



## Detection of hepatotoxicity potential with metabolite profiling (metabolomics) of rat plasma



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### ABSTRACT

While conventional parameters used to detect hepatotoxicity in drug safety assessment studies are generally informative, the need remains for parameters that can detect the potential for hepatotoxicity at lower doses and/or at earlier time points. Previous work has shown that metabolite profiling (metabonomics/metabolomics) can detect signals of potential hepatotoxicity in rats treated with doxorubicin at doses that do not elicit hepatotoxicity as monitored with conventional parameters. The current study extended this observation to the question of whether such signals could be detected in rats treated with compounds that can elicit hepatotoxicity in humans (i.e., drug-induced liver injury, DILI) but have not been reported to do so in rats. Nine compounds were selected on the basis of their known DILI potential, with six other compounds chosen as negative for DILI potential. A database of rat plasma metabolite profiles, MetaMap<sup>®</sup>Tox (developed by metanomics GmbH and BASF SE) was used for both metabolite profiles and mode of action (MoA) metabolite signatures for a number of known toxicities. Eight of the nine compounds with DILI potential elicited metabolite profiles that matched with MoA patterns of various rat liver toxicities, including cholestasis, oxidative stress, acetaminophen-type toxicity and peroxisome proliferation. By contrast, only one of the six non-DILI compounds showed a weak match with rat liver toxicity. These results suggest that metabolite profiling may indeed have promise to detect signals of hepatotoxicity in rats treated with compounds having DILI potential.

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## 1. Introduction

The clear goal of safety evaluation studies is to characterize the hazards posed by a novel chemical. This goal (hazard characterization) is well established for human and environmental risk assessment (Faustman and Omenn, 2008) and drug development (ICH Steering Committee, 2009). Because overt toxicity is preceded by a complex sequence of biochemical, cellular and physiological events (Gregus, 2008), exposures to novel chemicals are evaluated with a variety of parameters that monitor these events (Baldrick,

2008; Crissman et al., 2004; ICH Steering Committee, 2009; Weingand et al., 1996). Furthermore, multiple parameters need to be considered to evaluate the sequence of events leading up to any given organ toxicity. For example, increases in the blood levels of alanine aminotransferase (ALT) may presage clear liver histopathology and failure (Ennulat et al., 2010; Senior, 2009; Travlos et al., 1996).

An organ toxicity that remains of critical interest to drug development is that of hepatotoxicity (Corsini et al., 2012; Horner et al., 2013). Current preclinical safety assessment study designs and parameters are effective for identifying a large number of chemicals with hepatotoxic potential, yet examples still exist of drugs that reach the marketplace only to then elicit cases of drug-induced liver injury (DILI), i.e., hepatotoxicity (Peters, 2005).

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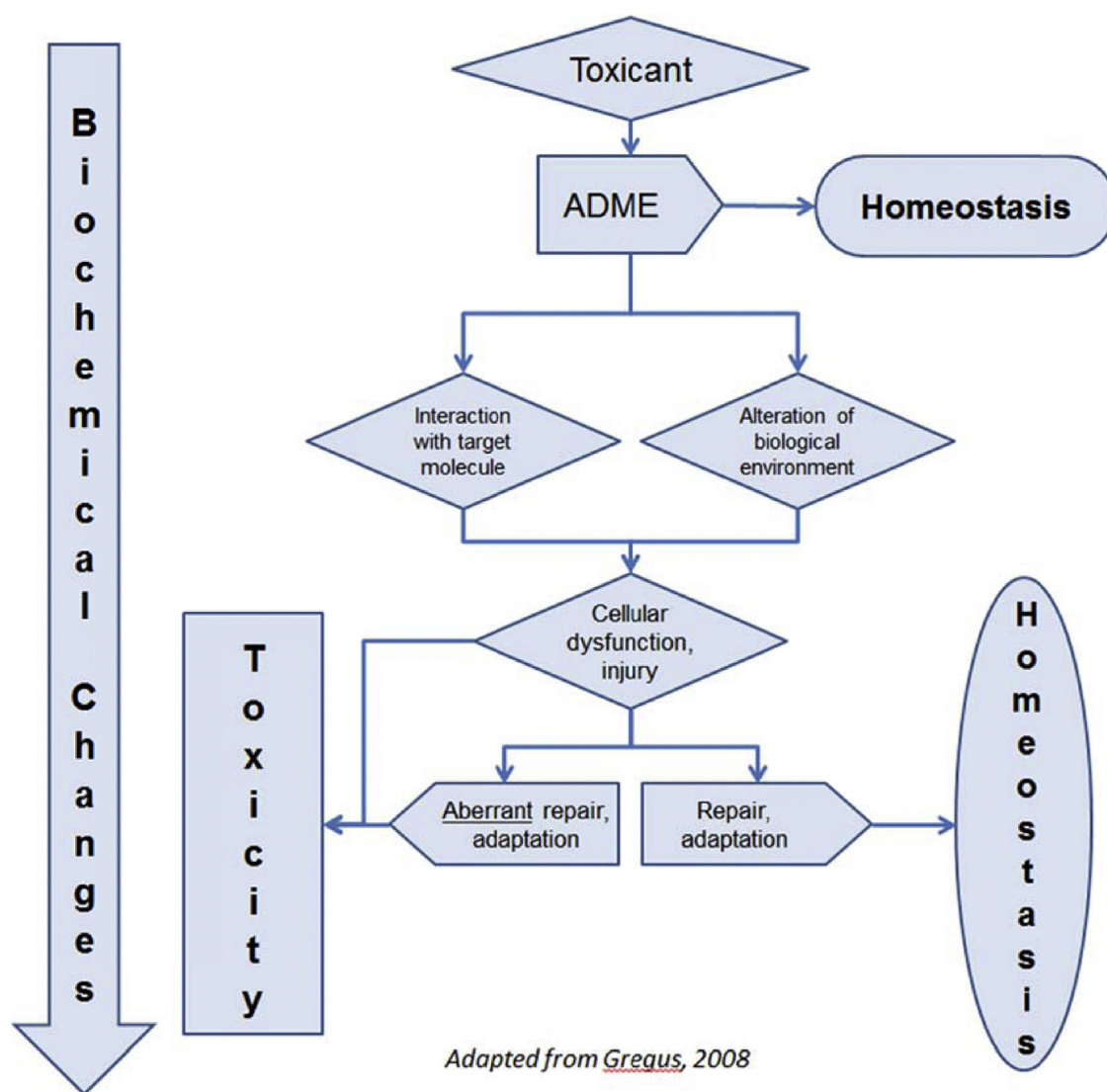
It is well understood that hepatotoxicity may have several different pathological expressions, due to different mechanisms (Jaeschke et al., 2002; Russmann et al., 2009), and the molecular events in microcystin (Campos and Vasconcelos, 2010) and acetaminophen (Jaeschke et al., 2012) hepatotoxicity point to the complexity in the sequence of events leading to liver toxicity. Accordingly, research continues to seek new parameters that will monitor early events in hepatotoxicity to aid in liver safety assessment (Antoine et al., 2009).

New discoveries and technologies often offer new parameters that may complement those conventionally used in existing testing strategies. Ideally such new parameters fill gaps in evaluating the multiple events leading to toxicity, particularly in the ability to monitor early events in the sequence leading up to overt toxicity. Such parameters would be expected to provide signals at lower doses and/or earlier time points than provided by conventional parameters (that may be monitoring events later in the sequence). As an example, Kim 1 was discovered as a protein detectable in urine following kidney injury and has been recognized as detecting nascent nephrotoxicity undetectable by conventional clinical pathology (Vaidya et al., 2010). Such signals

from the novel parameters in essence “predict” the signals (i.e., the toxicity) detected “later” by conventional parameters.

### 1.1. Metabolite profiling

One promising new technology that offers new parameters is that of metabolite profiling (metabonomics/metabolomics), the measurement in biological systems of the full complement of endogenous low-molecular-weight metabolites and their intermediates. Several technologies allow such a measurement from urine, plasma, or tissue extracts. This can offer a global view of the comprehensive metabolic response of a biological system to genetic or environmental modification (Clarke and Haselden, 2008). If such metabolic responses are occurring early in the sequence of events leading up to overt toxicity (Fig. 1), they could be used to detect the adverse potential of chemicals at an early stage in their development (Beger et al., 2010; van Ravenzwaay et al., 2012). Indeed, urine metabolites have been shown to detect cardiotoxicity, hepatotoxicity and nephrotoxicity induced by doxorubicin treatment at lower doses and earlier time points than conventional parameters (Wang et al., 2009). Similarly, urine



**Fig. 1.** An overview of the sequence of events following toxicant exposure.

Unique biochemical changes, reflected in metabolite profiles, may be expected at multiple stages in the sequence of events following toxicant exposure. Adapted from (Gregus, 2008).

metabolites could be used to detect nephrotoxicity after 1 day of treatment with a variety of agents at doses that only elicited signals of nephrotoxicity after 28 days of treatment as monitored with conventional parameters (Boudonck et al., 2009).

To explore this capability, the MetaMap<sup>®</sup>Tox database was developed by Metanomics Health and BASF's department for Experimental Toxicology to augment safety evaluation studies by adding metabolomic parameters. It represents a unique compendium of plasma metabolite profiles from rat studies using over 500 chemicals and drugs with well understood safety profiles and/or biochemical effects. Metabolite profiles common to chemicals sharing common toxicities or effects were used to create mechanistically based metabolite signatures that then can serve as parameters to augment conventional parameters in safety evaluation. A match of a metabolite profile to such signatures indicates a positive signal for that mode of action (MoA).

In previous work we examined doxorubicin, at 2 mg/kg sc weekly for 4 weeks, and while clinical chemistry showed no evidence of hepatotoxicity, plasma metabolic profiles of treated rats showed matches to MoA metabolite signatures indicative of liver toxicity (Mattes et al., 2013). On the other hand, doxorubicin has been clearly shown to elicit hepatotoxicity in rats at higher doses (Deepa and Varalakshmi, 2003; El-Sayyad et al., 2009; Yagmurca et al., 2007), indicating the capability of metabolomics to provide signals of rodent hepatotoxicity at doses lower than those required to elicit signals with conventional parameters. In our earlier work we have shown, that metabolomics allows for the identification of hepatotoxic effects of compounds known to have this property in rats as well as humans. This was demonstrated for “classical” chemicals such as carbon tetrachloride, or pharmaceuticals such as paracetamol (van Ravenzwaay et al., 2010). The purpose of the present study was to investigate if this technology is sufficiently sensitive to pick up signals of hepatotoxicity in rats treated with compounds that do not show pathological changes in the liver of rats but are known to elicit such toxicity in humans (so-called human DILI compounds). Therefore, the selection of compounds was based on the publication by Chen et al. (2011). As a control, the metabolite profiles of rats treated with compounds that have not been associated with either rodent or human toxicity were also examined. In order to avoid additional animal testing, the evaluations were conducted for compounds that were already in the data base MetaMap<sup>®</sup>Tox. The results in this study suggest that metabolite signatures can indeed indicate signals of hepatotoxicity even with compounds that do not ultimately elicit signals of such injury by conventional parameters.

## 2. Methods

### 2.1. Compound selection

Fifteen compounds were selected on the basis of existing data in the MetaMap<sup>®</sup>Tox database and their classification by Chen et al. (Chen et al., 2011), who used FDA labeling: phenytoin, flutamide, propylthiouracil, lamivudine, methotrexate, captopril, nefazodone, nevirapine, valproic acid, and zidovudine were classified as having “Most DILI Concern,” while atropine, mannitol, neomycin, streptomycin, and vancomycin were classified as having “No DILI Concern.” These classifications were subsequently confirmed by reports in the LiverTox<sup>™</sup> database (Hoofnagle et al., 2013) as well as extensive searching for literature reports of DILI associated with the drug's use. In the case of lamivudine the FDA warning label was, in fact, a warning for the class of nucleoside anti-retrovirals. No literature report could be found of hepatotoxicity due to the use of lamivudine alone, consistent with the conclusions in the LiverTox<sup>™</sup> database, and therefore it was re-classified for this study having “No DILI Concern.” None of the

fifteen compounds has been used to establish the patterns for liver toxicity (see Section 2.4).

### 2.2. Ethics statement

The studies were approved by the BASF Animal Welfare Body, and were performed according to the German Animal Welfare Act and EU Directive 2010/63, with the permission of the local authority, the Landesuntersuchungsamt Rheinland-Pfalz (permission number 23 177-07/G08-3-001). The laboratory is AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International) certified.

### 2.3. Animal treatment, examinations, clinical pathology and histopathology

Animal handling, compound treatment, clinical examinations, and clinical pathology (hematology and clinical chemistry) have been described earlier (Kamp et al., 2012; van Ravenzwaay et al., 2007, 2012). Briefly, Wistar rats (CrI:WI(Han)) were supplied by Charles River, Germany and were approx. 70 days old at the beginning of the studies. The diet and drinking water were available ad libitum (except before blood sampling) and regularly assayed for chemical contaminants and the presence of micro-organisms. Compound dose-levels and routes of administration (based on literature and range-finding data) are summarized in Table 1. While all studies incorporated a control, low dose level, and high dose level for each compound, results for captopril are focused on only the low dose treatment in this report. All animals were checked daily for mortality and clinical signs. Food consumption was determined on study days 7, 14, 21 and 28. Body weight was determined before the start of the administration period in order to randomize the animals and on study days 0, 4, 7, 14, 21 and 28. At the end of the treatment period, the animals were sacrificed by decapitation under isoflurane anesthesia. For all compounds, clinical pathology (hematology and clinical chemistry) was performed on blood collected at sacrifice from 5 animals of each sex in control, low dose, and high dose groups. For 12 (phenytoin, captopril, nefazodone, nevirapine, valproic acid, zidovudine, atropine, lamivudine, mannitol, neomycin, streptomycin, or vancomycin) of the compounds, liver was collected at necropsy and fixed in 10% neutral buffered formalin from 5 animals of each sex in control and high dose groups. After fixation, liver tissues were processed to hematoxylin and eosin stained glass slides as described previously (Kamp et al., 2012) and examined by light microscopy.

**Table 1**

Compounds used, dose levels and routes of administration.

Treatment	Dose	Route
Phenytoin	2400 PPM	Diet
Flutamide	100 mg/kg/day	po
Propylthiouracil	10 mg/kg/day	po
Methotrexate	1 mg/kg/day	ip
Captopril	20 mg/kg/day	po
Captopril	200 mg/kg/day	po
Nefazodone hydrochloride	300 mg/kg/day	po
Nevirapine	40 mg/kg/day	po
Valproic acid	500 mg/kg/day	po
Zidovudine	1000 mg/kg/day	po
Atropine sulfate monohydrate	62.5 mg/kg/day	po
Mannitol	50000 PPM	Diet
Neomycin sulfate	250 mg/kg/day	po
Streptomycin sulfate	360 mg/kg/day	sc
Vancomycin hydrochloride	200 mg/kg/day	ip
Lamivudine	300 mg/kg/day	po

po: oral administration by gavage, ip: intraperitoneal injection, sc: subcutaneous injection, diet: administration in feed.

## 2.4. MetaMap<sup>®</sup>Tox methodology

As described by Kamp et al. (Kamp et al., 2012), K-EDTA samples were taken from the retro-orbital sinus in all rats under isoflurane anesthesia on study days 7, 14 and 28 for mass spectrometry-based metabolite profiling analysis and extracted by a proprietary method. Two types of mass spectrometry-based metabolite profiling analysis were applied to all samples: GC–MS (gas chromatography–mass spectrometry) and LC–MS/MS (liquid chromatography–MS/MS) were used for broad profiling and hormone measurement as described in van Ravenzwaay et al. (van Ravenzwaay et al., 2007). The method resulted in 225 semi-quantitative analytes, 171 of which are chemically identified and 54 are structurally unknown. The changes in these analytes induced by the various treatments are given in the Supporting Table.

MetaMap<sup>®</sup>Tox is a unique database of biochemical profiles from rat plasma and comprehensive pharmacological and toxicological data based on more than 500 pharmaceuticals, chemicals and agrochemicals after 7, 14 and 28 days of test substance treatment. Studies used groups of CrI:WI (Han) rats, 5 of each sex in control, low dose, and high dose groups. Discriminating metabolite patterns for various toxicological modes of action (MoAs) were developed from the metabolite profiles in the MetaMap<sup>®</sup>Tox database (van Ravenzwaay et al., 2012). Briefly, these metabolite patterns are usually based on the data from at least three different chemicals included in the MetaMap<sup>®</sup>Tox database and which share a common toxicological mode of action (reference compounds). The establishment of a specific metabolite pattern associated with a particular MoA or form of toxicity starts with the identification of common metabolite changes for these reference compounds. Iteratively, the resulting list of metabolites is checked for consistency and modified based on mechanistic knowledge by an expert panel of experienced toxicologists. During this process metabolites might be added or removed from the pattern, as well as given special weight according to the importance for the predictivity of the pattern. Finally, the resulting pattern is checked for sensitivity and selectivity against the reference data in the

database: a pattern should correctly identify at least one further reference compound sharing the same mode of action, which has not been used to establish the pattern. Furthermore, reference compounds in MetaMap<sup>®</sup>Tox which do not share this particular toxicity should not be identified.

The pattern ranking itself is a two-step process. Firstly, an algorithm used in the database yields a ranking list based on similarity of the test compound metabolic profile compared to the specific patterns in MetaMap<sup>®</sup>Tox using a median *r* value metric. Secondly, the metabolite changes are evaluated by an expert panel of experienced toxicologists to determine what may be described as “confirmed” matches. Generally, based on the number of commonly changed metabolites, a match prerequisites approx. 90% or more of metabolites significantly changed as defined by the pattern (weak matches: approx. 75–90%; equivocal findings: approx. 50–75%; mismatches: <50%). Furthermore, the quality and importance of the metabolite changes for a certain toxicological mode of action is considered for this evaluation. For example, metabolites which are based on perturbations of specific biochemical pathways, and which can be connected to the toxicity observed, are particularly evaluated. For some patterns, we have defined so-called “anchor metabolites”, these metabolites are essential components of the biochemical pathway related to the mode of action, and these have to be significantly changed in the right direction in order to result in a matching pattern. The second step of the process can then result in a determination that a pattern with a seemingly high median *r* value may not be considered a confirmed match.

## 3. Results

### 3.1. Food consumption, body weight, clinical pathology and histopathology

Results of our studies with phenytoin have already been reported (Kamp et al., 2012); briefly, the high dose treatment with phenytoin produced statistically significant body weight decreases in both sexes, along with the microscopic findings of minimal to

**Table 2**  
Significant food consumption, body weight, clinical pathology and histopathology results.

Treatment	Summary findings
Flutamide	Decreased body weights in males and decreased food consumption in both males and females
Propylthiouracil	Decreased body weights and decreased food consumption in both males and females
Methotrexate	Decreased body weights in females and decreased food consumption in both males and females; males were sacrificed after 14 days of treatment
Captopril (high dose)	Slightly decreased creatinine levels in both males and females; increased serum GGT in males
Nefazodone	Increased inorganic phosphate and white blood cell counts in females; increased BUN in males; slightly increased magnesium in both males and females
hydrochloride	
Nevirapine	Increased cholesterol, triglycerides and total protein in females; slightly increased platelets in females; decreased white blood cells in males
Valproic acid	Decreased food consumption, globulin, hemoglobin, platelets, red blood cell counts, white blood cell counts in both males and females; increased triglycerides and slightly increased inorganic phosphate and total bilirubin (1.29× and 1.21× respectively) in both males and females
Zidovudine	Increased cholesterol and slightly increased mean corpuscular hemoglobin content, and mean corpuscular volume, in males and females; slightly decreased hematocrit, hemoglobin, and red blood cell counts in males and females; slightly increased urea and total bilirubin (1.42×) in males and white blood cell counts in females
Atropine sulfate	Slightly increased total bilirubin (1.4×) in males and females; increased urea in males; decreased food consumption and body weights in males; increased triglycerides and inorganic phosphate and slightly increased ALT (1.3×) in females; decreased urinary creatinine in females
monohydrate	
Mannitol	Slightly increased total bilirubin (1.2×) in males; slightly increased ALT (1.23×) in females; decreased food consumption and urinary creatinine in females
Neomycin sulfate	Increased cholesterol and slightly increased inorganic phosphate in males and females; decreased food consumption and alkaline phosphatase in males and females; decreased magnesium in females
Streptomycin sulfate	Slightly increased inorganic phosphate and urea in males and females; increased triglycerides and slightly increased white blood cell counts in females
Lamivudine	Slightly increased platelets in males; decreased food consumption, body weights, and alkaline phosphatase in males; decreased white blood cell counts and slightly decreased total bilirubin (0.7×) in females
Vancomycin	Decreased food consumption in males and females; increased triglycerides and urea in males; decreased body weights and slightly decreased alkaline phosphatase in males; slightly increased inorganic phosphate in females.
hydrochloride	Microscopic finding of mild to moderate chronic-active inflammation confined to the hepatic capsule in males and females attributed to peritonitis from intraperitoneal administration of the compound without compound-related microscopic findings in the hepatic parenchyma.



slight centrilobular hepatocellular hypertrophy. A summary of the statistically significant changes elicited by the other compounds is given in Table 2. Mild or modest changes in food consumption, body weight, clinical chemistry, and/or hematological parameters were seen in all studies. Parameters suggestive of liver injury (i.e., elevated ALT, AST or total bilirubin) were unchanged in most of these studies; serum ALT levels were very slightly, albeit statistically significantly, increased (1.2–1.3 fold) in the atropine and mannitol studies, while total bilirubin was slightly increased (1.2–1.4 fold) in the valproic acid, zidovudine, atropine and mannitol studies. The microscopic finding of mild to moderate chronic-active inflammation confined to the hepatic capsule was considered to be related to the high dose administration of vancomycin in both sexes. The finding was present in all high dose vancomycin treated male and female animals and absent in all male and female control animals. The inflammation of the hepatic capsule was consistent with peritonitis and was considered to be the result of the intraperitoneal administration of the compound. There were no microscopic findings involving the hepatic parenchyma that were considered related to the high dose administration of vancomycin. Similarly for the high dose

administration of captopril, nefazodone, nevirapine, valproic acid, zidovudine, atropine, lamivudine, mannitol, neomycin, and streptomycin there were no microscopic findings in the liver that were considered to be treatment related.

### 3.2. Metabolomic data

Comparison of the metabolite profiles of the fifteen compounds to the MetaMap<sup>®</sup>Tox database resulted in matches to a number of validated patterns corresponding to various toxicological and pharmacological modes of action (MoAs). Results for phenytoin have been reported earlier (Kamp et al., 2012), where the high-dose phenytoin resulted in a metabolic profile matching numerous database patterns associated with liver enzyme induction, liver cell cytotoxicity, liver cell oxidative stress (i.e., paracetamol-like liver toxicity), liver cholestasis as well as those associated with indirect effects on the thyroid due to increased conjugation and excretion of thyroxine. Human hepatotoxicity of phenytoin has been known for some time (Russo et al., 2004; Smythe and Umstead, 1989), but it should be noted that in the current study the strong metabolomic data elicited by phenytoin treatment of rats is accompanied by only

Rank no.	Pattern Name	Median r	Assessment
1	Liver_cholestasis_female high dose (putative)	0,92	Weak Match
2	Liver_enzyme induction_female high dose	0,91	Match
3	Liver_toxicity_female high dose	0,91	Match
4	Liver_cholestasis_female low and high dose (putative) <high dose>	0,91	Weak Match
5	Adrenals_steroid synthesis Inhibition_female high dose	0,88	Match
6	Hormones_antandrogen_male high dose	0,88	Match
7	Liver_paracetamol-derived_female high dose	0,88	Match
8	Liver_enzyme induction_female high dose day 14	0,85	Match
9	Thyroid_indirect, liver enzyme induction_female high dose	0,85	Match
10	Hormones_antandrogen receptor antagonist prostate_male high dose	0,84	Match
11	Hormones_GnRH agonist_female high dose (putative)	0,83	Equivocal
12	Liver_enzyme induction_male high dose	0,82	Match
13	Liver_cholestasis_female high and low dose (putative) <low dose>	0,80	Weak Match
14	Liver_peroxisome proliferation_female high dose	0,78	Mis-match
15	Nervous system_serotonin receptor antagonist_female high dose	0,77	Mis-match
16	Duodenum_iron-deficiency_female high dose	0,77	Mis-match
17	Adrenals_cortex dihydrotestosterone-reducer_female high dose (putative)	0,76	Mis-match
18	Liver_peroxisomal proliferation_female high dose day 28	0,75	Mis-match
19	Liver_enzyme induction_female high dose day 7	0,75	Match
20	Spleen_Methemoglobinemia_male high dose	0,74	Equivocal
21	Nutrition biomarkers of effect_male day 7 and 14	0,74	Mis-match
22	Energy metabolism_decreased biomarker of effect_male high dose	0,74	Mis-match
23	Thyroid_indirect, liver enzyme induction_male high dose	0,73	Match

**Fig. 2.** Ranking of patterns matching the metabolite changes induced by flutamide treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Based on similarity analysis comparing the metabolic profile produced by flutamide treatment with the mode of action (MoA) profiles in the MetaMap<sup>®</sup>Tox database; the algorithm produces an *r* value metric. The shaded boxes indicate the subsequent expert assessment of the match with the respective pattern: solid green – confirmed match, light green – weak match, yellow – equivocal match (no conclusion possible), red – confirmed non-match.

minimal evidence of liver enzyme induction (i.e., minimal centrilobular hepatocellular hypertrophy).

The high dose flutamide treatment (a non-steroidal antiandrogen with a well-known history of human hepatotoxicity (Wysowski and Fourcroy, 1996)) resulted in a metabolic profile with confirmed matches to database patterns corresponding to adrenal steroid synthesis inhibition and antiandrogen receptor antagonist (Fig. 2) consistent with its pharmacological mode of action (Rouquie et al., 2009). However, flutamide treatment also resulted in a number of confirmed matches to MoA patterns corresponding to various modes of liver toxicity, including liver toxicity, paracetamol-like toxicity, and cholestasis. These patterns represent distinct, but somewhat overlapping, metabolite profiles associated with treatments producing liver pathology.

Propylthiouracil at the high dose produced a metabolic profile that matched MoA patterns indicating a direct effect on the thyroid (Fig. 3), entirely consistent with this drug's pharmacological action (Ma et al., 2007). While the MetaMap<sup>®</sup>Tox database lacks MoA patterns for anti-retroviral pharmacology, it did have MoA pattern matches for the metabolite profile elicited by high dose zidovudine

treatment. These included MoAs for platelet and adrenal effects and kidney toxicity, effects (Fig. 4), documented in literature reports (Omar et al., 1996; Ragni et al., 1992; Tortorella et al., 2009), as well as MoAs associated with bone marrow suppression; this toxicity is well known for this drug (D'Andrea et al., 2008). Relevant to this investigation however, both propylthiouracil and zidovudine treatments produced metabolite profiles that matched MoA patterns associated with liver toxicity (Figs. 3 and 4, respectively), such as liver oxidative stress for propylthiouracil and and liver enzyme induction as well as paracetamol-like toxicity for zidovudine. And while the in vivo findings for rats treated with these compounds showed little evidence of hepatotoxicity, human hepatotoxicity for both compounds has been well-established (Chen et al., 1992; Russo et al., 2004).

By contrast, treatment with vancomycin or lamivudine (Figs. 5 and 6, respectively) produced metabolite profiles that matched only a small number of MoA patterns associated with toxicity, none being associated with liver toxicity. While vancomycin treatment has not been associated with anemia (as might be expected by porphyrin inhibition), lamivudine treatment has been associated

Rank no.	Pattern Name	Median r	Assessment
1	Thyroid_direct effect strong_female high dose	0,93	Match
2	Thyroid_direct inhibition strong_male high dose	0,88	Match
3	Pancreas_endocrine modulation_female high dose	0,84	Mis-match
4	Liver_oxidative stress_male high dose day 28 (putative)	0,84	Mis-match
5	liver_oxidative stress_male low and high dose (putative)	0,78	Match
6	Eye_HPPD-inhibition_male high dose	0,76	Mis-match
7	Liver_peroxisomal proliferation_male high dose	0,68	Weak Match
8	liver_oxidative stress_male low and high dose (putative)	0,68	Weak Match
9	Blood_porphyrin inhibitor_male high dose day 28	0,68	Mis-match
10	Spleen_haematopoiesis_male high dose	0,68	Mis-match
11	CNS_antipsychotic drugs_dopamine receptor block_male high dose	0,67	Mis-match
12	Nervous system_serotonine reuptake inhibition_male high dose	0,64	Mis-match
13	Bone_mineralisation_male high dose day 28	0,63	Mis-match
14	Phthalates_long-chain_male high dose (putative)	0,62	Mis-match
15	Testis_degeneration_male high dose	0,62	Mis-match
16	Liver_enzyme induction_male high dose day 7	0,59	Mis-match
17	Blood_porphyrin inhibition_male high dose	0,59	Mis-match
18	Liver_fibrate phthalate phenoxy_male high dose day 28	0,58	Mis-match
19	Kidney direct tubular effect_male high dose day 28	0,58	Mis-match
20	Adrenals_hypocortisolism_male high dose day 28	0,55	Mis-match
21	Nervous System_dopamin agonist_female high dose day 28	0,54	Mis-match
22	Phthalates_short-chain_male high dose (putative)	0,54	Mis-match
23	Liver_PPAR gamma agonist_male high dose	0,53	Mis-match
24	Nutrition biomarker of feed exposure_males day 7 and day 14	0,53	Mis-match

**Fig. 3.** Ranking of patterns matching the metabolite changes induced by propylthiouracil treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

See legend for Fig. 2.

Rank no.	Pattern Name	Median r	Assessment
1	Liver_enzyme induction_female high dose	0,86	Weak Match
2	Kidney_tubular necrosis_female high dose	0,84	Match
3	Thyroid_indirect, liver enzyme induction_male high dose	0,84	Match
4	Liver_enzyme induction_male high dose	0,82	Weak Match
5	Blood_platelet aggregation inhibition_female high dose (putative)	0,81	Match
6	Thyroid_indirect, liver enzyme induction_female high dose	0,80	Weak Match
7	Duodenum_iron-deficiency_female high dose	0,80	Equivocal
8	Liver_paracetamol-derived_male high dose	0,79	Match
9	Bone marrow_suppression_female high dose day 28 (putative)	0,79	Weak Match
10	Adrenals_cortex_synthesis inhibition_male high dose	0,75	Weak Match
11	Kidney_tubular defect_female high dose	0,74	Mis-match
12	Hormones_antiandrogen_male high dose	0,74	Mis-match
13	Nervous system_serotonin receptor antagonist_female high dose	0,73	Mis-match
14	Kidney_interstitial nephritis_male high dose	0,72	Mis-match
15	Kidney_glomerular-tubular defect_male high dose	0,72	Mis-match
16	Spleen_methemoglobinemia_female high dose	0,71	Mis-match
17	Blood_aplastic anemia_male high dose	0,71	Mis-match
18	Bone marrow_suppression platins_female low dose	0,71	Mis-match
19	Kidney_diuretik-like_female high dose day 28	0,70	Mis-match
20	Thyroid_direct inhibition strong_male high dose	0,70	Mis-match

**Fig. 4.** Ranking of patterns matching the metabolite changes induced by zidovudine treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

See legend for Fig. 2.

with bone mineral content (Tsekas et al., 2002), a side effect of gonadotropin-releasing hormone (GnRH) agonism (Choi and Lee, 2011). Atropine treatment did result in a metabolite profile that had matches to patterns of kidney and bone marrow toxicity, as well as liver toxicity and liver enzyme induction (Fig. 7), but liver enzyme induction is generally viewed as a compensatory and not adverse response. None of these adverse effects have been reported for atropine, but it is conceivable that the CNS effects of this drug may overshadow any conventional measures of these toxicities.

Methotrexate treatment, while affecting both body weight and food consumption (Table 2), elicited a metabolite profile that only matched a pattern for kidney tubular necrosis (Supporting Fig. 1), an adverse effect well known for this drug (Widemann and Adamson, 2006). On the other hand, the low dose treatment with captopril elicited a metabolite profile with numerous matches to patterns associated with kidney, bone marrow and liver toxicity (Supporting Fig. 2). All of these toxicities have been described for captopril (Andrade et al., 2006; Hoitsma et al., 1991; Pillans and Koopowitz, 1991) and the metabolite profile for the high dose captopril treatment was used to establish the pattern for kidney tubular defects. It should be noted that while the signals for bone marrow suppression and kidney toxicity are seen at both dose levels (data not shown) and clinical pathology effects were observed only at the high dose, the patterns for liver toxicities only matched the metabolite profile elicited by low dose captopril treatment (Supporting Fig. 2). Such an “inverse” dose response has been seen for other effects of captopril, namely the effect of this drug on water drinking where captopril enhanced drinking at low doses but inhibited it at high doses (Evered and Robinson, 1984).

However, a mechanistic basis for such an inverse response in either case is speculative at this point.

Treatment of rats with the anti-depressant nefazodone elicited a metabolic profile that matched a MoA pattern associated with adrenal steroid synthesis Inhibition (Supporting Fig. 3); this drug has been reported to indeed have an effect on the hypothalamic–pituitary–adrenal axis in rats (Matheson et al., 1997). Both nefazodone and nevirapine treatment elicited metabolic profiles that matched MoA patterns for various liver toxicities (Supporting Figs. 3 and 4), including paracetamol-like hepatotoxicity (both drugs) and peroxisome proliferation (nevirapine). And while neither drug treatment resulted in *in vivo* signs of hepatotoxicity (Table 2), both drugs are associated with human liver injury (Chu et al., 2010; Stewart, 2002); nefazodone was withdrawn from the market due to its risk (Edwards, 2003).

Valproic acid treatment at the high dose yielded a metabolic profile that matched an MoA pattern for adrenals steroid synthesis Inhibition (Supporting Fig. 5), as might be expected given this compound’s inhibition of steroid hormone metabolism (Morrell, 2003). However, there were also matches to multiple MoA patterns for peroxisome proliferation, a liver pathology. The relationship of this rodent pathology to human pathologies is not clear, as multiple receptors can be activated by the same compound. Nonetheless, valproic acid is well known to be capable of causing human hepatotoxicity.

Treatment with neomycin sulfate produced a plasma metabolome that could be matched to MoA patterns for kidney tubular necrosis, bone marrow suppression, and liver enzyme induction (Supporting Fig. 6). While kidney toxicity is recognized for system neomycin exposure (Masur et al., 1976), and bone marrow effects



Rank no.	Pattern Name	Median r	Assessment
1	Blood_porphyrin inhibition_male high dose	0,83	Weak Match
2	Blood_porphyrin inhibitor_male high dose day 28	0,82	Weak Match
3	Adrenals_hypocortisolism_male high dose day 28	0,78	Equivocal
4	Neurotox_PNS nicotinic receptor agonist_males high dose (putative)	0,77	Equivocal
5	Energy metabolism_decreased biomarker of effect_male high dose	0,76	Equivocal
6	Reduced feed consumption_male day 7	0,74	Equivocal
7	Hormones_antandrogen_male high dose	0,72	Mis-match
8	Kidney_tubular necrosis_female high dose	0,70	Equivocal
9	Bone marrow_suppression platins_female low dose	0,69	Mis-match
10	Immune System_Immunsuppression_male low dose day 28	0,68	Mis-match
11	Skeletal muscle_innervation stimulation_male high dose	0,63	Mis-match
12	Kidney_glomerular-tubular defect_female high dose	0,62	Mis-match
13	Thyroid_indirect, liver enzyme induction_male high dose	0,61	Mis-match
14	Nervous system_serotonine reuptake inhibition_male high dose	0,58	Mis-match
15	Reduced food consumption_female day 7	0,57	Mis-match
16	Liver_enzyme induction_male high dose	0,56	Mis-match
17	Liver_PPAR gamma agonist_male high dose	0,55	Mis-match
18	Spleen_haematopoiesis_male high dose	0,54	Mis-match
19	CNS_psycoanaleptics_male high dose	0,52	Mis-match
20	CNS_GABA receptor agonist_male low dose day 28	0,51	Mis-match

**Fig. 5.** Ranking of patterns matching the metabolite changes induced by vancomycin treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

See legend for Fig. 2.

have been seen in dogs treated intramuscularly (Nelson et al., 1951), there are no reports of liver enzyme induction or toxicity. Treatment of rats with either mannitol or streptomycin sulfate, while resulting in modest in vivo effects (Table 2), produced metabolite profiles that lacked matches to established MoA patterns (Supporting Figs. 7 and 8, respectively).

As noted in Section 2.4, and as can be seen in the pattern ranking displays, several specific patterns may be associated with a general mechanism of toxicity, such as cholestasis or paracetamol-type toxicity. Hence, for each compound treatment, the numbers of such common patterns can be summed. For example, the metabolite profile induced by high-dose phenytoin treatment matched the MoA profiles of “Liver\_toxicity\_female high dose” and “Liver\_toxicity\_male high dose” (data taken from reference (Kamp et al., 2012)). Based on the type of pathology associated with these MoAs, both of these MoA patterns can be considered “Liver, cell damage” and were counted as two matches to this common MoA. Such an analysis of common patterns of liver toxicity is presented in Table 3 and Fig. 8. Of course, any given general mechanism of toxicity may have more patterns associated with it than another, so that a greater count of pattern matches would not necessarily equate to a greater degree of hepatotoxic potential. Nevertheless, the results do indicate that with the exception of methotrexate, those compounds with clear literature evidence for human DILI do produce metabolic profiles that contain matches for metabolite patterns associated with hepatotoxicity. By contrast, of the six compounds lacking clear literature evidence for human DILI, only atropine produced metabolic profiles that contained matches for one metabolite profile associated with hepatotoxicity. As noted

earlier, it is conceivable that the CNS effects of this drug may overshadow any conventional measures of liver toxicity.

#### 4. Discussion

Exposure of any cell or organism to a toxicant leads to a cascade of events that may or may not lead to overt toxicity as evinced by organ failure or death. As Gregus has noted (Gregus, 2008), several steps must take place for an adverse outcome (Fig. 1). The molecule must first be absorbed into the system, after which it may be bio-transformed into a molecule with either greater or lesser toxicity. The parent or transformed molecule may then be excreted, removing it from the biological system, or delivered to a target site. At the target site the parent toxicant or its metabolite may either interact with a target molecule or alter the biological environment to produced cellular dysfunction or injury. The toxicant may also interact with multiple targets to effect toxicity, as is seen for gastrointestinal injury induced by nonsteroidal anti-inflammatory drugs (Boelsterli et al., 2013). At this point, repair or adaptation may take place so as to return the biological system to homeostasis, or such processes may lead to toxicity instead of repair. Importantly, biochemical changes occur at every step in this cascade (Fig. 1).

Previous reports have described a mass spectrometry-based approach to metabolite profiling, wherein all individual metabolites are identified in a given sample (van Ravenzwaay et al., 2007). Coupled with a reference database of metabolite profiles (MetaMap<sup>®</sup>Tox) (van Ravenzwaay et al., 2012), where patterns associated with known mechanisms of toxic and pharmacologic



Rank no.	Pattern Name	Median r	Assessment
1	Nutrition biomarkers of feed exposure_females day 7 and day 14	0,95	Mis-match
2	Hormones_GnRH agonist_female high dose (putative)	0,78	Match
3	Immune System_Immunsuppression_male low dose day 28	0,76	Mis-match
4	Bone marrow_suppression platins_female low dose	0,73	Mis-match
5	Adrenals_hypocortisolism_male high dose day 28	0,70	Mis-match
6	Bone marrow_suppression_male high dose day 28	0,70	Mis-match
7	Kidney_tubular necrosis_female high dose	0,69	Mis-match
8	Nervous System_dopamin agonist_female high dose day 28	0,69	Mis-match
9	Spleen_haematopoiesis_male high dose	0,66	Mis-match
10	Adrenals_cortex_steroid synthase inhibition_males high dose	0,64	Mis-match
11	Reduced feed consumption_male day 7	0,64	Mis-match
12	vehicle oil_females	0,62	Mis-match
13	Bone marrow_suppression_female high dose day 28 (putative)	0,58	Mis-match
14	CNS_GABA receptor agonist_male low dose day 28	0,58	Mis-match
15	CNS_receptor block serotoninine-dopamine_female high dose	0,54	Mis-match
16	Hormones_testosterone_female high dose	0,54	Mis-match
17	Spleen_methemoglobinemia_female high dose	0,53	Mis-match
18	Spleen-methemoglobinemia_female high dose	0,53	Mis-match
19	Adrenals_aromatase inhibitor_male low dose	0,53	Mis-match
20	Bone_osteoblast inhibitor_male high dose	0,52	Mis-match
21	Bone marrow supression platin_male low dose day 28	0,50	Mis-match

**Fig. 6.** Ranking of patterns matching the metabolite changes induced by lamivudine treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

See legend for Fig. 2.

action (MoAs) have been elucidated, this approach allows for the detection of MoAs following treatment with a novel compound. Exploring the possibility that biochemical changes may indeed represent parameters responsive to events early in the development of toxicity, previous studies examined whether metabolite profiling may indicate signals of toxicity in the absence of signals from conventional parameters (Kamp et al., 2012; Mattes et al., 2013). As a clear example, MoA's indicative of hepatotoxicity were detected in the metabolite profiles of rats treated with doxorubicin at doses that did not elicit signals from conventional parameters of liver injury. The current study extended the question of enhanced signal detection with metabolomics to compounds known to produce toxicity (hepatotoxicity) in humans, but not reported to do so in rats.

Under the conditions of this current study, treatment of rats with compounds clearly associated with human DILI generally resulted in plasma metabolite profiles that, when queried against the MetaMap<sup>®</sup>Tox database, matched metabolite patterns associated with mechanisms of hepatotoxicity. By contrast, treatment of rats with compounds not associated with human DILI generally resulted in plasma metabolite profiles that, when queried against the MetaMap<sup>®</sup>Tox database, did not match metabolite patterns associated with mechanisms of hepatotoxicity. In this study, 8 of 9 DILI-positive compounds were correctly identified; 5 of 6 DILI-negative compounds were correctly identified as such, giving an overall sensitivity and specificity of 89% and 83%, respectively (albeit the total number of compounds examined is rather small).

That hepatotoxicity is a multi-step process, involving pathways of both damage and repair, is well accepted. Indeed, the stochastic

nature of disease and/or toxicity (Coggon and Martyn, 2005) suggests that any given treatment or stimulus is unlikely to always result in the terminal response culminating a multistep process. What is known is that not all drugs have the potential for eliciting human DILI even in large populations. The results presented in this paper suggest that treatment of rats with compounds that do have the potential for human DILI creates an initial event with a clear metabolic response consistent with a multi-step process (Fig. 1). The subsequent steps that would lead to DILI in a clinical situation are either lacking in the rat model or abrogated by repair pathways in the healthy rat that allow it to recover and return to homeostasis from the initial insult (Fig. 1) without showing clinical or histopathology signals. Nonetheless, the essential characteristic of a compound with DILI-potential, i.e., the initiating event, is present at some level in the preclinical studies we evaluated and are detectable by changes in the overall metabolome in blood.

The exact molecular pathway connecting any observed DILI pathology with an initiating event has yet to be determined. One can only speculate as to how such a pathway may differ between humans and rats in the case where the drug-induced pathology is seen in humans but not in rats. A clear difference between the human situation as compared to the rat model is that polymorphisms in e.g., drug metabolism occur with much higher impact in humans as compared to the commercially bred rat strain used in this study. It would seem equally speculative to compare drug-induced pathologies seen in the clinic for the compounds described here with the MoAs matching the metabolite profile elicited by those compounds in treated rats, which themselves are not displaying those pathologies. Nonetheless, it is intriguing to

Rank no.	Pattern Name	Median r	Assessment
1	Adrenals_steroid synthesis Inhibition_female high dose	0,91	Mis-match
2	Kidney_tubular necrosis_female high dose	0,85	Match
3	Phthalates_short-chain_male high dose (putative)	0,85	Mis-match
4	Bone marrow_suppression_male high dose day 28	0,85	Match
5	Duodenum_iron-deficiency_female high dose	0,82	Mis-match
6	Liver_enzyme induction_female high dose	0,82	Weak Match
7	Hormones_antandrogen_male high dose	0,80	Mis-match
8	Liver_toxicity_female high dose	0,80	Weak Match
9	Nervous system_serotonin receptor antagonist_female high dose	0,79	Mis-match
10	Liver_enzyme induction_female high dose day 14	0,77	Equivocal
11	Liver_cholestasis_female low and high dose (putative)	0,77	Equivocal
12	Liver_cholestasis_female high dose (putative)	0,77	Equivocal
13	Liver_paracetamol-derived_female high dose	0,76	Equivocal
14	Liver_Tox_male high dose	0,76	Mis-match
15	Blood_porphyrin inhibition_male high dose	0,75	Mis-match
16	Bone marrow_suppression_female high dose day 28 (putative)	0,74	Mis-match
17	Thyroid_indirect, liver enzyme induction_female high dose	0,73	Mis-match
18	Liver_paracetamol-derived_male high dose	0,73	Mis-match
19	Blood_anemia_female high dose	0,72	Equivocal
20	Hormones_antandrogen receptor antagonist prostate_male high dose	0,70	Mis-match

**Fig. 7.** Ranking of patterns matching the metabolite changes induced by atropine treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

See legend for Fig. 2.

note that both phenytoin and flutamide treatment, clinically known for mixed hepatocellular and cholestatic injury in humans (Zimmerman, 1999), produced metabolite profiles that matched liver MoA patterns corresponding to “cell damage” “cholestasis”, and “paracetamol-like toxicity.” Zidovudine treatment, clinically known for hepatocellular damage (Zimmerman, 1999), produced a metabolite profile that matched “paracetamol-like toxicity.” On the other hand, propylthiouracil treatment, clinically known for hepatocellular damage (Zimmerman, 1999), matched MoAs of “peroxisome proliferation” and “oxidative stress”; captopril treatment, clinically known for mixed hepatocellular–cholestatic

damage, matched the MoA of “oxidative stress”; and valproic acid treatment, clinically known for hepatocellular damage (Zimmerman, 1999), matched the MoA of “peroxisome proliferation.” Again, the metabolite profile may only hint at the molecular pathway and resulting pathology.

In conclusion, these results show that the MetaMap<sup>®</sup>Tox database and associated mechanistic metabolite patterns can detect signals of hepatotoxicity in rats treated with compounds under conditions where signals of hepatotoxicity are not seen with conventional parameters. These compounds include those that have been reported to elicit overt hepatotoxicity in humans, but

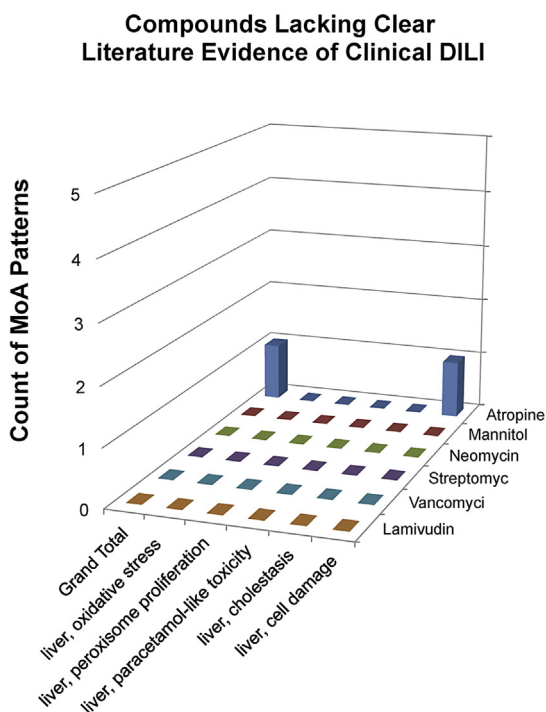
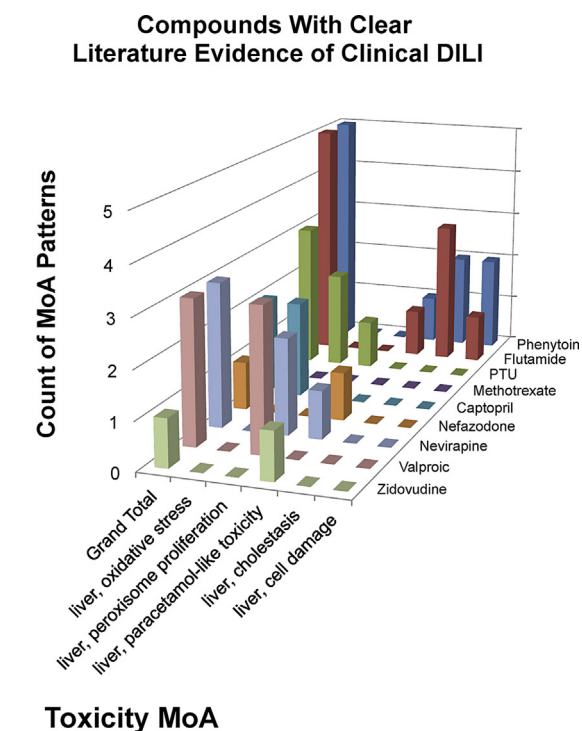
**Table 3**

Count of patterns mapping to a given toxicity MoA for the test compounds.

Toxicity MoA	Clear literature evidence of clinical DILI								
	Phenytoin	Flutamide	PTU	Methotrexate	Captopril	Nefazodone	Nevirapine	Valproic	Zidovudine
Liver, cell damage	2	1	0	0	0	0	0	0	0
Liver, cholestasis	2	3	0	0	0	0	0	0	0
Liver, paracetamol-like toxicity	1	1	0	0	0	1	1	0	1
Liver, peroxisome proliferation	0	0	1	0	0	0	2	3	0
Liver, oxidative stress	0	0	2	0	2	0	0	0	0
Grand total	5	5	3	0	2	1	3	3	1

Toxicity MoA	No clear literature evidence of clinical DILI					
	Atropine	Mannitol	Neomycin	Streptomycin	Vancomycin	Lamivudine
Liver, cell damage	1	0	0	0	0	0
Liver, cholestasis	0	0	0	0	0	0
Liver, paracetamol-like toxicity	0	0	0	0	0	0
Liver, peroxisome proliferation	0	0	0	0	0	0
Liver, oxidative stress	0	0	0	0	0	0
Grand total	1	0	0	0	0	0



**Fig. 8.** Liver-related toxicity MoA pattern matches per compound. Summary counts of common patterns of liver toxicity for each compound treatment. High dose treatments were evaluated, except for those of captopril, where the low dose treatment was evaluated. Abbreviations: PTU: propylthiouracil. Data taken from Table 3.

not in rats. The specific molecular pathways resulting in DILI have yet to be determined, but the observation that compounds with DILI potential produce a metabolome response with common characteristics holds promise for their discovery.

## Conflict of interest

The authors declare that there are no conflicts of interest.

## Transparency document

The Transparency document associated with this article can be found in the online version.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.tox-let.2014.07.021>.

## References

- Andrade, R.J., Lucena, M.I., Kaplowitz, N., Garcia-Munoz, B., Borraz, Y., Pachkoria, K., Garcia-Cortes, M., Fernandez, M.C., Pelaez, G., Rodrigo, L., Duran, J.A., Costa, J., Planas, R., Barriocanal, A., Guarner, C., Romero-Gomez, M., Munoz-Yague, T., Salmeron, J., Hidalgo, R., 2006. Outcome of acute idiosyncratic drug-induced liver injury: long-term follow-up in a hepatotoxicity registry. *Hepatology* 44, 1581–1588.
- Antoine, D.J., Mercer, A.E., Williams, D.P., Park, B.K., 2009. Mechanism-based bioanalysis and biomarkers for hepatic chemical stress. *Xenobiotica* 39, 565–577.
- Baldrick, P., 2008. Safety evaluation to support first-in-man investigations II: toxicology studies. *Regul. Toxicol. Pharmacol.* 51, 237–243.
- Beger, R.D., Sun, J., Schnackenberg, L.K., 2010. Metabolomics approaches for discovering biomarkers of drug-induced hepatotoxicity and nephrotoxicity. *Toxicol. Appl. Pharmacol.* 243, 154–166.
- Boelsterli, U.A., Redinbo, M.R., Saitta, K.S., 2013. Multiple NSAID-induced hits injure the small intestine: underlying mechanisms and novel strategies. *Toxicol. Sci.* 131, 654–667.
- Boudonck, K.J., Mitchell, M.W., Nemet, L., Keresztes, L., Nyska, A., Shinar, D., Rosenstock, M., 2009. Discovery of metabolomics biomarkers for early detection of nephrotoxicity. *Toxicol. Pathol.* 37, 280–292.
- Campos, A., Vasconcelos, V., 2010. Molecular mechanisms of microcystin toxicity in animal cells. *Int. J. Mol. Sci.* 11, 268–287.
- Chen, M., Vijay, V., Shi, Q., Liu, Z., Fang, H., Tong, W., 2011. FDA-approved drug labeling for the study of drug-induced liver injury. *Drug Discov. Today* 16, 697–703.
- Chen, S.C., Barker, S.M., Mitchell, D.H., Stevens, S.M., O'Neill, P., Cunningham, A.L., 1992. Concurrent zidovudine-induced myopathy and hepatotoxicity in patients treated for human immunodeficiency virus (HIV) infection. *Pathology* 24, 109–111.
- Choi, S., Lee, A.K., 2011. Efficacy and safety of gonadotropin-releasing hormone agonists used in the treatment of prostate cancer. *Drug Healthc. Patient Saf.* 3, 107–119.
- Chu, K.M., Boule, A.M., Ford, N., Goemaere, E., Asselman, V., van Cutsem, G., 2010. Nevirapine-associated early hepatotoxicity: incidence, risk factors, and associated mortality in a primary care ART programme in South Africa. *PLoS One* 5, e9183.
- Clarke, C.J., Haselden, J.N., 2008. Metabolic profiling as a tool for understanding mechanisms of toxicity. *Toxicol. Pathol.* 36, 140–147.
- Coggon, D.I., Martyn, C.N., 2005. Time and chance: the stochastic nature of disease causation. *Lancet* 365, 1434–1437.
- Corsini, A., Ganey, P., Ju, C., Kaplowitz, N., Pessayre, D., Roth, R., Watkins, P.B., Albassam, M., Liu, B., Stancic, S., Suter, L., Bortolini, M., 2012. Current challenges and controversies in drug-induced liver injury. *Drug Saf.* 35, 1099–1117.
- Crissman, J.W., Goodman, D.G., Hildebrandt, P.K., Maronpot, R.R., Prater, D.A., Riley, J. H., Seaman, W.J., Thake, D.C., 2004. Best practices guideline: toxicologic histopathology. *Toxicol. Pathol.* 32, 126–131.
- D'Andrea, G., Brisdelli, F., Bozzi, A., 2008. AZT: an old drug with new perspectives. *Curr. Clin. Pharmacol.* 3, 20–37.
- Deepa, P.R., Varalakshmi, P., 2003. Protective effect of low molecular weight heparin on oxidative injury and cellular abnormalities in adriamycin-induced cardiac and hepatic toxicity. *Chem. Biol. Interact.* 146, 201–210.
- Edwards, I.R., 2003. Withdrawing drugs: nefazodone, the start of the latest saga. *Lancet* 361, 1240.
- El-Sayyad, H.I., Ismail, M.F., Shalaby, F.M., Abou-El-Magd, R.F., Gaur, R.L., Fernando, A., Raj, M.H., Ouhit, A., 2009. Histopathological effects of cisplatin, doxorubicin and 5-fluorouracil (5-FU) on the liver of male albino rats. *Int. J. Biol. Sci.* 5, 466–473.
- Ennulat, D., Magid-Slav, M., Rehm, S., Tatsuoaka, K.S., 2010. Diagnostic performance of traditional hepatobiliary biomarkers of drug-induced liver injury in the rat. *Toxicol. Sci.* 116, 397–412.
- Evered, M.D., Robinson, M.M., 1984. Increased or decreased thirst caused by inhibition of angiotensin-converting enzyme in the rat. *J. Physiol.* 348, 573–588.

- Faustman, E.M., Omenn, G., 2008. Risk assessment. In: Klaassen, C.D. (Ed.), Casarett and Doull's Toxicology. New York, McGraw-Hill, pp. 107–128.
- Gregus, Z., 2008. Mechanisms of toxicity. In: Klaassen, C.D. (Ed.), Casarett and Doull's Toxicology: The Basic Science of Poisons. McGraw Hill, New York, pp. 45–106.
- Hoitsma, A.J., Wetzels, J.F., Koene, R.A., 1991. Drug-induced nephrotoxicity. Aetiology clinical features and management. *Drug Saf.* 6, 131–147.
- Hoofnagle, J.H., Serrano, J., Knoben, J.E., Navarro, V.J., 2013. LiverTox: a website on drug-induced liver injury. *Hepatology* 57, 873–874.
- Horner, S., Ryan, D., Robinson, S., Callander, R., Stamp, K., Roberts, R.A., 2013. Target organ toxicities in studies conducted to support first time in man dosing: an analysis across species and therapy areas. *Regul. Toxicol. Pharmacol.* 65, 334–343.
- ICH Steering Committee, 2009. Guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals. vol. M3. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use 31.
- Jaeschke, H., Gores, G.J., Cederbaum, A.I., Hinson, J.A., Pessayre, D., Lemasters, J.J., 2002. Mechanisms of hepatotoxicity. *Toxicol. Sci.* 65, 166–176.
- Jaeschke, H., McGill, M.R., Ramachandran, A., 2012. Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: lessons learned from acetaminophen hepatotoxicity. *Drug Metab. Rev.* 44, 88–106.
- Kamp, H., Fabian, E., Groeters, S., Herold, M., Krennrich, G., Looser, R., Mattes, W., Mellert, W., Prokoudine, A., Ruiz-Noppinger, P., Strauss, V., Walk, T., Wiemer, J., van Ravenzwaay, B., 2012. Application of in vivo metabolomics to preclinical/toxicological studies: case study on phenytoin-induced systemic toxicity. *Bioanalysis* 4, 2291–2301.
- Ma, R.C., Kong, A.P., Chan, N., Tong, P.C., Chan, J.C., 2007. Drug-induced endocrine and metabolic disorders. *Drug Saf.* 30, 215–245.
- Masur, H., Whelton, P.K., Whelton, A., 1976. Neomycin toxicity revisited. *Arch. Surg.* 111, 822–825.
- Matheson, G.K., Knowles, A., Guthrie, D., Gage, D., Weinzapfel, D., Blackbourne, J., 1997. Actions of serotonergic agents on hypothalamic–pituitary–adrenal axis activity in the rat. *Gen. Pharmacol.* 29, 823–828.
- Mattes, W.B., Kamp, H.G., Fabian, E., Herold, M., Krennrich, G., Looser, R., Mellert, W., Prokoudine, A., Strauss, V., van Ravenzwaay, B., Walk, T., Naraoka, H., Omura, K., Schuppe-Koistinen, I., Nadanaciva, S., Bush, E.D., Moeller, N., Ruiz-Noppinger, P., Piccoli, S.P., 2013. Prediction of clinically relevant safety signals of nephrotoxicity through plasma metabolite profiling. *BioMed. Res. Int.* 2013, 12.
- Morrell, M.J., 2003. Reproductive and metabolic disorders in women with epilepsy. *Epilepsia* 44 (Suppl. 4), 11–20.
- Nelson, A.A., Radowski, J.L., Hagan, E.C., 1951. Renal and other lesions in dogs and rats from intramuscular injection of neomycin. *Fed. Proc.* 10, 366–367.
- Omar, R.F., Gourde, P., Desormeaux, A., Tremblay, M., Beauchamp, D., Bergeron, M.G., 1996. In vivo toxicity of foscarnet and zidovudine given alone or in combination. *Toxicol. Appl. Pharmacol.* 139, 324–332.
- Peters, T.S., 2005. Do preclinical testing strategies help predict human hepatotoxic potentials? *Toxicol. Pathol.* 33, 146–154.
- Pillans, P.I., Koopowitz, A., 1991. Captopril-associated agranulocytosis. A report of 3 cases. *S Afr. Med. J.* 79, 399–400.
- Ragni, M.V., Miller, B.J., Whalen, R., Ptachcinski, R., 1992. Bleeding tendency, platelet function, and pharmacokinetics of ibuprofen and zidovudine in HIV(+) hemophilic men. *Am. J. Hematol.* 40, 176–182.
- Rouquie, D., Friry-Santini, C., Schorsch, F., Tinwell, H., Bars, R., 2009. Standard and molecular NOAELs for rat testicular toxicity induced by flutamide. *Toxicol. Sci.* 109, 59–65.
- Russmann, S., Kullak-Ublick, G.A., Grattagliano, I., 2009. Current concepts of mechanisms in drug-induced hepatotoxicity. *Curr. Med. Chem.* 16, 3041–3053.
- Russo, M.W., Galanko, J.A., Shrestha, R., Fried, M.W., Watkins, P., 2004. Liver transplantation for acute liver failure from drug induced liver injury in the United States. *Liver Transpl.* 10, 1018–1023.
- Senior, J.R., 2009. Monitoring for hepatotoxicity: what is the predictive value of liver function tests? *Clin. Pharmacol. Ther.* 85, 331–334.
- Smythe, M.A., Umstead, G.S., 1989. Phenytoin hepatotoxicity: a review of the literature. *DICP* 23, 13–18.
- Stewart, D.E., 2002. Hepatic adverse reactions associated with nefazodone. *Can. J. Psychiatry* 47, 375–377.
- Tortorella, C., Guidolin, D., Petrelli, L., De Toni, R., Milanese, O., Ruga, E., Rebuffat, P., Bova, S., 2009. Prolonged zidovudine administration induces a moderate increase in the growth and steroidogenic capacity of the rat adrenal cortex. *Int. J. Mol. Med.* 23, 799–804.
- Travlos, G.S., Morris, R.W., Elwell, M.R., Duke, A., Rosenblum, S., Thompson, M.B., 1996. Frequency and relationships of clinical chemistry and liver and kidney histopathology findings in 13-week toxicity studies in rats. *Toxicology* 107, 17–29.
- Tsekos, G., Chrysos, G., Douskas, G., Paraskeva, D., Mangafas, N., Giannakopoulos, D., Papanikolaou, M., Georgiou, E., Lazanas, M.C., 2002. Body composition changes in protease inhibitor-naïve HIV-infected patients treated with two nucleoside reverse transcriptase inhibitors. *HIV Med.* 3, 85–90.
- Vaidya, V.S., Ozer, J.S., Dieterle, F., Collings, F.B., Ramirez, V., Troth, S., Muniappa, N., Thudium, D., Gerhold, D., Holder, D.J., Bobadilla, N.A., Marrer, E., Perentes, E., Cordier, A., Vonderscher, J., Maurer, G., Goering, P.L., Sistare, F.D., Bonventre, J.V., 2010. Kidney injury molecule-1 outperforms traditional biomarkers of kidney injury in preclinical biomarker qualification studies. *Nat. Biotechnol.* 28, 478–485.
- van Ravenzwaay, B., Coelho-Palermo Cunha, G., Fabian, E., Herold, M., Kamp, H., Krennrich, G., Krotzky, A., Leibold, E., Looser, R., Mellert, W., Prokoudine, A., Strauss, V., Tretheway, R., Walk, T., Wiemer, J., 2010. The use of metabolomics in cancer research. In: Cho, W.C.S. (Ed.), *An Omics Perspective of Cancer*. Springer science + media B. V. Dordrecht, pp. 141–166.
- van Ravenzwaay, B., Cunha, G.C., Leibold, E., Looser, R., Mellert, W., Prokoudine, A., Walk, T., Wiemer, J., 2007. The use of metabolomics for the discovery of new biomarkers of effect. *Toxicol. Lett.* 172, 21–28.
- van Ravenzwaay, B., Herold, M., Kamp, H., Kapp, M.D., Fabian, E., Looser, R., Krennrich, G., Mellert, W., Prokoudine, A., Strauss, V., Walk, T., Wiemer, J., 2012. Metabolomics: a tool for early detection of toxicological effects and an opportunity for biology based grouping of chemicals—from QSAR to QBAR. *Mutat. Res.* 746, 144–150.
- Wang, J., Reijmers, T., Chen, L., van der Heijden, R., Wang, M., Peng, S., Hankemeier, T., Xu, G., van der Greef, J., 2009. Systems toxicology study of doxorubicin on rats using ultra performance liquid chromatography coupled with mass spectrometry based metabolomics. *Metabolomics* 5, 407–418.
- Weingand, K., Brown, G., Hall, R., Davies, D., Gossett, K., Neptun, D., Waner, T., Matsuzawa, T., Salemin, P., Froelke, W., Provost, J.P., Dal Negro, G., Batchelor, J., Nomura, M., Groetsch, H., Boink, A., Kimball, J., Woodman, D., York, M., Fabianson-Johnson, E., Lupart, M., Melloni, E., 1996. Harmonization of animal clinical pathology testing in toxicity and safety studies. The Joint Scientific Committee for International Harmonization of Clinical Pathology Testing. *Fundam. Appl. Toxicol.* 29, 198–201.
- Widemann, B.C., Adamson, P.C., 2006. Understanding and managing methotrexate nephrotoxicity. *Oncologist* 11, 694–703.
- Wysowski, D.K., Fourcroy, J.L., 1996. Flutamide hepatotoxicity. *J. Urol.* 155, 209–212.
- Yagmurca, M., Bas, O., Mollaoglu, H., Sahin, O., Nacar, A., Karaman, O., Songur, A., 2007. Protective effects of erdosteine on doxorubicin-induced hepatotoxicity in rats. *Arch. Med. Res.* 38, 380–385.
- Zimmerman, H.J., 1999. *Hepatotoxicity: The Adverse Effects of Drugs and Other Chemicals on the Liver*. Lippincott Williams & Wilkins, Philadelphia, pp. 789.