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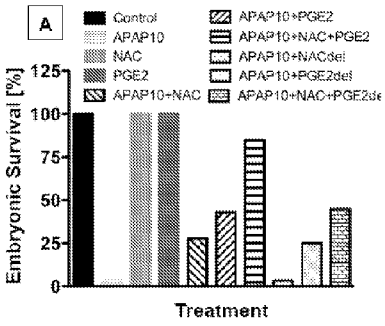
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(54) **Title**: Methods For Treating Toxicity

FIGURES 5(A)



(57) **Abstract**: Provided herein are, *inter alia*, methods and compositions for treating acetaminophen toxicity.

Methods For Treating Toxicity

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Serial No. 61/270,636, filed on July 10, 2009, the entire contents of which are hereby incorporated by reference.

TECHNICAL FIELD

This invention relates to methods and compositions for treating acetaminophen toxicity.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under grant number 5 K08 DK071940-03 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

Acetaminophen (APAP) toxicity is one of the most common drug-induced causes of acute liver failure in the U.S. The only available treatment, N-acetylcysteine (NAC), 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 (more than about 12 hours after ingestion) do not derive the same benefit from treatment and exhibit up to 15-fold increased death rates (Schmidt et al., (2002), Hepatology 35(4):876-882).

SUMMARY

The present invention is based, in part, on the discovery that administration of prostaglandin E2 (PGE2), particularly when combined with administration of N-acetylcysteine (NAC), mitigated the effects of acetaminophen (APAP) toxicity. Accordingly, provided herein are, *inter alia*, methods and compositions for treating APAP toxicity in a subject.

In one aspect, provided herein are uses of a compound that increases prostaglandin E2 (PGE2) level or activity for the treatment of acetaminophen (APAP) toxicity in a subject.

In another aspect, methods for treating acetaminophen (APAP) toxicity in a subject are described herein. The methods comprise: selecting a subject who is suffering APAP toxicity; and administering to the subject an effective amount of a compound that increases prostaglandin E2 (PGE2) level or activity.

In some embodiments, the methods and uses further comprises administering to the subject an effective amount of N-acetylcysteine (NAC).

In yet another aspect, described herein are uses of a composition comprising a compound that increases prostaglandin E2 (PGE2) level or activity and N acetylcysteine (NAC) for the treatment of acetaminophen (APAP) toxicity in a subject.

In one aspect, methods for treating acetaminophen (APAP) toxicity in a subject are provided herein. The methods comprise: selecting a subject who is suffering APAP toxicity; and administering to the subject a composition comprising an effective amount of a compound that increases prostaglandin E2 (PGE2) level or activity and an effective amount of N-acetylcysteine (NAC).

In some embodiments, the compound that increases PGE2 level or activity is PGE2 or a derivative thereof, e.g., 16,16-dimethyl-PGE2 (dmPGE2). In some embodiments, a compound that increases PGE2 level or activity is a PGE2 receptor agonist. For example, the PGE2 receptor agonist can be a EP2 or EP4 agonist described herein.

In another aspect, provided herein are uses of a compound that increases Wnt signaling for the treatment of acetaminophen (APAP) toxicity in a subject.

In one aspect, described herein are methods for treating acetaminophen (APAP) toxicity in a subject, the methods comprise: selecting a subject who is suffering APAP toxicity; and administering to the subject an effective amount of a compound that increases Wnt signaling, e.g., BIO.

In some embodiments, an effective amount of N-acetylcysteine (NAC) is administered together with the compound that increases Wnt signaling.

In some cases, the compositions and compounds described herein can administered to the subject 12 hours or more, e.g., 18 hours, after the subject has ingested APAP.

In another aspect, the invention provides compositions, e.g., pharmaceutical compositions, comprising a compound that increases prostaglandin E2 (PGE2) level or activity and N-acetylcysteine (NAC). In some embodiments, the compound that increases PGE2 level or activity is PGE2 or a derivative thereof, e.g., 16,16-dimethyl-PGE2 (dmPGE2). In some embodiments, the compound that increases PGE2 level or activity is a PGE2 receptor agonist, e.g., a EP2 or EP4 agonist.

As used herein, "treatment" means any manner in which one or more of the symptoms of a disease or disorder are ameliorated or otherwise beneficially altered. As used herein, amelioration of the symptoms of a particular disorder refers to any lessening, whether permanent or temporary, lasting or transient that can be attributed to or associated with treatment by the compositions and methods of the present invention.

The terms "effective amount" and "effective to treat," as used herein, refer to an amount or a concentration of one or more compounds or a pharmaceutical composition described herein utilized for a period of time (including acute or chronic administration and periodic or continuous administration) that is effective for treating acetaminophen toxicity.

Effective amounts of one or more compounds or a pharmaceutical composition for use in the present invention include amounts that treat acetaminophen toxicity, e.g., prevent or delay the onset, delay or halt the progression, ameliorate the effects of, or generally improve the prognosis of a subject diagnosed with e.g., acetaminophen toxicity. For example, in the treatment of acetaminophen toxicity, a compound which improves survival or limits liver damage to any degree or delays or arrests any symptom of acetaminophen toxicity would be therapeutically effective. A therapeutically effective amount of a compound is not required to cure a disease but will provide a treatment for a disease.

The term "subject" is used throughout the specification to describe an animal, human or non-human, to whom treatment according to the methods of the present invention is provided. Veterinary and non-veterinary applications are contemplated. The term includes, but is not limited to, birds and mammals, e.g., humans, other

primates, pigs, rodents such as mice and rats, rabbits, guinea pigs, hamsters, cows, horses, cats, dogs, sheep and goats. Typical subjects include humans, farm animals, and domestic pets such as cats and dogs.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

FIGs. 1A-D show data demonstrating that acetaminophen causes liver toxicity in adult zebrafish. Adult zebrafish were exposed to APAP for 24 hours. (A) Total liver glutathione (GSH) was significantly diminished following exposure to 10mM APAP; *t-test, $p \leq 0.001$, $n \geq 4$. (B) Serum alanine aminotransferase (ALT) levels were measured 6 hours pose exposure (hpe) to 5, 10, and 25 mM APAP; *significant vs. control, **significant vs. 5 mM, ANOVA, $p < 0.01$, n = 20/sample x 3 replicates. (C) ALT levels measured over time after exposure to 5 and 10mM APAP rose over the first 24 hpe ($n \geq 10$/measurement). (D) Survival analysis showed APAP caused progressive death at doses $\geq$ 10 mM.

FIGs. 2A-B illustrate data showing that N-acetylcysteine prevents liver toxicity. (A) Survival was assessed in adult zebrafish exposed to 5, 10, or 25 mM APAP and 10 μM NAC as indicated (n = 10 per group). (B) APAP-induced elevation of ALT is reversed by addition of NAC (*ANOVA, $p < 0.05$, 15-20 fish/3 replicates).

FIGs. 3A-D show proteomic and transcriptional changes in response to APAP toxicity. (A) SDS page revealed fifty-four spots excised manually from the Coomassie stained gel. (B) Summary of all protein identities analyzed by iTRAQ demonstrated differential response patterns. (C) Representative responsive proteins;

those marked with an asterisk were found previously to be APAP-regulated. (D) qPCR was performed for genes representing each cluster on dissected liver samples at 12, 24 and 48hpe (ANOVA, n = 4). *transferrin* expression declined over the entire time course; *significant vs. 12 and 24 hpe, $p < 0.05$). *lfabp* and *ceruloplasmin* expression changed in a biphasic manner; *significant vs. 12 and 48 hpe, $p < 0.01$. *Lactate dehydrogenase* expression increased over time; * all time points significant, $p < 0.001$.

FIGs. 4A-D show data demonstrating that prostaglandin E2 inhibits APAP toxicity in the embryo by enhancing proliferation and inhibiting apoptosis. (A) PGE2 (10 μM) increases embryonic liver growth (at 120 hpf) and diminishes 10mM APAP toxicity in *lfabp:GFP* embryos. (B) Embryonic *lfabp* expression at 96hpf, 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.

FIGs. 5A-B represents data showing that PGE2 acts synergistically with NAC to improve APAP toxicity. (A) Embryo survival is dependent on treatment parameter. (B) Caspase 3/7 activity, as indicator of apoptosis, was measured in a luminometric assay, and normalized to controls; *ANOVA, n = 10/ treatment x3 replicates, $p < 0.001$.

FIGs. 6A-D illustrate data showing that 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, PGE2, or a combination either concomitantly or 18 hours later. (A) Hepatic GSH (nmoles/ whole liver) was improved by combined exposure to NAC and dmPGE2; *ANOVA, n = 3-4, $p < 0.01$. (B) Relative levels of caspases 3/7 were elevated after APAP and improved with immediate NAC and/or PGE2; delayed combinatorial therapy was still beneficial (*ANOVA, n = 4-5, $p < 0.001$). (C) Serum ALT levels (n = 10-20 fish/treatment at 24hpe) show combined NAC and PGE2 prevented hepatocyte damage by APAP, even after delay. (D) Effect of NAC and dmPGE2 treatment on adult zebrafish survival (n = 9-10/treatment) at 48hpe; combination treatment led to improved survival.

FIGs. 7A-B illustrate data showing effect of EtOH on APAP toxicity. (A) Serum ALT levels rose in response to 10 mM APAP and 0.5% EtOH treatment alone,

with additive negative effects of combined treatment (n = 15-20). (B) Exposure of adult zebrafish to 0.5% EtOH for 24 hrs did not affect survival alone or in combination with 5 mM APAP. It did not worsen survival rates in combination with 10 mM APAP, but negatively affected the ability of NAC to rescue APAP toxicity.

FIG. 8 is a set of graphs showing effect of APAP treatment on serum protein concentration by iTRAQ analysis. Zebrafish serum was collected 12, 24, and 48 hours post APAP (5 mM) exposure and subjected to iTRAQ analysis. The individual relative protein concentration changes over time are depicted for all four clusters identified.

FIGs. 9A-B illustrate data showing that APAP toxicity is dose- and time-dependent in zebrafish embryos. (A) Graphical representation of the fraction of zebrafish with diminished liver size at increasing APAP doses (n≥20 embryos/x3 replicates). (B) The fraction of zebrafish with decreased liver size, declines with decreasing exposure time; n > 20 embryos/group with 3 repeats per treatment group.

FIGs 10 is a schematic representation of the fraction of zebrafish with decreased liver size, analyzed by in situ hybridization for *lfabp*, showing data demonstrating that the interaction between APAP, NAC, and EtOH is conserved in zebrafish embryos.

FIG. 11 illustrates a schematic of the pilot chemical toxin modifier screen in zebrafish embryos. Liver reporter zebrafish were mated and the offspring was distributed into multiwell plates (10 embryos/well). Embryos were exposed to 10 mM APAP and concomitantly to 20 μM of the screening drug at 48 hpf. Liver size was assessed by *in vivo* fluorescence microscopy at 96 hpf and confirmed by *in situ* hybridization for *lfabp*.

FIG. 12 shows Survival after APAP exposure is affected by wnt and PGE2 modulation. WT adult zebrafish were exposed to 10 mM APAP and indomethacin (10μM), NAC (10μM), and BIO (0.5μM). BIO improved survival if given immediately, either alone or with NAC. If given with 18 hrs delay, 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.

DETAILED DESCRIPTION

Described herein are, *inter alia*, characterization of a zebrafish model for acetaminophen (APAP) liver toxicity and identification of novel therapeutics which work cooperatively with N-acetylcysteine (NAC) to treat APAP toxicity. Data show that survival and histological evidence of liver injury in zebrafish were APAP dose-dependent. Progressive APAP injury was inhibited by NAC, making zebrafish an excellent pre-clinical model. Data also demonstrate that liver-specific APAP toxicity was conserved in zebrafish embryos. Using zebrafish embryos, a screen identified prostaglandin E2 (PGE2) as a potential therapeutic for APAP toxicity. In the adult zebrafish, data show that PGE2 worked synergistically with NAC to limit hepatic APAP injury and enhance survival, extending the therapeutic window for intervention. PGE2 enhanced wnt pathway activity, a known regulator of liver regeneration, after APAP injury to improve outcome in zebrafish embryos and adults. These results demonstrate the therapeutic relevance of modeling organ-specific toxicity in zebrafish and point to novel clinical strategies to reduce APAP-mediated liver damage.

Acetaminophen Toxicity

Acetaminophen (N-acetyl-p-aminophenol; APAP) is a commonly used analgesic and antipyretic. While 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 U.S. (Lai, et al. (2006), Clin Toxicol (Phila) 44(6-7):803-932). The only antidote in clinical use is N-acetylcysteine (NAC) (Heard (2008), N Engl J Med 359(3):285-292), which reduces mortality by ~20-28%, (Harrison et al.(1990), Lancet 335(8705):1572-1573); however, the time interval of effective intervention after ingestion is typically < 12 hours (hrs), and delayed or prolonged treatment can negatively impact clinical outcome and survival (Athuraliya and Jones (2009), Crit Care 13(3):144; Whyte et al. (2007), Curr Med Res Opin 23(10):2359-2368). 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, (Manyike et al. (2000), Clin Pharmacol Ther 67(3):275-282; Lee et al. (1996), J Biol Chem 271(20):12063-12067). At therapeutic doses, NAPQI is efficiently inactivated in the liver by glutathione (GSH) conjugation (Mitchell et al. (1973), J Pharmacol Exp Ther 187(1):211-217). At toxic doses, excess production of NAPQI depletes hepatic GSH. Unconjugated NAPQI causes dysfunction of critical liver proteins, oxidative stress and mitochondrial damage, (Kon et al. (2004), Hepatology 40(5):1170-1179; Ruepp et al. (2002), Toxicol Sci 65(1):135-150; Welch et al. (2005), Chem Res Toxicol 18(6):924-933). 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 (Brok et al. (2006), Cochrane Database Syst Rev (2):CD003328), therapeutic benefits have been demonstrated in mammalian models for other antioxidants that function to restore GSH levels (Terneus et al. (2008), Toxicology 244(1):25-34; Oz et al. (2004), J Biochem Mol Toxicol 18(6):361-368). 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 (Latchoumycandane et al. (2007), Hepatology 45(2):412-421). However, *in vivo* screens for novel compounds to act synergistically with NAC have not been performed.

Zebrafish

Zebrafish (*Danio rerio*) have been utilized previously to document water quality and identify potential toxins (Rubinstein (2006), Expert Opin Drug Metab Toxicol 2(2):231-240). Genetic conservation between zebrafish and mammals was documented in liver development, regeneration and carcinogenesis (Goessling et al. (2008), Dev Biol 320(1):161-174). Proteomic analysis of the zebrafish liver has revealed many proteins potentially indicative of xenobiotic responses (Wang et al. (2007), J Proteome Res 6(1):263-272), suggesting that zebrafish is well suited for translational toxicological studies. Zebrafish embryos are uniquely amenable to chemical screening approaches for the identification of novel therapeutics (Zon and Peterson (2005), Nat Rev Drug Discov 4(1):35-44). It has been demonstrated that findings in embryonic screens can be extended to adult zebrafish physiology and

translated into therapeutic applications (North et al. (2007), Nature 447(7147):1007-1011). The data described herein further demonstrate that zebrafish is a physiologically relevant model of APAP hepatotoxicity.

Therapeutic Methods

The data described herein demonstrate that administering a compound that increases prostaglandin E2 (PGE2) level or activity can work synergistically with NAC to treat APAP toxicity. The data further suggest that modulating the wnt pathway can also work synergistically with NAC. In particular, the therapeutic methods provided herein can be used to extend the therapeutic window, e.g., the period post-ingestion of APAP during which treatment can still be effective. Thus, provided herein are methods for treating APAP toxicity that can include selecting a subject in need of such treatment, and administering to the subject an effective amount of a compound that increases PGE2 level or activity and an effective amount of NAC. In some embodiments, the compound that increases PGE2 level or activity and NAC are administered simultaneously, e.g., in a combined dosage formulation. In another aspect, a compound that increases wnt signaling and NAC are administered to the subject.

PGE2

PGE2 is a prostanoid that is synthesized from arachidonic acid (AA). AA is converted to an intermediate (PGH2) by cyclooxygenase (COX) -1 and COX-2. The PGH2 intermediate may then be converted to PGE2 by specific prostaglandin synthases (see, e.g., Kudo and Murakami (2005), J. Biochem. Mol. Biol. 38(6): 633-638).

PGE2 has been implicated in a number of biological processes and exerts its action through G-protein-coupled receptors. There are four PGE2 receptor subtypes, EP1, EP2, EP3 and EP4. It has been shown that the various EP subtypes mediate different downstream signaling events, e.g., increased cAMP levels and protein kinase A (PKA) activation, and diverse biological processes, e.g., bone formation and inflammation. For a review of PGE2 receptors and their functions, see Sugimoto and Narumiya (2007), J. Biol. Chem. 282(16): 11613-11617.

Wnt Signaling

Wnt proteins are a family of highly conserved secreted signaling molecules that regulate cell-to-cell interactions during vertebrate embryogenesis. Binding of Wnt to its receptor initiates a number of downstream signaling events involving, e.g., β-catenin and glycogen synthase kinase 3b (Gsk3b) (Goessling et al. (2009), Cell 136:1136-1147).

Data demonstrate that Wnt has a role in liver regeneration and hemotopoetic stem cell formation. It has also been shown that PGE2 acts through cAMP/PKA signaling to modulate the Wnt signaling pathway by enhancing β-catenin levels (Goessling et al. (2009), Cell 136:1136-1147).

Increasing PGE2 level or activity

PGE2 level in a subject can be increased by methods known in the art. For example, a PGE2 analog or derivative can be administered to the subject. PGE2 level can also be increased by enhancing production of endogenous PGE2, e.g., by modulating the activity of the enzymes (e.g., COX-2) in the PGE2 biosynthetic pathway, or increasing the level of a precursor of PGE2.

As used herein, PGE2 activity refers to a biological activity or a downstream signaling event mediated by PGE2, e.g., by the binding of PGE2 to one of its receptors. For example, PGE2 activities can include events in the PGE2 signaling pathway, such as an elevation in the concentration of a second messenger (e.g., cAMP or Ca^{2+}), or activation of PKA or phosphatidylinositol 3-kinase.

PGE2 analogs and PGE2 receptor agonists are known in the art and can include butaprost, sulprostone, 16,16-dimethyl PGE_2, 11-deoxy-16,16-dimethyl PGE_2, 17-phenyl trinor PGE_2, ONO-DI-004 (Ono Pharmaceutical Co., Japan), ONO-AE1-259 (Ono Pharmaceutical Co., Japan), ONO-AE-248 (Ono Pharmaceutical Co., Japan), ONO-AE1-329 (Ono Pharmaceutical Co., Japan), ONO-4819CD (Ono Pharmaceutical Co., Japan), L-902688 (Cayman Chemical), CAY10598 (Cayman Chemical), CP-533536 (Pfizer), and those described in WO99/19300, US2003/0166631, WO03/77910, WO03/45371, WO03/74483, WO03/09872, WO04/37813, WO04/37786, WO04/19938, WO03/103772, WO03/103664, WO03/40123, WO03/47513, WO03/47417, WO03/77919, and U.S. Pat. Nos. 6,410,591, 6,747,037. 7,696,235, 7,662,839, 7,652,063, 7622,475, and 7,608,637.

Compounds that increase or activate an event in the PGE2 signaling pathway can also be used in the treatment methods described herein to increase PGE2 activity. For example, compounds that can increase cAMP level, e.g., an adenylate cyclase activator, can be used. Examples of adenylate cyclase activators known in the art include forskolin and its derivatives, e.g., 6-acetyl-7-deacetyl-forskolin, 7-deacetyl-forskolin, and 7-deacetyl-6-(N-acetylglycyl)-forskolin.

Increasing Wnt Signaling

Wnt signaling can be increased by, e.g., increasing or activating a component or event of the Wnt signaling pathway, for example, by increasing β-catenin level or activity. Compounds that can modulate, e.g., activate, the Wnt pathway are known in the art, for example, Wnt ligands (DSH/DVL1, 2, 3, LRP6ΔN, WNT3A, WNT5A, and WNT3A, 5A), Qs11 (Zhang et al.(2007), PNAS, 104(18): 7444-7448), WAY-316606 (Bodine et al.(2009), Bone, 44(6):1063-8), lithium chloride (LiCl), Purvalanol A, olomoucine, alsterpaullone, kenpaullone, benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8), 2-thio(3-iodobenzyl)-5-(1-pyridyl)-[1,3,4]-oxadiazole (GSK3 inhibitor II), 2,4-dibenzyl-5-oxothiadiazolidine-3-thione (OTDZT), α-4-Dibromoacetophenone (i.e., Tau Protein Kinase I (TPK I) Inhibitor), 2-Chloro-1-(4,5-dibromo-thiophen-2-yl)-ethanone, N-(4-Methoxybenzyl)-N′-(5-nitro-1,3-thiazol-2-yl)urea (AR-A014418), and indirubins (e.g., indirubin-5-sulfonamide; indirubin-5-sulfonic acid (2-hydroxyethyl)-amide indirubin-3'-monoxime; 5-iodo-indirubin-3'-monoxime; 5-fluoroindirubin; 5, 5'-dibromoindirubin; 5-nitroindirubin; 5-chloroindirubin; 5-methylindirubin; 5-bromoindirubin; (2′Z,3′E)-6-Bromoindirubin-3′-oxime (BIO)), α-4-Dibromoacetophenone (i.e., Tau Protein Kinase I (TPK I) Inhibitor), 2-Chloro-1-(4,5-dibromo-thiophen-2-yl)-ethanone, N-(4-Methoxybenzyl)-N′-(5-nitro-1,3-thiazol-2-yl)urea (AR-A014418), and H-KEAPPAPPQSpP-NH2 (L803) or its cell-permeable derivative Myr-N-GKEAPPAPPQSpP-NH2 (L803-mts). Additional Wnt/β-catenin pathway activators and inhibitors are reviewed in the art (Moon et al., Nature Reviews Genetics, 5:689-699(2004))

Subject Selection

The methods, compounds, and compositions described herein can be used for treating subjects who are suffering APAP toxicity, e.g., from ingesting, accidentally or intentionally, a toxic amount of APAP. A person of ordinary skill in the art would be able to diagnose APAP toxicity in a subject and determine whether the subject needs treatment for APAP toxicity using routine methods,

While it is preferred that the treatment methods provided herein are administered as soon as possible after a subject has ingested a toxic amount of APAP, the present methods can be administered up to, e.g., 18 hours, after ingestion.

Pharmaceutical Compositions and Methods of Administration

The compounds and compositions described herein can be administered to a subject, e.g., a subject identified as being in need of treatment, using a systemic route of administration. Systemic routes of administration can include, but are not limited to, parenteral routes of administration, *e.g.*, intravenous injection, intramuscular injection, and intraperitoneal injection; enteral routes of administration, *e.g.*, administration by the oral route, lozenges, compressed tablets, pills, tablets, capsules, drops (*e.g.*, ear drops), syrups, suspensions and emulsions; transdermal routes of administration; and inhalation (*e.g.*, nasal sprays).

In some embodiments, the modes of administration described above may be combined in any order and can be simultaneous or interspersed.

The methods described herein include the manufacture and use of pharmaceutical compositions, which include compounds that increase PGE2 level or activity or wnt signaling as active ingredients. Also included are the pharmaceutical compositions themselves. In some embodiments, the compositions also include NAC. In some embodiments, the compositions comprise PGE2 and NAC as active ingredients.

Pharmaceutical compositions typically include a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" includes saline, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration.

Pharmaceutical compositions are typically formulated to be compatible with its intended route of administration. Examples of routes of administration include

parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration.

Methods of formulating suitable pharmaceutical compositions are known in the art, see, e.g., the books in the series Drugs and the Pharmaceutical Sciences: a Series of Textbooks and Monographs (Dekker, NY). For example, solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use can include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, CREMOPHOR EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition.

Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle, which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying, which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules, e.g., gelatin capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, PRIMOGEL, or corn starch; a lubricant such as magnesium stearate or STEROTES; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

Systemic administration of a therapeutic compound as described herein can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The pharmaceutical compositions can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the therapeutic compounds are prepared with carriers that will protect the therapeutic compounds against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Such formulations can be prepared using standard techniques, or obtained commercially, e.g., from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to selected cells with monoclonal antibodies to cellular antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

The compositions described herein can be administered one from one or more times per day to one or more times per week; including once every other day. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of the therapeutic compounds described herein can include a single treatment or a series of treatments.

Dosage, toxicity and therapeutic efficacy of the therapeutic compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit high therapeutic indices are preferred.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies

preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

EXAMPLES

Example 1: APAP causes liver toxicity in adult zebrafish

APAP exposure in mammals leads to the rapid depletion of GSH stores (Potter et al. (1974), Pharmacology 12(3):129-143). Similarly, in the adult zebrafish liver, GSH was significantly reduced by 75% in liver homogenates 24hrs post exposure (hpe) to APAP (10mM) (513.4±18.0 vs. 130.5±13.6 nmoles/liver, t-test, $p \leq 0.001$, n = 5; Figure 1A). Baseline serum alanine aminotransferase (ALT) levels, used to detect liver injury clinically, were higher in zebrafish than in mice and humans, consistent with prior reports (Murtha et al.(2003), Comp Med 53(1):37-41). (Figure 1B); within 6hpe, ALT levels were elevated in a dose-dependent fashion and continued to rise over the first 24hrs (Figure 1C). To establish toxicity thresholds for APAP-induced liver injury, survival after exposure to increasing doses of APAP was recorded (Figure 1D). Doses up to 5 mM had no impact on survival, while 10 mM APAP caused progressive death in approximately 50% of fish, beginning by 20 hpe. Exposure to 25-50 mM APAP caused rapid death in almost all fish by 10 hpe. Bolus treatment with 50 mM APAP for 3 hrs, to mimic suicidal overdose, resulted in liver toxicity comparable to extended 10mM exposure. Histological evaluation revealed minimal effects with 1mM APAP. 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 was seen as early as 12hpe following exposure to 10 mM APAP. To visualize the extent of liver damage *in vivo*, fluorescent liver fatty acid binding protein, *lfabp:GFP,* zebrafish, was

used; APAP caused a time-dependent reduction in fluorescence, suggestive of hepatocyte death and corresponding with the histological analysis.

Example 2: NAC prevents liver toxicity

To document the applicability of the zebrafish model to human physiology, APAP-treated fish was exposed concurrently to NAC. Zebrafish survival, and ALT serum concentration, was markedly improved by NAC (10 μM) (Figures 2A,B, $p < 0.05$). In cases of accidental or intentional overdose, NAC treatment is rarely initiated immediately following ingestion; up to 12hpe, delayed NAC administration resulted in comparable rescue rates as contemporaneous treatment, whereas later NAC intervention could no longer improve survival. These results are consistent with clinical observations for NAC. I*n vivo* fluorescence microscopy of *lfabp:GFP* fish demonstrated dramatic improvement in liver morphology following immediate NAC treatment, which was confirmed histologically. EtOH exposure worsened outcome after APAP injury (Figure 7).

Example 3: Proteomic analysis of zebrafish plasma

The liver regulates protein synthesis, glucose and lipid metabolism, and xenobiotic degradation. To demonstrate changes in liver function in response to APAP, alterations in serum protein concentration were assessed by mass spectrometry. Zebrafish serum was collected from 5mM APAP-exposed and control fish over the course of 48 hrs. After generation of a partial 2D-gel map of the plasma proteome (Figure 3A), comprehensive protein coverage was obtained by 2D-LC MS/MS, resulting in the identification of 223 high-confidence proteins. Most were highly evolutionarily conserved and present in human serum (Table 1, Figure 8). Relative changes in serum protein concentration were quantitated over time by iTRAQ, revealing four differential protein response patterns (Figure 3B). One group (71 proteins) steadily decreased over time (Figure 3C); 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 glutathione-S-transferase and carbonic anhydrase, were known to be affected by APAP treatment (Merrick et al. (2006), J Pharmacol Exp Ther 318(2):792-802). Cluster 3 (33

proteins) was characterized by the reverse response, with initial increase in concentration followed by falling levels; this cluster included structural and remodeling proteins. Cluster 4 (73 proteins) showed continuously increasing protein levels indicative of cellular damage. Several representative genes showed qPCR expression patterns consistent with the time-dependent protein responses observed by iTRAQ (Figure 3D) (Ruepp et al. (2002), Toxicol Sci 65(1):135-150). These results are comparable to prior observations in murine models of APAP toxicity (Merrick et al. (2006), J Pharmacol Exp Ther 318(2):792-802; Amacher et al (2005), Clin Chem 51(10):1796-1803), indicating conservation of the xenobiotic response.

Example 4: Conserved effects of APAP in zebrafish embryos

Zebrafish embryos are amenable to unbiased chemical genetic screens (North et al. (2007), Nature 447(7147):1007-1011). To discern whether APAP produced similar effects on embryonic and larval hepatocytes, *lfabp:GFP* embryos were exposed to increasing doses (or time) of APAP; lfabp serves as an ideal tool for these studies, as proteomic analysis indicated it was highly regulated during injury and recovery. Liver size and fluorescence progressively diminished in a dose- and time-dependent fashion following APAP treatment (Figure 9A). The effect of NAC was also conserved, as evidenced by the larger liver in more than 50% of APAP-treated embryos (Figure 9B). This analysis was confirmed by *in situ* hybridization for *lfabp* and replicated in zebrafish larvae.

Example 5: An embryonic APAP chemical modifier screen identifies PGE2

Given the conserved embryonic effects of APAP, a pilot chemical screen for modifiers of xenobiotic-induced toxicity was performed. *lfabp:GFP* embryos were exposed to APAP from 48-96hpf (hours post fertilization) concurrently with individual compounds with known embryonic liver-specific bioactivity; Figure 11). Embryonic APAP toxicity, over this time frame, resulted in substantial death (> 90%) and dramatically reduced liver size. Prostaglandin E2 (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, 10μM) substantially increased liver size at 96hpf (screen: 10 increased/12; retest: 59/66, Figure 4A) and partially corrected the APAP phenotype (screen: 8 normal/12; retest:

37/58). These results were confirmed by *lfabp* qPCR (Figure 4B). To determine how PGE2 mitigated APAP-induced hepatotoxicity, proliferation and apoptosis were examined by BrdU and TUNEL staining, respectively. dmPGE2 enhanced proliferation (20 increased/29), whereas APAP reduced BrdU incorporation (19 decreased/24). 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). To determine if the effect of PGE2 was maintained during APAP-mediated liver injury in the adult, survival following single-agent and combinatorial exposure was examined. By 24hpe, dmPGE2 almost completely reversed in APAP mortality (Figure 4C). QPCR for *lfapb* confirmed that APAP-induced loss of liver-specific transcription was limited by PGE2 (Figure 4D).

Example 6: dmPGE2 acts synergistically with NAC to limit embryonic liver damage

To determine whether dmPGE2 could act in concert with NAC to reduce liver damage, embryos were exposed to drug combinations and assessed for alterations in *lfabp* expression. APAP alone inhibited liver growth (47 decreased/48), while dmPGE2 increased liver size at 96hpf (56 increased/59). dmPGE2 (42 normal/66) and NAC (37 normal/61) each partially rescued the negative effects of APAP with synchronous exposure. When given 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). Combinatorial treatment affected embryonic survival (Figure 5A, 85% for combined immediate treatment) as well as apoptosis, as measured by combined caspase 3/7 activity (Figure 5B, $p < 0.001$). In clinical practice, NAC is typically administered with some delay after APAP ingestion. While NAC alone had minimal effects on survival when given with 12hrs delay, dmPGE2 rescued liver size (7/29) and embryo survival (25%) in a modest proportion of embryos (Figure 5A). The combination of NAC and dmPGE2, after delay, caused marked improvement in survival (45%) and *lfabp* expression (26/48). Caspase analysis confirmed the effects of combinatorial treatment were mediated, in part, by a reduction in apoptosis (Figure 5B).

Example 7: PGE2 and NAC improves clinical parameters of APAP injury

To demonstrate applicability, adult zebrafish were exposed to single agent or combinatorial therapy concomitantly with APAP or with delay, and clinical parameters of liver injury were recorded (Figure 6A-D). Hepatic GSH was significantly diminished by APAP-treatment and corrected by immediate administration of NAC or combinatorial therapeutics (Figure 6A-C). dmPGE2 alone improved caspase and ALT levels, but not hepatic GSH, indicating that it improved 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. Combinatorial dmPGE2 and NAC treatment of APAP-mediated damage despite delay still vastly improved liver parameters, cell death, survival and histology (Figure 6D). These results indicate the potential synergistic therapeutic benefit for use of dmPGE2 and NAC as standard treatment for liver APAP toxicity.

Example 8: PGE2 enhances Wnt activity to improve APAP liver toxicity

PGE2 was previously shown to regulate wnt signaling during liver regeneration (Goessling et al. (2009), Cell 136:1136-1147), wnt activation has been observed after both surgical and toxic liver injury (Goessling et al. (2008) , Dev Biol 320(1):161-174; Monga et al. (2001), Hepatology 33(5):1098-1109; Apte et al. (2009), Am J Pathol 175(3):1056-1065). In order to document hepatic wnt activation in zebrafish, *TOP:dGFP* wnt reporter embryos were exposed to APAP at 48hpf. Markedly increased fluorescence was found in livers of treated fish at 72hpf (34 increased/48). To demonstrate that wnt signaling induced by APAP injury functioned to improve outcome, inducible *wnt8* transgenic embryos were heat-shocked and exposed to APAP at 48hpf. *wnt8* overexpression was hepatoprotective and rescued liver-specific fluorescence (wt APAP: 49 decreased/51; wnt8: 49 increased/55; wnt8+APAP: 53 rescued/67). To corroborate that dmPGE2 treatment worked at least in part by enhancing wnt activation following adult APAP toxicity, *TOP:dGFP* expression was analyzed: wnt activity was increased in response to APAP injury, consistent with recent results (Apte et al. (2009), Am J Pathol 175(3):1056-1065), and was further enhanced by dmPGE2 treatment. 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 BIO

(0.5μM). 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. Inhibition of PGE2 production by indomethacin had detrimental effects on both morphology and survival (Figure 12), 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.

Example 9: Materials and Methods

The following materials and methods were used in Examples 1-8.

Zebrafish husbandry

Zebrafish were maintained according to IACUC protocols and institutional guidelines. *lfabp:GFP, hs:wnt8-GFP, and TOP:dGFP* transgenic lines were described previously (Goessling et al. (2008), Dev Biol 320(1):161-174).

Drug exposure

Embryos and adult zebrafish were exposed to APAP, NAC, EtOH, dmPGE2, indomethacin, and BIO at doses and time intervals as described. Fish were transferred to fresh fish water at the end of exposure until time of analysis. For the chemical screening panel, *lfabp:GFP* zebrafish embryos were arrayed in multiwell plates (10 embryos/well) and exposed to 10mM APAP and contemporaneously to each test compound (~20μM) at 48hpf. At 96hpf, liver size and morphology were assessed by fluorescent microscopy and confirmed by in situ hybridization for *lfabp*.

qPCR

cDNA was obtained from pooled embryos (n = 20) or the dissected livers of adult fish. qPCR (60°C annealing) was performed using SYBR Green Supermix on the iQ5 Multicolor RTPCR Detection System (BioRad).

In situ hybridization and whole mount staining

PFA-fixed embryos were processed for *in situ* hybridization for *lfabp* using standard zebrafish protocols (http://zfin.org/ZFIN/Methods/ ThisseProtocol.html). Changes in expression are reported as the # altered/# scored per treatment; 3 independent experiments of n≥10 embryos were conducted per analysis. Whole

mount immunostaining for BrdU incorporation and TUNEL were performed as described (North et al. (2009), Cell 137(4):736-748).

GSH Assay

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

Caspase 3/7 Assay

A Caspase 3/7 was performed on homogenized tissue (4dpf zebrafish embryos (10/treatment) or adult livers at 24hpe) in 0.9x PBS according to manufacturer's (Promega) protocol.

Serum Alanine Aminotransferase Measurements

Zebrafish blood (pooled from 10-20 fish) was obtained by cardiac puncture. Serum was separated by centrifugation (2000g x 10min) and subjected to enzyme determination in the clinical laboratory.

Histology

Adult zebrafish (5 per treatment/2 replicates) fixed with PFA, were paraffin embedded and cut in 10μm serial step-sections for histological analysis. Hematoxylin/eosin staining was performed on alternate sections using standard techniques.

Fluorescence microscopy

Microscopy was performed on transgenic embryos and adults (10/treatment x 3 replicates); anesthetized with 0.04mg/ml Tricaine-S.

2D-gel electrophoresis

Isoelectric focusing was performed on an Amersham Ettan IPGPhor II system. Protein from 6 μL of pooled zebrafish plasma was isolated by acetone precipitation at -20°C. Precipitated proteins were pelleted by centrifugation at 12000 rpm, air dried, and dissolved in 200 μL of IPG strip rehydration buffer (2% CHAPS, 7M Urea, 2M thiourea, 0.002% bromophenol blue, 1% IPG buffer 3-10 L) containing 30 μg of DeStreak reagent (Amersham Bioscience). Samples were loaded onto an 11 cm, pH 3-10 L IPG strip by re-hydration. The IEF profile was 500-500 (1.5 hrs)-1000 (1hr)-6000 (2 hrs)-6000 V (0.40 hr) to a total of 18KVh. Immediately after IEF, proteins in the strip were reduced by shaking with 100 mg of DTT in 10mL of equilibration buffer (50mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 0.002%

bromophenol blue) for 20 minutes. Strips were briefly washed with equilibration buffer (without DTT) and the sulfhydryl groups of cysteines were capped by treating the IPG strips with a solution of 250 mg of iodoacetamide in 10mL of equilibration buffer for 20 minutes. Second-dimension SDS PAGE was performed on 8-16% Criterion gels (Bio-Rad) with tris glycine buffer at 20 mA constant current for 2.75hrs. Gels were stained with SIMPLYBLUE™ SAFESTAIN (Coomassie substitute, Invitrogen) according to the manufacturer's protocol.

In-gel protein digestion

Protein bands were excised manually, diced and washed with 1:1 methanol:0.1M ammonium bicarbonate solution in microcentrifuge tubes for 15 minutes or until colorless. Following this, gel pieces were treated sequentially with, 200 µL of 1:1 acetonitrile:0.1M ammonium bicarbonate (15 min x 2), 200 µL acetonitrile (10min) and were vacuum dried for 45 minutes. Gel pieces were rehydrated in 20 µL of 20 ng/mL trypsin, 0.1 M ammonium bicarbonate and 10 mM $CaCl_2$ for 2 minutes. Where necessary ammonium bicarbonate buffer was added to submerge the gel pieces and digestion was carried out at 37°C overnight. Digests were transferred to centrifuge tubes, gel pieces were washed with 8:1 acetonitrile:ammonium bicarbonate solution and the washes were pooled with the digest. Samples were redissolved in 0.1% TFA and peptides were extracted with C18 ZipTips according the manufacturer's protocol.

Protein digestion and SCX chromatography

Plasma (6 µL) was incubated under shaking with 100 µL of Protein A agarose beads in PBS for 20 min in UltrafreeMC 0.4 µm centrifugal filters. Unbound protein was extracted by washing the bed with 3 x 200 µL of PBS. Washes were pooled, spin dialyzed to ~10µL on 3000Da cut-off Microcon centrifugal tubes and vacuum dried. To this, 50 µL of 0.5 M TEABIC , 0.1% SDS, 10mM TCEP solution was added and heated at 60°C for 1 hour. After cooling to room temperature, 2 µL of 100 mM iodoacetamide was added and samples were incubated for 15min at room temperature. Proteins were precipitated by addition of ice-cold acetone, pelleted by centrifugation and the pellets were resuspended in 10µL of 0.5M TEAB, 0.1% SDS, sonicated for 10min and heated at 60°C for 5min. After cooling, 8mg of iodoacetamide was added, followed by incubation at room temperature for 15min. Proteins were then separated by acetone precipitation, re-suspended in 10µL of 0.5M TEAB, 0.5% SDS by heating

at 60°C for 10min and cooled to room temperature. Digestion was performed by adding 40µL of 0.5M TEAB, 1mM $CaCl_2$ buffer containing 10µg of modified porcine trypsin (Promega) and incubating the sample solution at 37°C overnight. Strong cation exchange HPLC (SCX) was performed on a Poly-SULFOETHYL A™ column (100 x 4.6mm, 5µm; 300 Å, PolyLC Inc.). Peptides were separated by a two-step gradient of 1M KCl (A = 25% CH3CN, 10mM phosphate, pH 2.9; B = 25% CH3CN, 10mM phosphate, 1M KCl, pH 2.9, 0.25 ml/min) and fractions were collected every minute.

LC-MS/MS analysis

Peptides from 2D-gel spots were analyzed on an Agilent LC-MSD ion-trap mass spectrometer equipped with a Chip-Cube Nano LC system. A 0-60 min H_2O-CH_3CN/0.5% AcOH linear gradient was used with a RP-C18 micro-fluidic chip containing both a trapping column and an analytical column.

SCX fractions were analyzed on an Applied Biosystems QSTAR-XL mass spectrometer under conditions similar to those reported earlier. (Kristiansson et al., J Proteome Res., 6:1735-44 (2007); Stevens et al., Mol Cell Proteomics, 8(7):1475-89 (2009)). Briefly, samples were desalted with C18-ZipTips prior to the analysis and the second dimension RP-C18 LC was performed on an in-house prepared fused-silica capillary column (0.075 x 150 mm, 5 µm, 300 Å) interfaced to the QSTAR with a nano-electrospray ion-source (Proxeon Biosystems). The information-dependent acquisition sequence included a 1-second survey scan followed by 3-second MS/MS acquisitions on the three most-abundant ions in the survey spectrum. The precursors from a given survey scan were actively excluded for the next 60 seconds.

Isobaric tag for relative and absolute quantitation (iTRAQ) labeling and MS/MS analysis

The iTRAQ system in these experiments is based on isobaric amine-reactive reagents that - in collision-induced-decomposition (CID) experiments - release 4 different isotopically-labeled reporter ions at *m/z* 114.1, 115.1, 116.1 and 117.1 (Ross et al., Mol Cell Proteomics, 3:1154-69(2004)). Peptides from four experiments can be mixed and will then chromatograph in a single run with identical retention times and with identical masses that can allow relative quantitation at the MS/MS level. Proteins from 10 µL each of serum from zebrafish exposed to 5 mM APAP for 0, 12, 24 and 48 hrs were processed according to the protocol described above in the SCX

chromatography section. Briefly, proteins were passed through Protein A agarose beads, unbound proteins were collected and concentrated on 3000 Da centrifugal filters. Cysteines were reduced and alkylated with iodoacetamide as mentioned above. After trypsin digestion, peptides from samples for exposures of 0, 12, 24 and 48 hours were labeled with iTRAQ reporters 114 Da, 115 Da, 116 Da, and 117 Da, respectively, according to the manufacturer's protocol. Excess label was quenched by addition of water, and peptides from all samples were pooled. iTRAQ-labeled peptide mixtures were fractionated by SCX chromatography and the fractions were analyzed by RP-18 nano-LC-MS/MS on the ABI QSTAR-XL mass spectrometer.

Database search and protein Identification

For qualitative global proteomics surveys, MS/MS spectra were searched with Agilent Spectrum Mill software against the NCBInr zebrafish protein sequence database containing 56,700 sequences. Results were auto-validated with a score threshold of > 20 and the results were further curated manually. For MS/MS spectra from iTRAQ-labeled samples, Protein Pilot software (Applied Biosystems) was used. Only proteins that matched a confidence of identification ≥ 95% were used for further analysis. (Bhat et al., J Proteome Res., 4:1814-25 (2005); Stevens et al., Arthritis Rheum., 58:489-500 (2008)).

Protein clustering and data analysis

iTRAQ intensities from the 12-, 24-, and 48-hour time-points, as ratios with respect to control population (zero time-point) for 223 proteins identified by Protein Pilot software, were used for the k-means cluster analysis. Clustering analysis was performed with SPOTFIRE software, yielding the optimal result when k was set to 4.

TABLE 1. Select zebrafish serum proteins with identified human correlates.

#	Protein Name	Spectrum Mill Score	# Unique peptides	Percent coverage	NCBI accession #
1	parvalbumin	150.33	10	62	45387577
2	complement component 6	123.22	9	12	41055345
3	tropomyosin 3	119.65	7	22	41393141
4	betaine-homocysteine methyltransferase	93.65	6	26	60279651
5	heat shock protein 8	83.6	6	11	56790264
6	phosphoglycerate kinase 1	80.39	6	14	47087077
7	complement component 9	77.7	6	21	66773068
8	lymphocyte cytosolic plastin 1	76.96	6	11	41281615
9	catalase	71.43	4	10	56744251
10	coagulation factor II (thrombin)	71.27	5	8	47087349
11	sex hormone binding globulin	65.29	4	13	55742376
12	angiotensinogen	60.57	5	10	37620189
13	retinol binding protein 4, plasma	46.38	3	20	18859309
14	heat shock protein 90-beta	43.57	3	4	18858875
15	eukaryotic translation initiation factor 4A, isoform 1A	42.26	3	9	38198643
16	eukaryotic translation elongation factor 1 gamma	39	3	7	27545277
17	phosphoglycerate mutase 2 (muscle)	37.73	3	17	41056123
19	proteasome (prosome, macropain) 26S subunit, non-ATPase, 2	34.77	2	3	41054527
20	hypoxanthine phosphoribosyltransferase 1	31.75	2	13	47085697
21	glutathione peroxidase 1	28.53	2	20	55925454
22	regucalcin	28.36	2	6	45387895
23	myristoylated alanine-rich C kinase substrate 2	27.96	2	12	47086029
24	2-peptidylprolyl isomerase A	26.67	2	14	47086533
25	aldehyde dehydrogenase 1 family, member A2	26.58	2	6	18858265
26	calreticulin	22.09	2	4	18858381
27	protocadherin 1 alpha 8	22.09	2	2	66773370
28	proteasome activator subunit 2	21.06	2	9	18859281
29	PREDICTED: similar to complement component C5-1	20.06	2	0	68369422

Comparison is based on gene ontology database (world wide web at geneontology.org). Human plasma proteins were identified in a human plasma proteome database (world wide web at plasmaproteomedatabase.org). The proteins are sorted by cluster number.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. Use of a compound that increases prostaglandin E2 (PGE2) level or activity for the treatment of acetaminophen (APAP) toxicity in a subject.

2. A method for treating acetaminophen (APAP) toxicity in a subject, the method comprises:

selecting a subject who is suffering APAP toxicity; and

administering to the subject an effective amount of a compound that increases prostaglandin E2 (PGE2) level or activity.

3. Use of a composition comprising a compound that increases prostaglandin E2 (PGE2) level or activity and N-acetylcysteine (NAC) for the treatment of acetaminophen (APAP) toxicity in a subject.

4. A method for treating acetaminophen (APAP) toxicity in a subject, the method comprises:

selecting a subject who is suffering APAP toxicity; and

administering to the subject a composition comprising an effective amount of a compound that increases prostaglandin E2 (PGE2) level or activity and an effective amount of N-acetylcysteine (NAC).

5. The use of claim 1 or 3 or the method of claim 2 or 4, wherein the compound that increases PGE2 level or activity is PGE2 or a derivative thereof.

6. The use or method of claim 5, wherein the PGE2 derivative is 16,16-dimethyl-PGE2 (dmPGE2).

7. The use of claim 1 or 3 or the method of claim 2 or 4, wherein the compound that increases PGE2 level or activity is a PGE2 receptor agonist.

8. The use or the method of claim 7, wherein the PGE2 receptor agonist is a EP2 or EP4 agonist.

9. The use of claim 1 or the method of claim 3, wherein the method further comprises administering to the subject an effective amount of N-acetylcysteine (NAC).

10. The use of claim 2 or the method of claim 4, wherein the composition is administered to the subject 12 hours or more after the subject has ingested APAP.

11. The use or method of claim 9, wherein the compound that increases PGE2 level or activity and NAC are administered to the subject 12 hours or more after the subject has ingested APAP.

12 Use of a compound that increases Wnt signaling for the treatment of acetaminophen (APAP) toxicity in a subject.

13. A method for treating acetaminophen (APAP) toxicity in a subject, the method comprises:

selecting a subject who is suffering APAP toxicity; and

administering to the subject an effective amount of a compound that increases Wnt signaling.

14. The use of claim 12 or the method of claim 13, the method further comprises administering to the subject an effective amount of N-acetylcysteine (NAC).

15. The use or method of claim 14, wherein the compound that increases Wnt signaling and NAC are administered to the subject 12 hours or more after the subject has ingested APAP.

16. The use of claim 12 or the method of claim 13, wherein the compound that increases Wnt signaling is (2′Z,3′E)-6-Bromoindirubin-3′-oxime (BIO).

17. A composition comprising a compound that increases prostaglandin E2 (PGE2) level or activity and N-acetylcysteine (NAC).

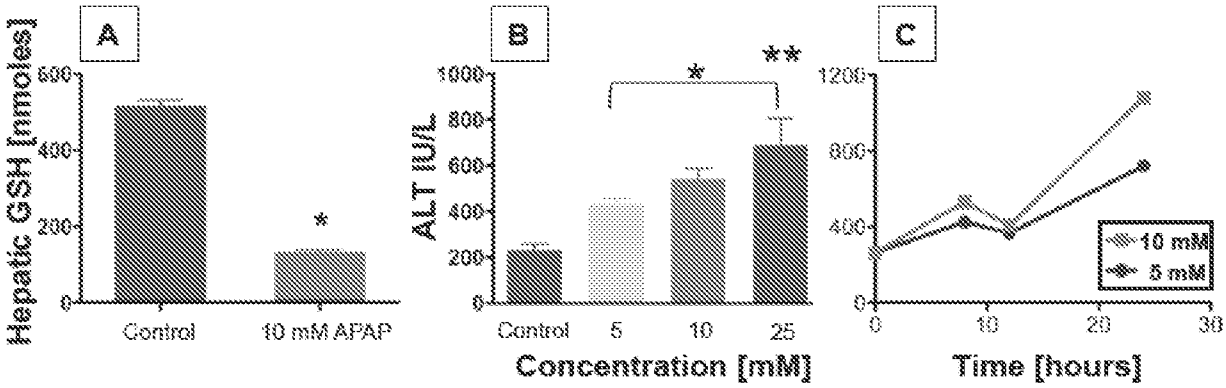
18. The composition of claim 17, wherein the compound that increases PGE2 level or activity is PGE2 or a derivative thereof.

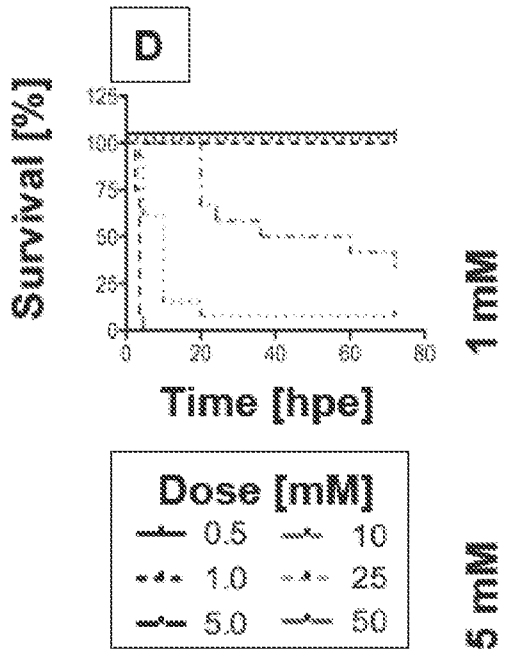
19. The composition of claim 18, wherein the PGE2 derivative is 16,16-dimethyl-PGE2 (dmPGE2).

20. The composition of claim 17, wherein the compound that increases PGE2 level or activity is a PGE2 receptor agonist.

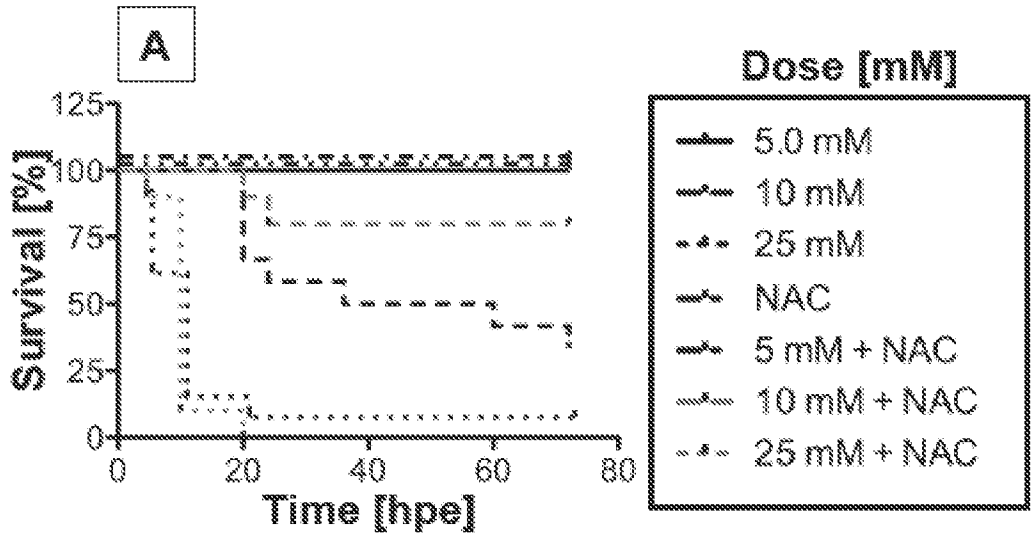
21. The composition of claim 20, wherein the PGE2 receptor agonist is a EP2 or EP4 agonist.

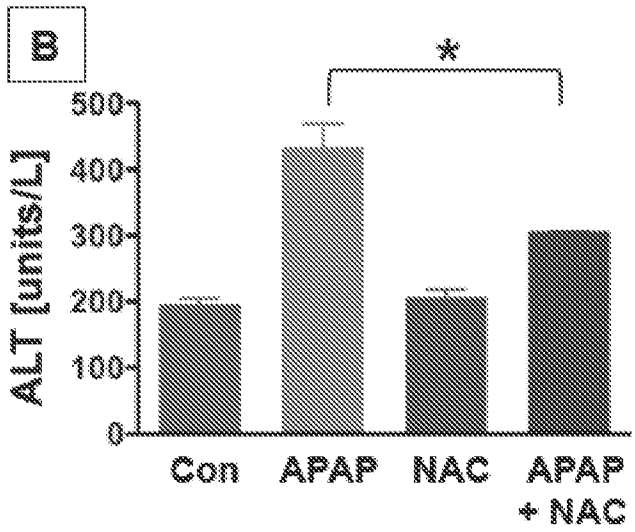
FIGURES 1(A)-(D)


A
Hepatic GSH [nmoles]
600
400
200
0
*
Control
10 mM APAP
B
ALT IU/L
1000
800
600
400
200
0
*
**
Control
5
10
25
Concentration [mM]
C
1200
800
400
0
10 mM
5 mM
0
10
20
30
Time [hours]

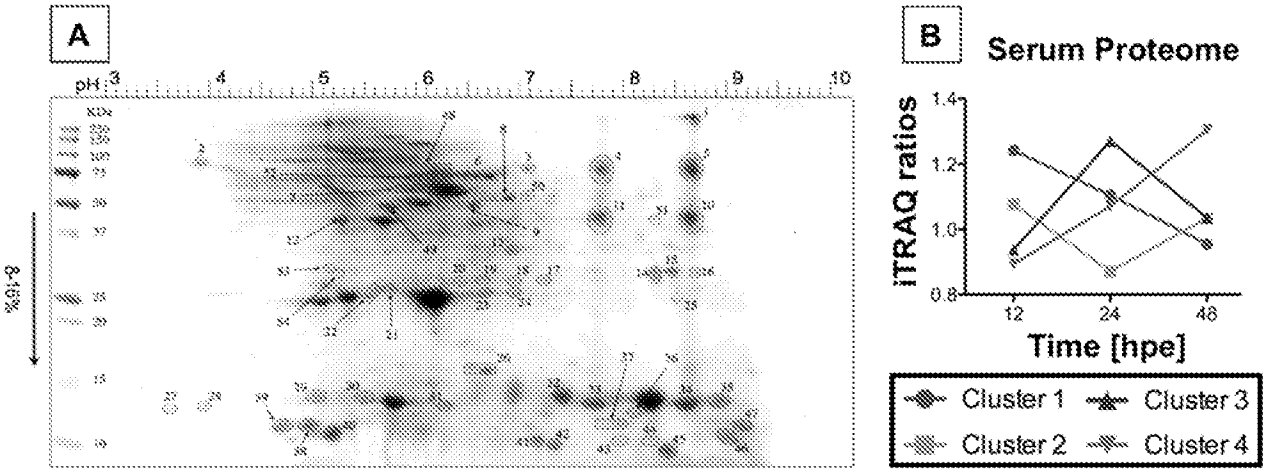

D
Survival [%]
125
100
75
50
25
0
0
20
40
60
80
Time [hpe]
1 mM
Dose [mM]
0.5
10
1.0
25
5.0
50
5 mM

FIGURES 2(A)-(B)


A
Survival [%]
125
100
75
50
25
0
0
20
40
60
80
Time [hpe]
Dose [mM]
5.0 mM
10 mM
25 mM
NAC
5 mM + NAC
10 mM + NAC
25 mM + NAC

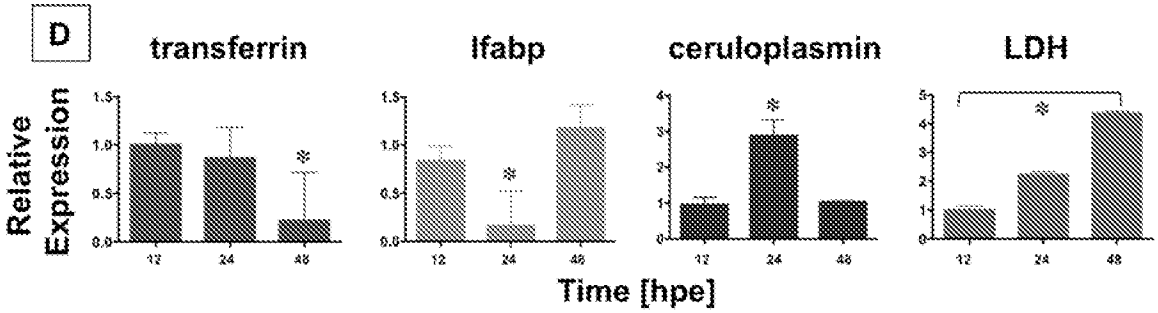

B
ALT [units/L]
500
400
300
200
100
0
*
Con
APAP
NAC
APAP + NAC

FIGURES 3(A)-(D)

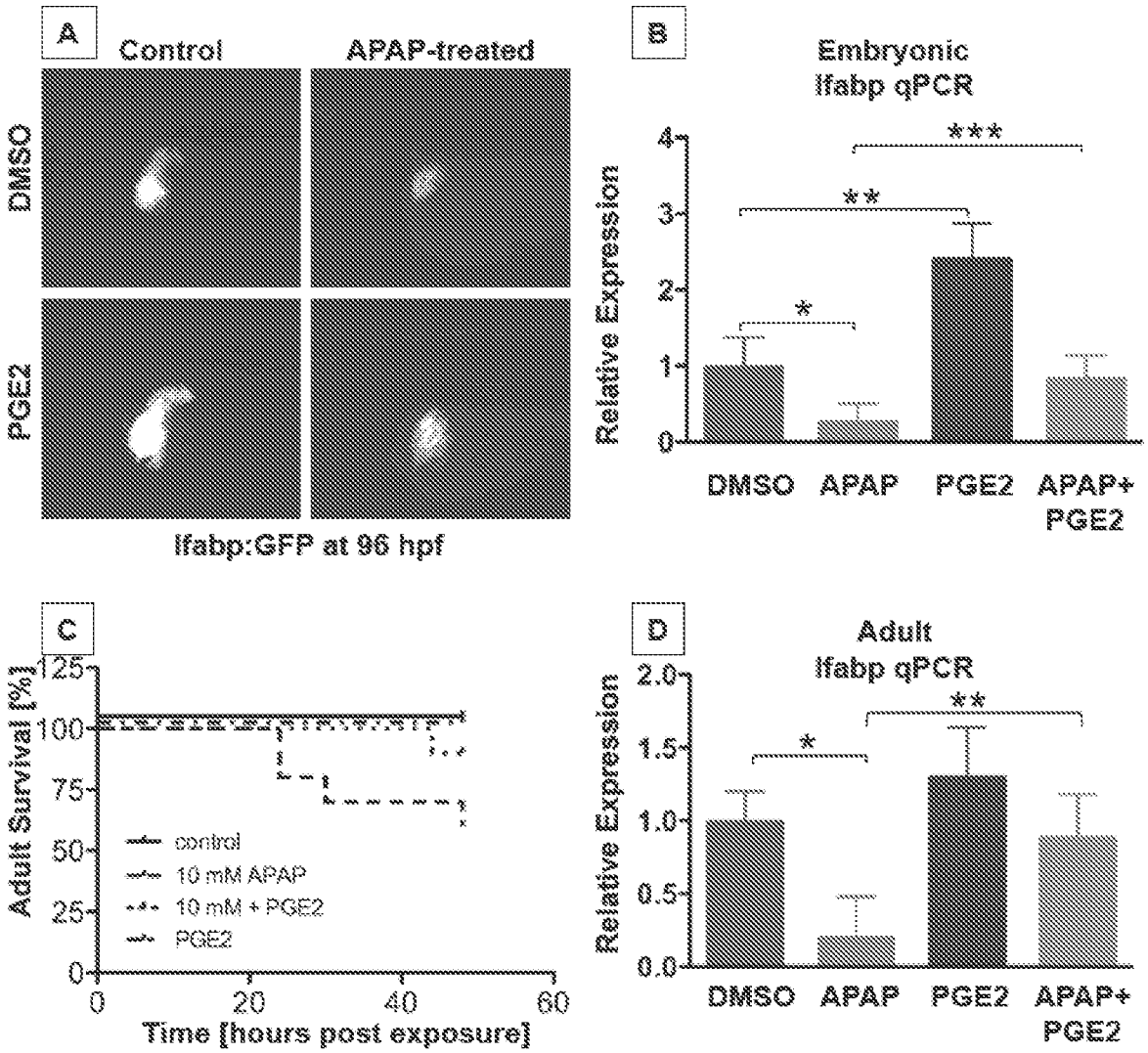


C

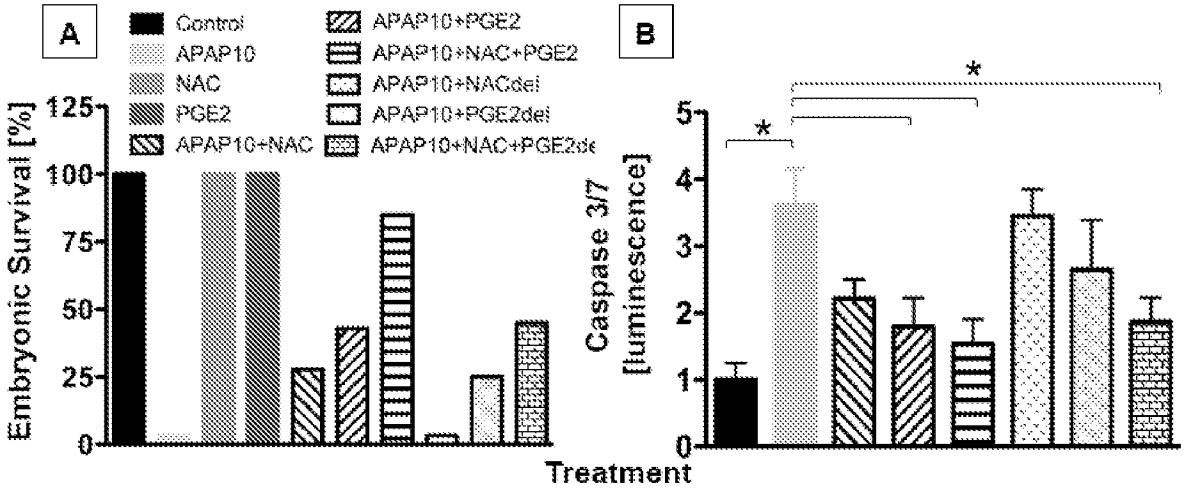
Cluster 1 (71 proteins)	Cluster 2 (46 proteins)	Cluster 3 (33 proteins)	Cluster 4 (73 proteins)
transferrin *	glutathione S transferase	ceruloplasmin *	aldehyde dehydrogenase *
apolipoprotein related *	lfabp *	parvalbumin related	lactate dehydrogenase
vitellogenin	fibrinogen *	collagen related	glyceraldehyde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



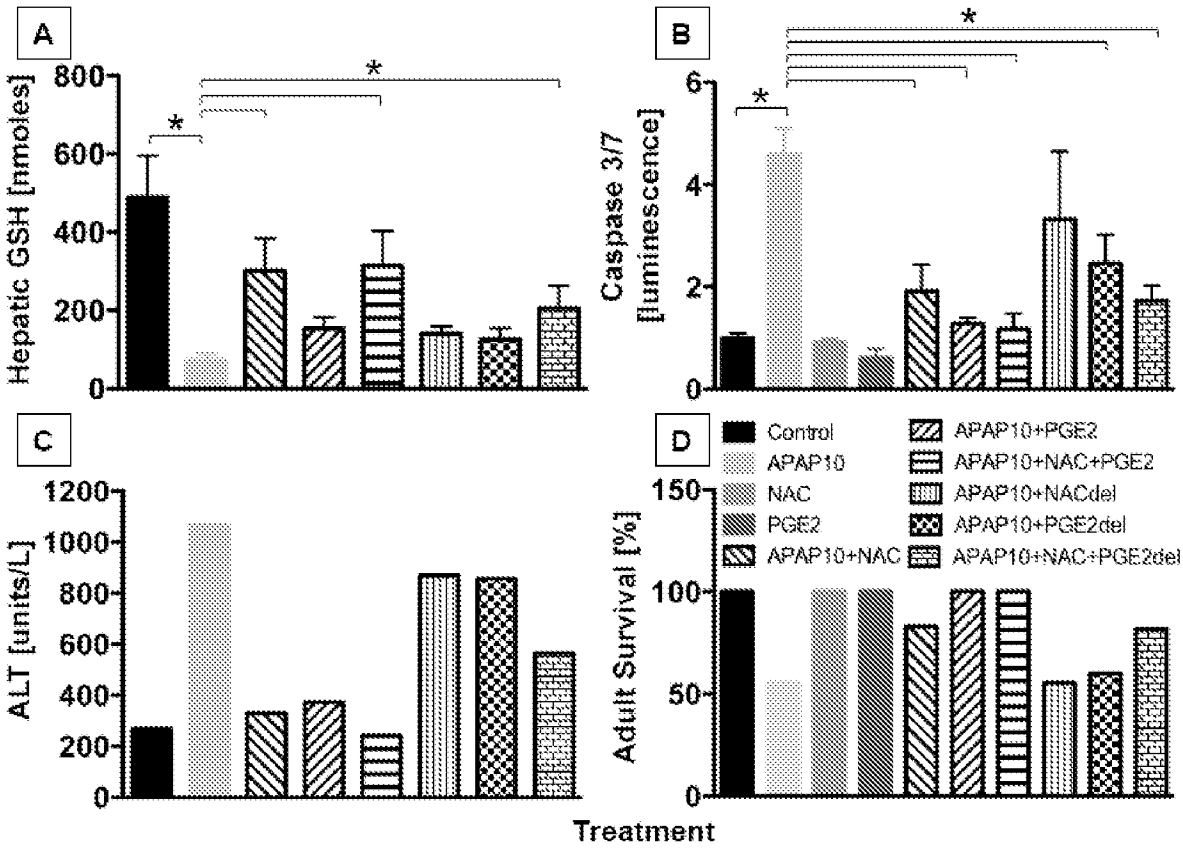
FIGURES 4(A)-(D)


A
Control
APAP-treated
DMSO
PGE2
lfabp:GFP at 96 hpf
B
Embryonic
lfabp qPCR
Relative Expression
***
**
*
DMSO
APAP
PGE2
APAP+ PGE2
C
Adult Survival [%]
control
10 mM APAP
10 mM + PGE2
PGE2
Time [hours post exposure]
D
Adult
lfabp qPCR
Relative Expression
**
*
DMSO
APAP
PGE2
APAP+ PGE2

FIGURES 5(A)-(B)


A
Control
APAP10
NAC
PGE2
APAP10+NAC
APAP10+PGE2
APAP10+NAC+PGE2
APAP10+NACdel
APAP10+PGE2del
APAP10+NAC+PGE2de
Embryonic Survival [%]
125
100
75
50
25
0
B
Caspase 3/7
[luminescence]
5
4
3
2
1
0
*
*
Treatment

FIGURES 6(A)-(D)


A
Hepatic GSH [nmoles]
800
600
400
200
0
*
*
B
Caspase 3/7 [luminescence]
6
4
2
0
*
*
C
ALT [units/L]
1200
1000
800
600
400
200
0
D
Adult Survival [%]
150
100
50
0
Control
APAP10
NAC
PGE2
APAP10+NAC
APAP10+PGE2
APAP10+NAC+PGE2
APAP10+NACdel
APAP10+PGE2del
APAP10+NAC+PGE2del
Treatment

FIGURES 7(A)-(B)

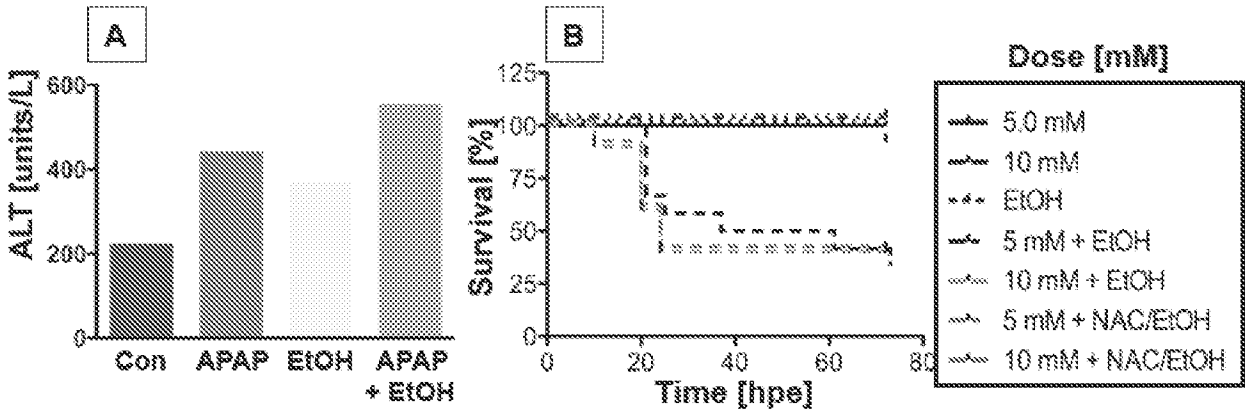
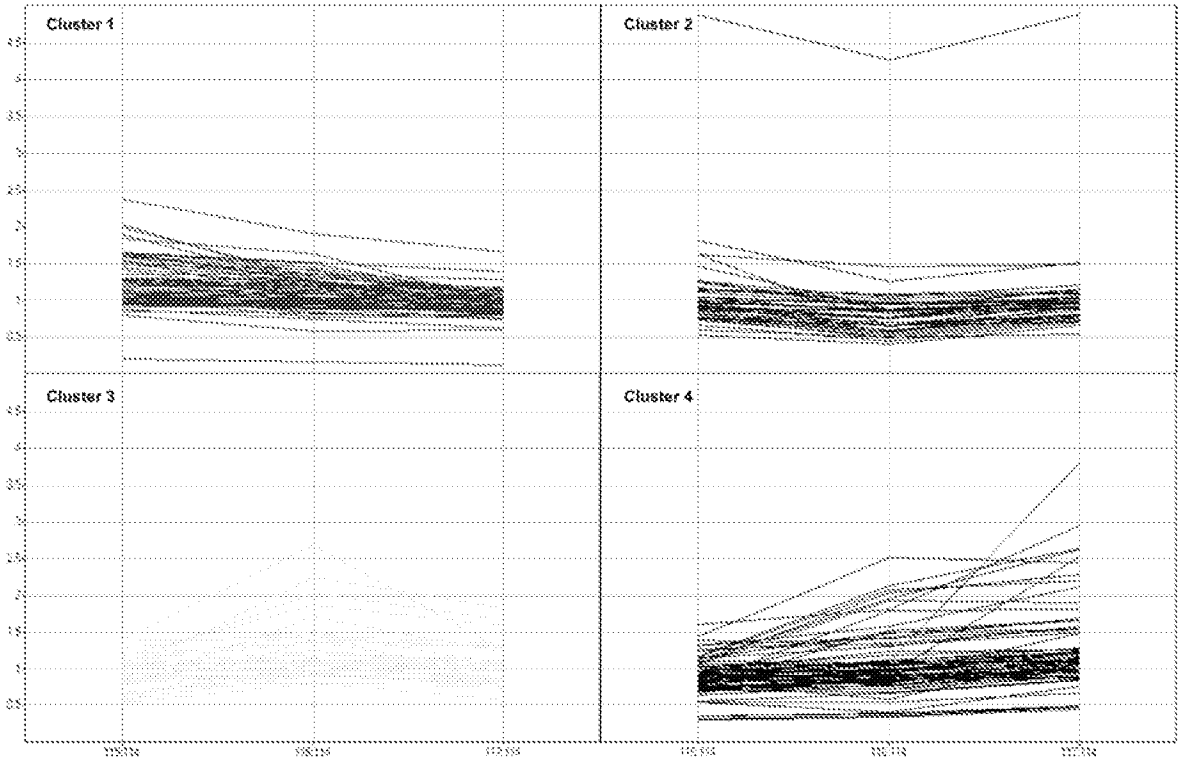

A
ALT [units/L]
600
400
200
0
Con
APAP
EtOH
APAP + EtOH
B
Survival [%]
125
100
75
50
25
0
0
20
40
60
80
Time [hpe]
Dose [mM]
5.0 mM
10 mM
EtOH
5 mM + EtOH
10 mM + EtOH
5 mM + NAC/EtOH
10 mM + NAC/EtOH

FIGURE 8



FIGURES 9(A)-(B)

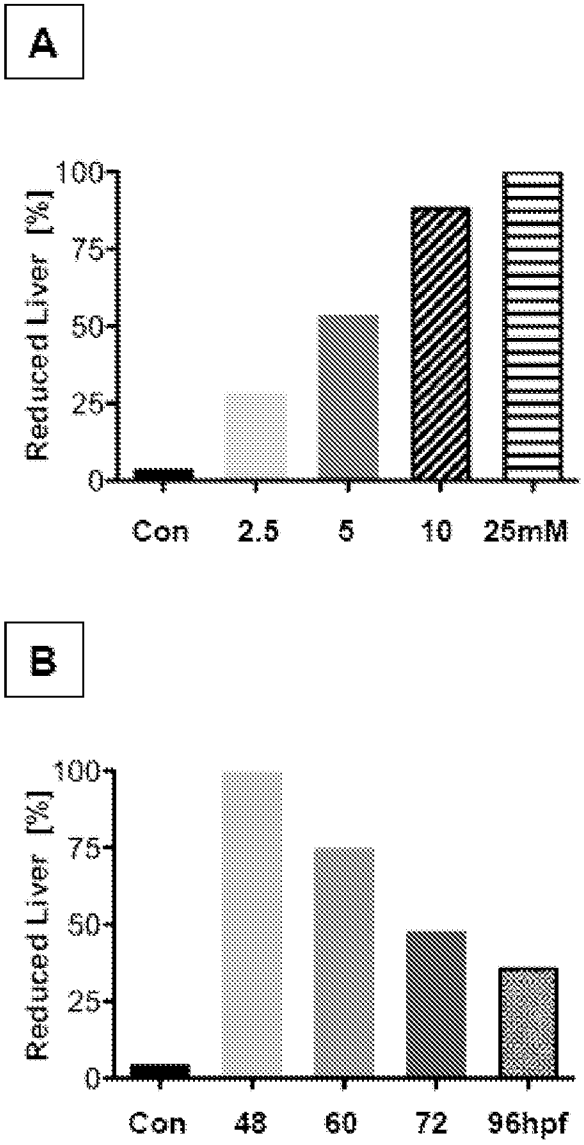

A
Reduced Liver [%]
100
75
50
25
0
Con
2.5
5
10
25mM
B
Reduced Liver [%]
100
75
50
25
0
Con
48
60
72
96hpf

FIGURE 10

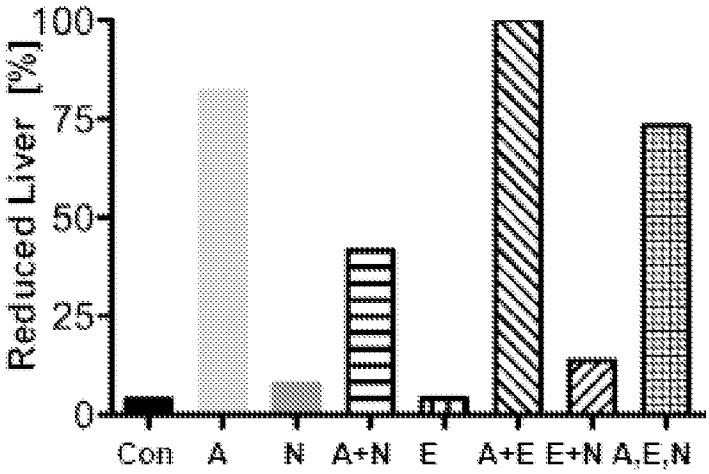


FIGURE 11

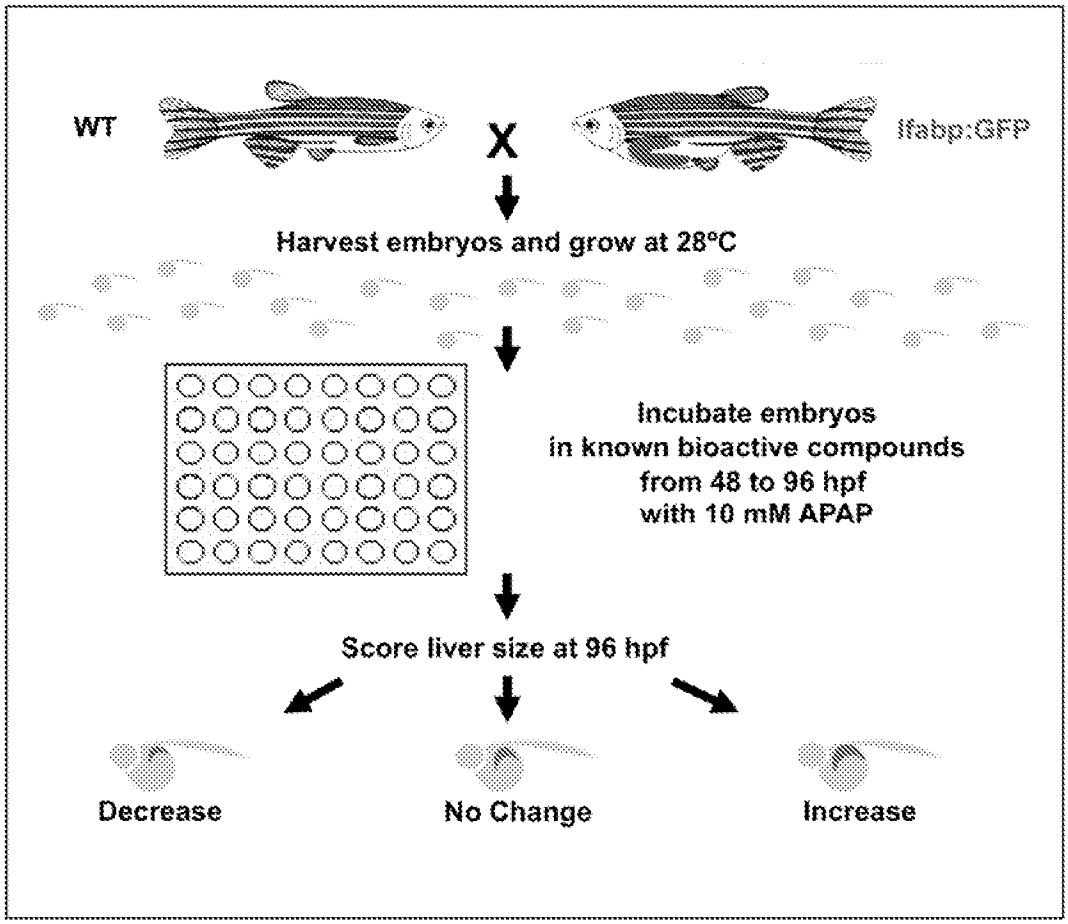
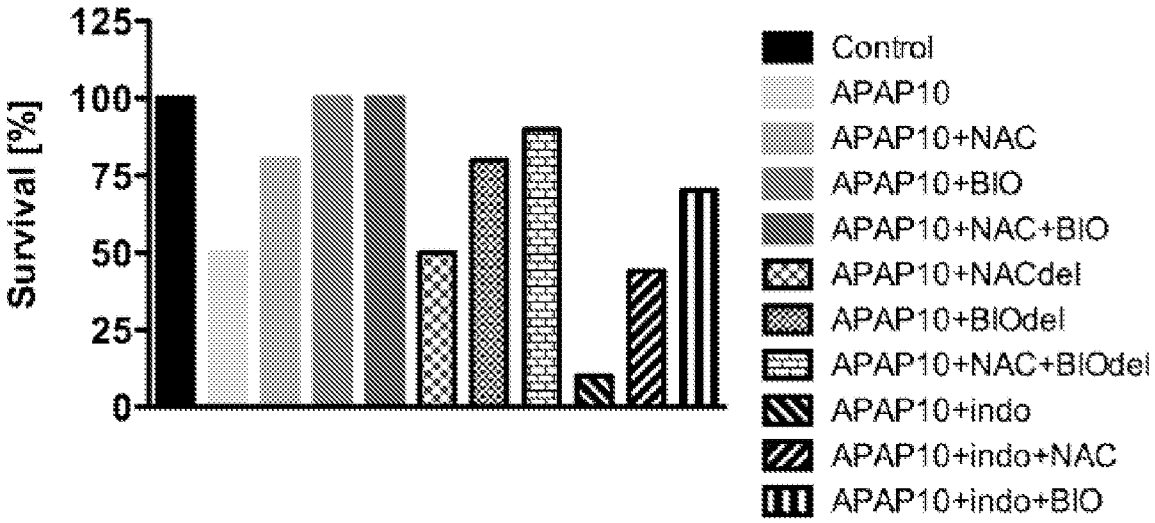

WT
X
lfabp:GFP
Harvest embryos and grow at 28°C
Incubate embryos
in known bioactive compounds
from 48 to 96 hpf
with 10 mM APAP
Score liver size at 96 hpf
Decrease
No Change
Increase

FIGURE 12


Survival [%]
125
100
75
50
25
0
Control
APAP10
APAP10+NAC
APAP10+BIO
APAP10+NAC+BIO
APAP10+NACdel
APAP10+BIOdel
APAP10+NAC+BIOdel
APAP10+indo
APAP10+indo+NAC
APAP10+indo+BIO