24 hpe ($n \geq 10$/measurement). (*C*) Effects of increasing APAP doses on liver histology; hepatocyte necrosis, sinusoidal widening, and focal hemorrhage can be seen.

by 75% in liver homogenates 24 h postexposure (hpe) to APAP (10 mM; 513.4 ± 18.0 vs. 130.5 ± 13.6 nmols/liver, *t* test, $P \leq 0.001$, $n = 5$) (Fig. 1*A*). Baseline ALT levels, used to detect liver injury clinically, were higher in zebrafish than in mice and humans, consistent with prior reports (16) (Fig. 1*B*); within 6 hpe, ALT levels were elevated in a dose-dependent fashion and continued to rise over the first 24 h (Fig. 1*B Inset*). To establish toxicity thresholds, we recorded survival after exposure to increasing doses of APAP (Fig. 2*A*). Doses up to 5 mM had no impact on survival, whereas 10 mM APAP caused progressive death in ~50% of fish, beginning by 20 hpe. Exposure to 25 mM APAP caused rapid death in almost all fish by 10 hpe. Bolus treatment with 50 mM APAP for 3 h, to mimic suicidal overdose, resulted in liver toxicity comparable with extended 10-mM exposure. Histological evaluation revealed minimal effects with 1 mM APAP (Fig. 1*C*); 5 mM APAP did not affect gross liver morphology at 24 hpe but by 48 hpe, caused increased hepatocyte necrosis and areas of focal hemorrhage. Widespread necrosis and sinusoidal hemorrhage were seen as early as 12 hpe after exposure to 10 mM APAP. To visualize the extent of liver damage in vivo, we used the transgenic liver fatty acid binding-protein reporter *Tg(-2.8fabp10:EGFP)*^{as3} (referred to as *lfabp:GFP*) (Fig. S1*A*) (17); APAP caused a time-dependent reduction in fluorescence, suggestive of hepatocyte death and corresponding with the histological analysis.

NAC Prevents Liver Toxicity. To document the applicability of the zebrafish model to human physiology, we exposed APAP-treated fish concurrently to NAC. Zebrafish survival and ALT levels after 10 mM APAP exposure were markedly improved by NAC (10 μM) compared with APAP-treated controls (Fig. 2 *A* and *B*) ($P < 0.05$). NAC could not improve survival after higher APAP doses (25 mM) (Fig. 2*A*); these results are consistent with clinical observations. In vivo fluorescence microscopy of *lfabp:GFP* fish showed dramatic improvement in liver morphology after immediate NAC treatment (Fig. S1*B*), which was confirmed histologically (Fig. 2*C*). Ethanol (EtOH) exposure worsened outcome after APAP injury (Fig. S2 *A* and *B*) and antagonized NAC efficacy.

Proteomic Analysis of Zebrafish Plasma. To show changes in liver function in response to APAP, serum protein concentrations were assessed by MS. After generation of a partial 2D-gel map of the plasma proteome (Fig. S3*A*), comprehensive protein coverage was obtained by 2D-liquid chromatography MS/MS, resulting in the identification of 223 high-confidence proteins. Most were highly evolutionarily conserved and present in human serum (Table S1). Relative changes in serum protein concentration over time were quantified by isobaric tag for relative and absolute quantitation (iTRAQ), revealing four differential protein response patterns (Fig. 3*A* and Fig. S3*B*). One group (71 proteins) steadily decreased over time (Fig. 3*B*); this cluster contained proteins associated with liver function, such as transferrin and thrombin, suggesting impaired synthetic capacity. Cluster 2 (46 proteins) exhibited a biphasic pattern, where protein concentration initially declined and then rose; several of these, such as GST and carbonic anhydrase, were previously shown to be regulated by APAP treatment (18). Cluster 3 (33 proteins) was characterized

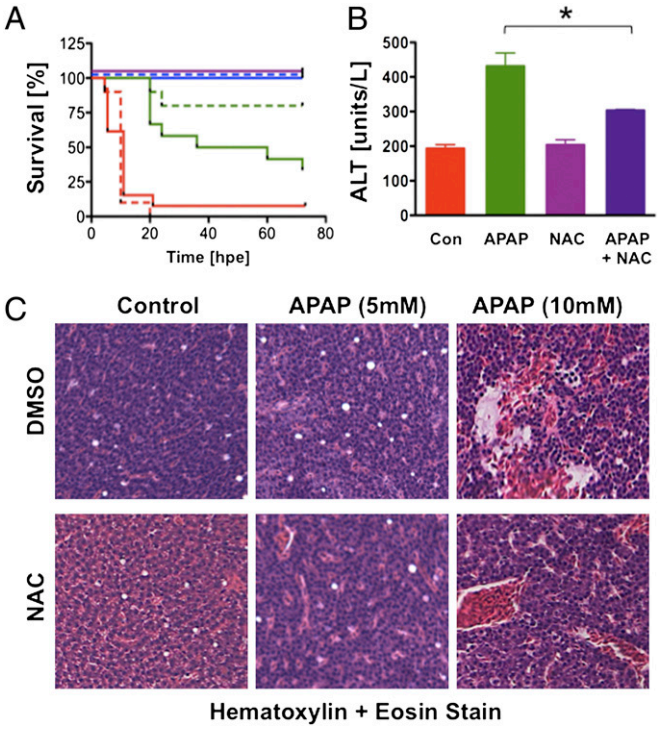
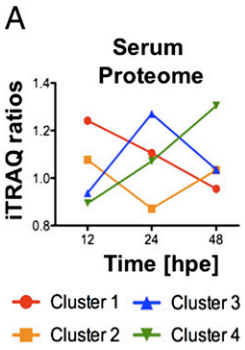


Fig. 2. *N*-acetylcysteine prevents liver toxicity. (*A*) Survival was assessed in adult zebrafish exposed to 5 (blue), 10 (green), or 25 (red) mM APAP alone (solid line) and with 10 μM NAC (dashed line; $n \geq 10$ per group). (*B*) APAP-induced elevation of ALT is reversed by the addition of NAC. *ANOVA, $P < 0.05$, 15–20 fish for three replicates). (*C*) Zebrafish were exposed to APAP and NAC at the indicated doses; NAC decreased the extent of necrosis after APAP.



B

Cluster 1 (71 proteins)	Cluster 2 (46 proteins)	Cluster 3 (33 proteins)	Cluster 4 (73 proteins)
transferrin *	glutathione S transferase *	ceruloplasmin	aldehyde dehydrogenase #○
apolipoprotein related #§	lfabp *	parvalbumin related	lactate dehydrogenase
vitellogenin	fibrinogen ¶	collagen related	glyceraldehyde dehydrogenase +
thrombin related §¶	carbonic anhydrase *#	complement related #§¶	integrin ¶

Fig. 3. Proteomic and transcriptional changes in response to APAP toxicity. (*A*) Schematic summary of all protein identities analyzed by iTRAQ showing differential response patterns. (*B*) Representative responsive proteins; those marked were found previously to be APAP-regulated in mammalian models. *, ref. 20. #, ref. 18. §, Ref. 19. +, ref. 21. ¶, ref. 22. ○, ref. 7.

by the reverse response; this cluster included structural and remodeling proteins. Cluster 4 (73 proteins) showed continuously increasing protein levels indicative of cellular damage. Several representative genes displayed quantitative PCR (qPCR) expression patterns consistent with iTRAQ protein responses (Fig. S3*C*). Although direct comparisons across model systems are difficult because of variation in experimental design, the results presented are broadly comparable with prior observations in murine models of APAP toxicity (7, 18–22), indicating conservation of the xenobiotic response.

Conserved Effects of APAP in Zebrafish Embryos. Zebrafish embryos are amenable to unbiased chemical genetic screens (14). To discern whether APAP produced similar effects on embryonic and larval hepatocytes, *lfabp:GFP* embryos were exposed to increasing doses of APAP. Liver size diminished in a dose- and time-dependent fashion after APAP treatment (Fig. S4 *A*–*D*). The effect of NAC was also conserved, as evidenced by the larger liver in more than 50% of APAP-treated embryos (Fig. S5 *A* and *B*). This analysis was confirmed by *lfabp* in situ hybridization and replicated in larvae.

Embryonic APAP Chemical Modifier Screen Identifies PGE2. Given the conserved embryonic effects of APAP, a targeted chemical screen for modifiers of xenobiotic-induced toxicity was performed. *lfabp:GFP* embryos were exposed to APAP from 48 to 96 hours postfertilization (hpf) concurrently with individual compounds with known embryonic liver-specific bioactivity (Fig. S6 *A* and *B*). APAP toxicity over this time frame resulted in substantial death (>90%) and dramatically reduced liver size. PGE2 mitigated the APAP effects in a significant fraction of embryos (screen: 6 normal/11 scored; retest: 26/54). Exposure to a long-acting derivative, 16,16-dimethyl-PGE2 (dmPGE2, 10 μM), substantially increased liver size at 96 hpf (screen: 10 increased/12; retest: 59/66) (Fig. 4*A*) and partially corrected the APAP phenotype (screen: 8 normal/12; retest: 37/58); these results were confirmed by qPCR (Fig. 4*B*). To determine how PGE2 mitigated APAP-induced hepatotoxicity, we examined proliferation and apoptosis by BrdU and TUNEL staining, respectively. dmPGE2 enhanced proliferation (20 increased/29), whereas APAP reduced BrdU incorporation (19 decreased/24) (Fig. S7*A*); concurrent dmPGE2 treatment partially restored cellular proliferation in APAP-treated embryos (11/27) and significantly reduced apoptosis (APAP: 31 enhanced/31; dmPGE2+APAP: 16/35) (Fig. S7*B*). To elucidate if the effect of PGE2 was maintained during APAP-mediated liver injury in the adult, we examined survival after single-agent and combinatorial exposure; by 24 hpe, dmPGE2 almost completely reversed APAP mortality (Fig. 4*C*). qPCR for *lfapb* confirmed that APAP-induced loss of liver-specific transcription was limited by PGE2 (Fig. 4*D*).

dmPGE2 Acts Synergistically with NAC to Limit Embryonic Liver Damage. To determine whether dmPGE2 acted in concert with NAC to reduce liver damage, embryos were exposed to drug combinations and assessed for alterations in *lfabp* expression. APAP inhibited liver growth (47 decreased/48), whereas dmPGE2 increased liver size at 96 hpf (56 increased/59) (Fig. 5*A*). dmPGE2 (42 normal/66) and NAC (37 normal/61) each partially rescued the negative effects of APAP with synchronous exposure. In combination, NAC and dmPGE2 had synergistic protective effects, where the majority of embryos did not exhibit signs of any APAP-mediated hepatocyte loss (52 normal/63). Immediate combinatorial treatment affected embryonic survival (Fig. 5*B*) (85%) and apoptosis, as measured by combined caspase 3/7 activity (Fig. 5*C*) ($P < 0.001$). In clinical practice, NAC is typically administered with some delay after APAP ingestion. Although NAC alone had minimal effects on survival when given with 12-h delay (del), dmPGE2 rescued liver size (7/29) and embryo survival (25%) in a modest proportion of embryos (Fig. 5 *A* and *B*); the combination of NAC and dmPGE2, after delay, caused marked improvement in survival (45%) and *lfabp* expression (26/48). Caspase analysis confirmed that the effects of combinatorial treatment were mediated, in part, by a reduction in apoptosis (Fig. 5*C*).

PGE2 and NAC Improve Clinical Parameters of APAP Injury. To show applicability, we exposed adult zebrafish to single agent or combinatorial therapy concomitantly with APAP or 18-h delay and

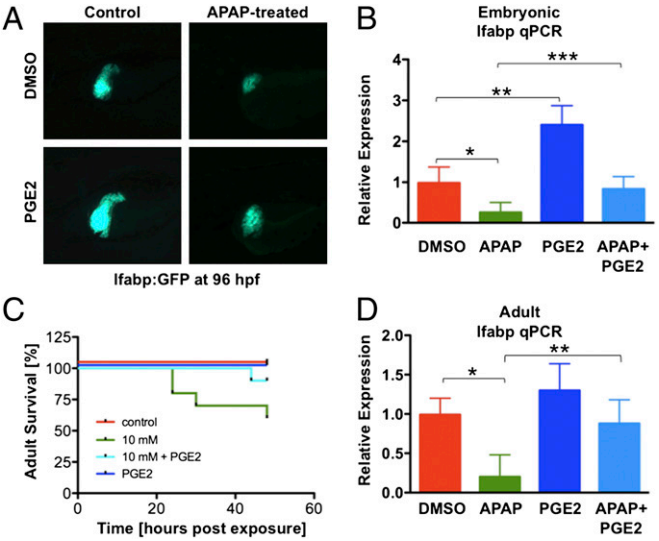


Fig. 4. Prostaglandin E2 (PGE2) inhibits APAP toxicity by enhancing proliferation and inhibiting apoptosis. (*A*) PGE2 (10 μM) increases embryonic liver growth (at 120 hpf) and diminishes 10 mM APAP toxicity in lfabp:GFP embryos. (*B*) Embryonic lfabp expression at 96 hpf, by qPCR, after the indicated treatment (ANOVA, $n = 5$). *$P = 0.06$. **$P < 0.001$. ***$P = 0.025$. (*C*) Survival in adult zebrafish ($n = 10$/treatment) after APAP is improved by dmPGE2. (*D*) lfabp expression in the adult liver, by qPCR, is reduced by APAP and rescued by dmPGE2 (ANOVA, $n = 4$). *$P = 0.002$. **$P = 0.0057$.

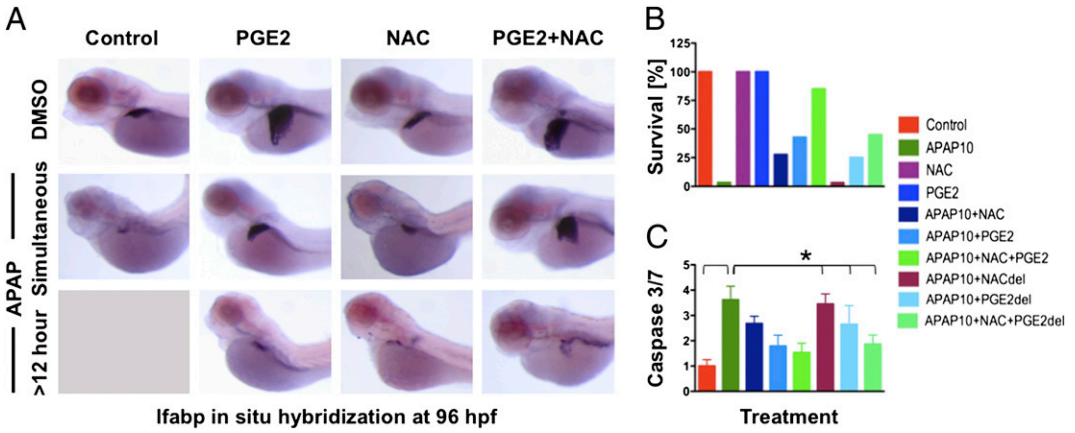


Fig. 5. PGE2 acts synergistically with NAC to improve APAP toxicity. (*A*) Zebrafish embryos were treated with APAP at 48 hpf and with dmPGE2 and/or NAC, either concomitantly or with 12-h delay (del). Combined NAC and PGE2 treatment improved liver morphology, assessed by in situ hybridization for *lfabp* at 96 hpf, even after delayed exposure. (*B*) Embryo survival is dependent on treatment parameter and is improved by NAC and PGE2 exposure. (*C*) Caspase 3/7 activity, as indicator of apoptosis, was measured in a luminometric assay and normalized to controls. *ANOVA, $n = 10$/ treatment for three replicates, $P < 0.001$.

recorded clinical parameters of liver injury (Fig. 6 *A–D*). Hepatic GSH was significantly diminished by APAP treatment and corrected by immediate administration of NAC or combinatorial therapeutics. dmPGE2 alone improved caspase and ALT levels, but not hepatic GSH, indicating it limited hepatotoxicity primarily through reducing apoptosis. Biochemical parameters of injury and cell death were unchanged between the APAP-exposed group and fish treated with significant delay (18 hpe) by NAC or dmPGE2 alone. Despite delay, combinatorial dmPGE2 and NAC treatment vastly improved liver parameters, cell death, survival, and histology (Fig. 6 *A–E*). These results indicate the potential synergistic therapeutic benefit of dmPGE2 and NAC as standard treatment for liver APAP toxicity.

PGE2 Enhances wnt Activity to Improve APAP Liver Toxicity. We have previously shown that PGE2 regulates wnt signaling during liver regeneration (23). Wnt activation has been observed after both surgical and toxic liver injury (12, 24, 25). To document hepatic wnt activation in zebrafish, Tg(*TOP:GFP*)^{w25} wnt reporter embryos (referred to as *TOP:GFP*) (26) were exposed to APAP at 48 hpf; markedly increased fluorescence was found in livers of treated fish at 72 hpf (Fig. S8*A*) (34 increased/48). To show that wnt signaling induced by APAP injury functioned to improve outcome, inducible *wnt8a* [*Tg(hsp70l:wnt8a-GFP)*^{w34}] embryos (27) were heat-shocked and exposed to APAP at 48 hpf. *wnt8a* overexpression was hepatoprotective and rescued liver-specific fluorescence (Fig. S8*B*) (wt APAP: 49 decreased/51; wnt8: 49 increased/55; wnt8+APAP: 53 rescued/67) (Fig. S8*C*). To corroborate that dmPGE2 treatment worked at least in part by enhancing wnt activation after adult APAP toxicity, *TOP:GFP* expression was analyzed. wnt activity was increased in response to APAP injury, consistent with recent results (25), and was further enhanced by dmPGE2 treatment (Fig. S8*D*). To determine whether pharmacologically elevating wnt signals could produce a biologically similar correlate to PGE2, adults were treated with APAP and exposed to the wnt-activator 6-bromoindirubin-3′-oxime (BIO) (0.5 μM). Gross morphological examination revealed that APAP-induced hemorrhage in the liver was greatly limited by BIO. As seen with PGE2, wnt activation dramatically improved liver appearance synergistically with NAC, even with delay (Fig. 7*A*). Inhibition of PGE2 production by indomethacin had detrimental effects on both morphology and survival (Fig. 7*B*), implying a role for PGE2 beyond the initiation of inflammatory responses in the setting of toxic injury. These effects were equilibrated by BIO treatment, highlighting the functional interaction between PGE2 and the wnt pathway in the nascent regenerative process after APAP liver injury.

Discussion

APAP liver toxicity remains a significant medical problem with multiple unmet clinical needs. NAC, the single available therapeutic option, is effective at preventing toxicity when given early after ingestion; however, it has a limited window of efficacy: patients who receive NAC with significant delay (>12 h) do not derive the same benefit from treatment and exhibit up to 15-fold increased death rates (28). The molecular mechanisms of APAP

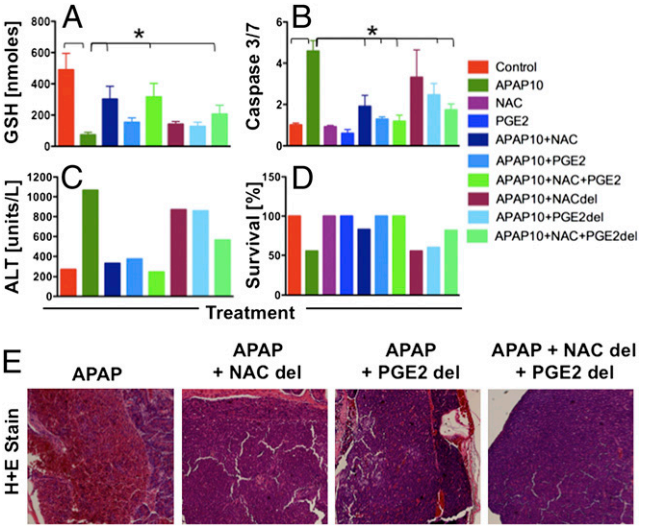


Fig. 6. Synergy between NAC and PGE2 limits toxicity and extends the therapeutic window in adult zebrafish after APAP. Adult zebrafish were exposed to APAP and NAC, dmPGE2, or a combination either concomitantly or with 18 h del. (*A*) GSH (nmoles/whole liver) was improved by combined exposure to NAC and dmPGE2. *ANOVA, $n = 3–4$, $P < 0.01$. (*B*) Relative caspase 3/7 levels were elevated after APAP and improved with immediate NAC and/or dmPGE2; delayed combinatorial therapy was still beneficial. *ANOVA, $n = 4–5$, $P < 0.001$. (*C*) Serum ALT levels ($n = 10–20$ fish/treatment at 24 hpe) show that combined NAC and dmPGE2 prevented hepatocyte damage by APAP, even after delay. (*D*) Combined NAC and dmPGE2 treatment led to improved adult zebrafish survival ($n = 9–10$/treatment) at 48 hpe. (*E*) Effect of delayed treatment on zebrafish liver histology at 24 hpe; sinusoidal hemorrhage and necrosis in APAP-treated adults could not be reversed with NAC or dmPGE2 alone but only with combined treatment.

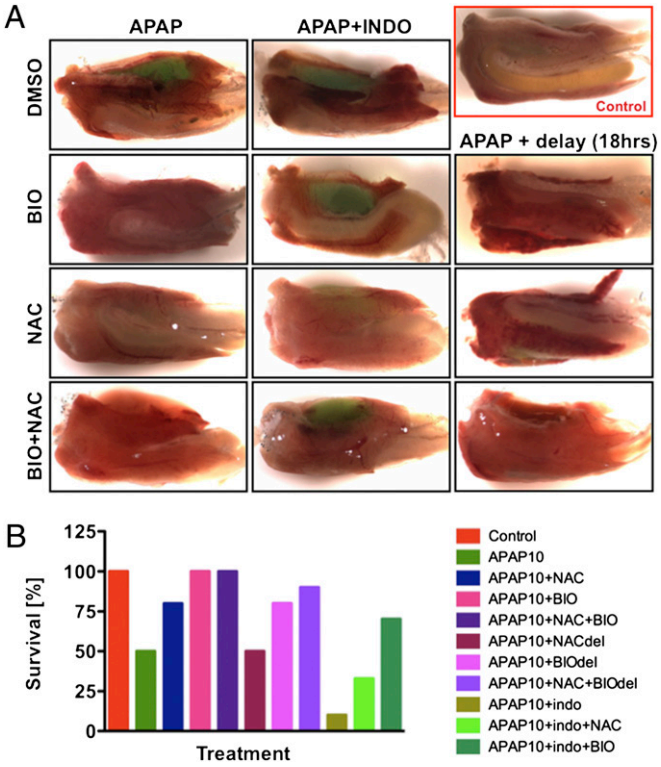


Fig. 7. Survival after APAP exposure is affected by wnt and PGE2 modulation. (*A*) Effect of wnt modulation on adult liver morphology and survival after APAP. Wild-type zebrafish were exposed to APAP and 6-bromoindirubin-3′-oxime (BIO) (0.5 μM) and/or NAC (10 μM), either concomitantly or with 18-h delay; BIO prevented liver hemorrhage and improved survival, particularly after delayed treatment. (*B*) wt adult zebrafish were exposed to 10 mM APAP and indomethacin (10 μM), NAC (10 μM), and BIO (0.5 μM). BIO improved survival if given immediately, either alone or with NAC. If given with 18-h delay (del), the combination of BIO and NAC acted synergistically. Indomethacin in combination with APAP was detrimental to survival, which was improved more substantially by BIO than by NAC.

toxicity have been elucidated in cell-culture studies and several animal models. Most treatment strategies are based on the same principles as NAC and work as a direct antidote through GSH repletion (9, 10). Here, we established zebrafish as a physiologically relevant model of APAP hepatotoxicity, amenable to compound screening; using this platform, we identified PGE2 as a synergistic hepatoprotective agent in combination with NAC.

The utility of in vivo animal models for human liver toxicity depends greatly on applicable assays to measure toxin-specific effects. Here, we use a variety of biochemical, histological, and clinical outcome measures to show the overall relevance of the zebrafish APAP toxicity model to human physiology. APAP significantly lowers hepatic GSH, which improves with NAC. Our findings are consistent with reports that an ortholog of the major APAP-metabolizing enzyme in humans, CYP3A4, is present in zebrafish. ALT levels, a standard test in clinical practice, were measured here to detect and quantify liver-specific toxicity and evaluate the effectiveness of therapeutic strategies in zebrafish. Histological parameters, especially hepatocyte necrosis, and survival are also consistent with clinical experiences.

For patients presenting with APAP-induced liver failure, the only reliable parameters to provide therapeutic guidance are APAP serum levels. To show conservation of the injury process across vertebrate species and discover potential biomarkers indicative of outcome, we performed the first analysis of the zebrafish serum plasma proteome. Prior proteomic studies in the zebrafish have focused on the embryo and adult liver (13, 29). Our analysis revealed an absence of albumin, the major mammalian serum transport protein, consistent with recent genome analyses (30); apolipoproteins and vitellogenin may serve as alternative carriers for hydrophobic compounds in zebrafish blood (31). We found distinct serum protein patterns of response to APAP and concordant transcriptional gene regulation. A subset of the proteins found to be regulated in our studies was also identified in mammalian models after APAP injury; many, such as catalase, aldehyde dehydrogenase, heat-shock proteins, complement, and transferrin, are involved in oxidative processes or inflammatory responses (18–20). These conserved reactions confirm the value of zebrafish as a relevant toxicological model for mammalian biology.

The feasibility of performing in vivo chemical screens in an intact vertebrate organism is a unique feature of the zebrafish (14). We used the conservation of hepatic metabolic function and gene expression to conduct a chemical toxicity modifier screen with direct clinical applicability. No prior in vivo studies have explored the potential to extend and enhance the therapeutic effects of NAC for APAP injury. Here, we showed that the combined use of dmPGE2 and NAC led to a significantly improved outcome in both embryos and adults after delayed treatment, whereas single agents had no impact. Previous investigations of the relationship between PGE2 and APAP in liver injury are controversial; some studies suggest that APAP directly impacts cyclooxygenase-2 function (32), whereas the majority of research indicates that APAP does not work as a nonsteroidal antiinflammatory drug. Recently, both PGE2 and prostacyclin (PGI2) were found to be hepatoprotective when given before or shortly after APAP exposure in the mouse (33, 34). Our work suggests that the greatly improved therapeutic outcome from combinatorial NAC and PGE2 administration after APAP injury, even after delay, results from the modulation of two different mechanisms of liver damage—the repletion of GSH mediated by NAC combined with a proproliferative, antiapoptotic signal from PGE2; it is unlikely, particularly with delayed treatment and given the failure of PGE2 alone to restore GSH levels, that exogenous PGE2 acts as an antidote to potential cyclooxygenase inhibition mediated by APAP or that it simply increases the pool of cells available to survive toxic injury.

Wnt activation is involved in many instances of organ repair, including liver regeneration after surgical resection (12, 24) or in the setting of mitochondrial dysfunction (35). Recently, Wnt signaling was found to be activated after APAP injury in murine livers (25), consistent with our work. Here, we showed that wnt activation can improve outcome from APAP toxicity in zebrafish, indicating that the proproliferative antiapoptotic effect of both PGE2 and wnt is not restricted to regeneration after mechanical injury. Modulation of wnt signaling may provide another therapeutic option for improving outcome of APAP poisoning; however, the current spectrum of wnt pathway activators is not easily tolerated and tends to have pleiotropic effects on organs such as the intestine and skin, where tissue turnover is regulated by wnt. In contrast, chemical modulation of PGE2 production and signaling is established and clinically well-tolerated. Additionally, the upcoming generation of therapeutics targeting PGE2 receptors should provide the necessary organ specificity to eliminate the majority of unwanted side effects associated with prolonged systemic use of these compounds.

The conservation of indicators of liver injury in zebrafish could lead to more universal applications of this model to assess drug-induced liver damage. The occurrence of liver toxicity, as a serious side effect, is a major problem for many candidate therapeutics in development, preventing them from reaching late clinical trial stages. Recently, complex in vitro assays have been developed to detect or predict hepatocyte toxicity (36); however, these may not completely replicate the interactions of the various liver cell types or physiologically mimic the metabolites produced in vivo. Our approach highlights the feasibility of performing whole-organism

liver-toxicity studies in adults that can be readily quantified and reflect mammalian physiology; without the space and cost commitment of murine studies, zebrafish could be used as a universal preclinical model organism to test hepatotoxic effects in vivo.

Materials and Methods

Zebrafish. Fish were maintained according to Institutional Animal Care and Use Committee protocols. *Tg(-2.8fabp10:GFP)^{as3}*, *Tg(hsp70l:wnt8a-GFP)^{w34}*, and *Tg(TOP:GFP)^{w25}* transgenic lines were described previously (17, 26, 27).

Drug Exposure and Chemical Screen. Embryos and adult zebrafish were exposed to APAP, NAC, EtOH, dmPGE2, indomethacin, and BIO at doses and times as described. After exposure, fish were transferred to fresh fish water until analysis. For the chemical screen, *Tg(-2.8fabp10:GFP)^{as3}* zebrafish embryos were arrayed in multiwell plates (10 embryos/well) and contemporaneously exposed to 10 mM APAP and each test compound (~10 μM) at 48 hpf; compounds were subselected from a prior screen for modifiers of lfabp+ liver development (Fig. S6 *A* and *B*). At 96 hpf, alterations in liver formation were qualitatively assessed by fluorescent microscopy compared with controls.

qPCR. qPCR (60 °C annealing) was performed with cDNA from pooled embryos (n = 20) or dissected adult livers using the iQ5 Multicolor RTPCR Detection System (BioRad).

In Situ Hybridization and Whole-Mount Staining. Paraformaldehyde (PFA)-fixed embryos were processed for *lfabp* in situ hybridization (http://zfin.org/ZFIN/Methods/ThisseProtocol.html). Expression changes are reported as the number of altered per number of scored per treatment (10 per treatment/3 replicates). Immunostaining for BrdU and TUNEL was performed as described (23).

GSH Assay. Total liver GSH content was determined in dissected livers after exposure of adult fish to either DMSO or APAP for 24 h, according to manufacturer's protocols (Sigma).

Caspase 3/7 Assay. Caspase 3/7 was measured on homogenized tissue [96 hpf zebrafish embryos (10 per treatment) or adult livers at 24 hpe] in 0.9× PBS, according to manufacturer's protocols (Promega).

Serum ALT Measurements. Zebrafish blood (pooled from 10 to 20 fish) was obtained by cardiac puncture. Serum was separated by centrifugation (2,000 × g for 10 min) and subjected to enzyme determination in the clinical laboratory.

Histology. Adult fish (5 per treatment/2 replicates) were fixed with PFA, paraffin embedded, cut for histological analysis, and stained with H&E using standard techniques.

Fluorescence Microscopy. Microscopy was performed on fish (10 per treatment/3 replicates) anesthetized with 0.04 mg/mL Tricaine-S.

Proteomic Analysis. Detailed experimental methods for the proteomic analysis are described in *SI Materials and Methods*.

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1. Lai MW, et al. (2006) 2005 Annual Report of the American Association of Poison Control Centers' national poisoning and exposure database. *Clin Toxicol (Phila)* 44:803–932.
2. Harrison PM, Keays R, Bray GP, Alexander GJ, Williams R (1990) Improved outcome of paracetamol-induced fulminant hepatic failure by late administration of acetylcysteine. *Lancet* 335:1572–1573.
3. Whyte IM, Francis B, Dawson AH (2007) Safety and efficacy of intravenous N-acetylcysteine for acetaminophen overdose: Analysis of the Hunter Area Toxicology Service (HATS) database. *Curr Med Res Opin* 23:2359–2368.
4. Lee SS, Buters JT, Pineau T, Fernandez-Salguero P, Gonzalez FJ (1996) Role of CYP2E1 in the hepatotoxicity of acetaminophen. *J Biol Chem* 271:12063–12067.
5. Mitchell JR, Jollow DJ, Potter WZ, Gillette JR, Brodie BB (1973) Acetaminophen-induced hepatic necrosis. IV. Protective role of glutathione. *J Pharmacol Exp Ther* 187:211–217.
6. Kon K, Kim JS, Jaeschke H, Lemasters JJ (2004) Mitochondrial permeability transition in acetaminophen-induced necrosis and apoptosis of cultured mouse hepatocytes. *Hepatology* 40:1170–1179.
7. Ruepp SU, Tonge RP, Shaw J, Wallis N, Pognan F (2002) Genomics and proteomics analysis of acetaminophen toxicity in mouse liver. *Toxicol Sci* 65:135–150.
8. Welch KD, et al. (2005) Proteomic identification of potential susceptibility factors in drug-induced liver disease. *Chem Res Toxicol* 18:924–933.
9. Terneus MV, Brown JM, Carpenter AB, Valentovic MA (2008) Comparison of S-adenosyl-L-methionine (SAMe) and N-acetylcysteine (NAC) protective effects on hepatic damage when administered after acetaminophen overdose. *Toxicology* 244: 25–34.
10. Oz HS, et al. (2004) Diverse antioxidants protect against acetaminophen hepatotoxicity. *J Biochem Mol Toxicol* 18:361–368.
11. Latchoumycandane C, Goh CW, Ong MM, Boelsterli UA (2007) Mitochondrial protection by the JNK inhibitor leflunomide rescues mice from acetaminophen-induced liver injury. *Hepatology* 45:412–421.
12. Goessling W, et al. (2008) APC mutant zebrafish uncover a changing temporal requirement for wnt signaling in liver development. *Dev Biol* 320:161–174.
13. Wang N, et al. (2007) Proteome profile of cytosolic component of zebrafish liver generated by LC-ESI MS/MS combined with trypsin digestion and microwave-assisted acid hydrolysis. *J Proteome Res* 6:263–272.
14. North TE, et al. (2007) Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature* 447:1007–1011.
15. Potter WZ, Thorgeirsson SS, Jollow DJ, Mitchell JR (1974) Acetaminophen-induced hepatic necrosis. V. Correlation of hepatic necrosis, covalent binding and glutathione depletion in hamsters. *Pharmacology* 12:129–143.
16. Murtha JM, Qi W, Keller ET (2003) Hematologic and serum biochemical values for zebrafish (Danio rerio). *Comp Med* 53:37–41.
17. Her GM, Chiang CC, Chen WY, Wu JL (2003) In vivo studies of liver-type fatty acid binding protein (L-FABP) gene expression in liver of transgenic zebrafish (Danio rerio). *FEBS Lett* 538:125–133.
18. Merrick BA, et al. (2006) Alterations in the rat serum proteome during liver injury from acetaminophen exposure. *J Pharmacol Exp Ther* 318:792–802.
19. Amacher DE, Adler R, Herath A, Townsend RR (2005) Use of proteomic methods to identify serum biomarkers associated with rat liver toxicity or hypertrophy. *Clin Chem* 51:1796–1803.
20. Yamamoto T, Kikkawa R, Yamada H, Horii I (2006) Investigation of proteomic biomarkers in in vivo hepatotoxicity study of rat liver: Toxicity differentiation in hepatotoxicants. *J Toxicol Sci* 31:49–60.
21. Dietze EC, Schäfer A, Omichinski JG, Nelson SD (1997) Inactivation of glyceraldehyde-3-phosphate dehydrogenase by a reactive metabolite of acetaminophen and mass spectral characterization of an arylated active site peptide. *Chem Res Toxicol* 10: 1097–1103.
22. Wetmore BA, et al. (2010) Quantitative analyses and transcriptomic profiling of circulating messenger RNAs as biomarkers of rat liver injury. *Hepatology* 51:2127–2139.
23. Goessling W, et al. (2009) Genetic interaction of PGE2 and Wnt signaling regulates developmental specification of stem cells and regeneration. *Cell* 136:1136–1147.
24. Monga SP, Pediaditakis P, Mule K, Stolz DB, Michalopoulos GK (2001) Changes in WNT/beta-catenin pathway during regulated growth in rat liver regeneration. *Hepatology* 33:1098–1109.
25. Apte U, et al. (2009) Beta-catenin activation promotes liver regeneration after acetaminophen-induced injury. *Am J Pathol* 175:1056–1065.
26. Dorsky RI, Sheldahl LC, Moon RT (2002) A transgenic Lef1/beta-catenin-dependent reporter is expressed in spatially restricted domains throughout zebrafish development. *Dev Biol* 241:229–237.
27. Weidinger G, Thorpe CJ, Wuennenberg-Stapleton K, Ngai J, Moon RT (2005) The Sp1-related transcription factors sp5 and sp5-like act downstream of Wnt/beta-catenin signaling in mesoderm and neuroectoderm patterning. *Curr Biol* 15:489–500.
28. Schmidt LE, Dalhoff K, Poulsen HE (2002) Acute versus chronic alcohol consumption in acetaminophen-induced hepatotoxicity. *Hepatology* 35:876–882.
29. Lucitt MB, et al. (2008) Analysis of the zebrafish proteome during embryonic development. *Mol Cell Proteomics* 7:981–994.
30. Noel ES, Reis MD, Arain Z, Ober EA (2010) Analysis of the Albumin/alpha-Fetoprotein/Afamin/Group specific component gene family in the context of zebrafish liver differentiation. *Gene Expr Patterns* 10:237–243.
31. Goessling W, Zucker SD (2000) Role of apolipoprotein D in the transport of bilirubin in plasma. *Am J Physiol Gastrointest Liver Physiol* 279:G356–G365.
32. Hinz B, Cheremina O, Brune K (2008) Acetaminophen (paracetamol) is a selective cyclooxygenase-2 inhibitor in man. *FASEB J* 22:383–390.
33. Cavar I, Kelava T, Heinzel R, Culo F (2009) The role of prostacyclin in modifying acute hepatotoxicity of acetaminophen in mice. *Coll Antropol* 33 (Suppl 2):25–29.
34. Cavar I, Kelava T, Vukojević K, Saraga-Babić M, Culo F (2010) The role of prostaglandin E2 in acute acetaminophen hepatotoxicity in mice. *Histol Histopathol* 25: 819–830.
35. Curado S, et al. (2010) The mitochondrial import gene tomm22 is specifically required for hepatocyte survival and provides a liver regeneration model. *Dis Model Mech* 3:486–495.
36. Sivaraman A, et al. (2005) A microscale in vitro physiological model of the liver: Predictive screens for drug metabolism and enzyme induction. *Curr Drug Metab* 6: 569–591.