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Proteomic profiling in incubation medium of mouse, rat and human precision-cut liver slices for biomarker detection regarding acute drug-induced liver injury

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ABSTRACT: Drug-induced liver injury is one of the leading causes of drug withdrawal from the market. In this study, we investigated the applicability of protein profiling of the incubation medium of human, mouse and rat precision-cut liver slices (PCLS) exposed to liver injury-inducing drugs for biomarker identification, using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. PCLS were incubated with acetaminophen (APAP), 3-acetamidophenol, diclofenac and lipopolysaccharide for 24–48 h. PCLS medium from all species treated with APAP demonstrated similar changes in protein profiles, as previously found in mouse urine after APAP-induced liver injury, including the same key proteins: superoxide dismutase 1, carbonic anhydrase 3 and calmodulin. Further analysis showed that the concentration of hepcidin, a hepatic iron-regulating hormone peptide, was reduced in PCLS medium after APAP treatment, resembling the decreased mouse plasma concentrations of hepcidin observed after APAP treatment. Interestingly, comparable results were obtained after 3-acetamidophenol incubation in rat and human, but not mouse PCLS. Incubation with diclofenac, but not with lipopolysaccharide, resulted in the same toxicity parameters as observed for APAP, albeit to a lesser extent. In conclusion, proteomics can be applied to identify potential translational biomarkers using the PCLS system. Copyright © 2013 John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

Keywords: Precision-cut liver slices; drug-induced liver injury; hepcidin; proteomics profiling; acetaminophen; diclofenac

Introduction

The most common adverse drug reaction leading to drug withdrawal is drug-induced liver injury (DILI) (Stirnimann *et al*., 2010). The incidence for DILI has been estimated to be at least 10–15 cases per 100 000 patient years (Tujios & Fontana, 2011). Despite the efforts to study DILI, for most drugs, the underlying mechanisms have not been fully elucidated yet. In addition, particular drugs can cause a rare and severe form of DILI, without a straightforward dose–effect relationship or a relation with their therapeutic mode of action. These adverse reactions are therefore described as idiosyncratic. Currently, there are no adequate biomarkers to detect idiosyncratic DILI in patients, in preclinical animal studies or in *in vitro* models during drug development (Stine & Lewis, 2011). Because of this, identification of novel biomarkers for DILI is difficult and new methods to address this issue are being explored (Fredriksson *et al*., 2011; Hadi *et al*., 2012). Although for non-idiosyncratic DILI more knowledge on potential mechanisms is available compared to idiosyncratic DILI, the prediction from preclinical data is limited and better preclinical prediction models with their accompanying biomarkers are needed.

Precision-cut liver slices (PCLS) of mouse, rat and human liver are being increasingly used to study the hepatotoxic effects of many compounds (Elferink *et al*., 2011, 2008; Hadi *et al*., 2012, 2013; Vickers & Fisher, 2005). The advantage of this *ex vivo* model over *in vitro* models, including cell cultures, is that the structure of the liver tissue is maintained in PCLS as well as the presence and interactions of all parenchymal and non-parenchymal cell types, including cell–matrix interaction (Lerche-Langrand & Toutain, 2000; van de Bovenkamp *et al*., 2006). Moreover, PCLS retain the expression and activity of phase I and II metabolizing enzymes well, which is comparable to the *in vivo* situation (De Graaf *et al*., 2002; Elferink *et al*., 2011; Ferrero & Brendel, 1997; van Midwoud *et al*., 2011; Vickers & Fisher, 2004). Furthermore, it has been shown that rat PCLS demonstrate the same responses to toxic compounds as observed *in vivo* based on microarray data (Elferink *et al*., 2008).

Recently, we have used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to assess

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and identify potential novel protein biomarkers for DILI (van Swelm *et al.*, 2012b). For this, mice were administered a single dose of acetaminophen (APAP) as a model compound for hepatocellular liver injury and 24 h urine samples were collected. From the urine samples, multiple proteins could be identified that were related to APAP-induced liver injury. In the present study, we investigated whether the PCLS system could be used to identify proteomic biomarkers for DILI. We compared APAP-induced changes in protein profile in the medium of mouse PCLS with toxicity-related protein profiles previously observed *in vivo*. In addition to APAP, we also investigated the protein profiles in the PCLS medium after exposure to 3-acetamidophenol (AMAP), diclofenac (DF) and lipopolysaccharide (LPS), and investigated potential species differences. AMAP was shown previously to induce species-specific toxicity in PCLS of human and rat, but not of mouse (Hadi *et al.*, 2013). DF is a non-steroidal anti-inflammatory drug that has been known to cause idiosyncratic DILI (Boelsterli, 2003) and to induce acute hepatotoxicity similar to APAP (Yano *et al.*, 2012), whereas LPS is known to induce inflammatory reactions in the liver (Callery *et al.*, 1992).

Methods

Animals for Precision-cut Liver Slice Studies

Female C57BL/6 mice weighing 20–24 g and male Wistar rats (HsdCpb:WU) weighing 300–350 g were obtained from Harlan (Horst, the Netherlands). The mice and rats were housed on a 12-h light/dark cycle in a temperature- and humidity-controlled room with food (Harlan chow no 2018) and tap water *ad libitum*. The animals were allowed to acclimatize for at least 7 days before experimentation. The experimental protocols were approved by the Animal Ethical Committee of the University of Groningen. Under isofluorane/O_2 anesthesia, the liver was excised and placed into ice-cold University of Wisconsin organ preservation solution (DuPont Critical Care, Waukegab, IL, USA).

Human Liver Tissue

Pieces of human liver tissue were obtained from patients undergoing partial hepatectomy for the removal of carcinoma or from liver tissue remaining as surgical waste after split liver transplantation, as described previously (Elferink *et al.*, 2011; van de Bovenkamp *et al.*, 2006). The experimental protocols were approved by the Medical Ethical Committee of the University Medical Center Groningen.

Preparation of the Precision-cut Liver Slices

PCLS were made as described previously (de Graaf *et al.*, 2010; Hadi *et al.*, 2012, 2013). In brief, cylindrical liver cores were made using a 5 mm biopsy punch (Kai Industries, Seki, Japan). These cores were sliced with a Krumdieck tissue slicer (Alabama R&D, Munford, AL, USA) in ice-cold Krebs–Henseleit buffer saturated with carbogen (95% O_2 and 5% CO_2). PCLS (5 mm diameter, 200–300 μm thick and ≈ 4.5–5.5 mg wet weight) were stored in ice-cold University of Wisconsin solution until incubation.

Incubation of the Precision-cut Liver Slices

Incubation of PCLS in 12-well plates (Greiner bio-one GmbH, Frickenhausen, Austria) was performed as described before (de Graaf *et al.*, 2010). In brief, PCLS were preincubated at 37 °C for 1 h individually in 1.3 ml Williams' medium E with glutamax-1 (Gibco, Paisley, UK), supplemented with 25 mM D-glucose and 50 μg ml^{-1} gentamicin (Gibco) (WEGG medium) in a 12-well plate with shaking (90 times min^{-1}) under saturated carbogen atmosphere. Preincubation allows the PCLS to restore their ATP levels. After preincubation, PCLS were transferred to fresh WEGG medium and incubated with vehicle, APAP, AMAP, DF or LPS for a further 24 h (final concentration of DMSO during incubation ≤ 0.5%). The human PCLS were incubated with APAP or AMAP for 48 h instead of 24 h, as no changes were observed after 24 h in protein profiles compared to control slices. A concentration of the compounds were selected because of their minor to moderate induction of damage, as determined by histology and PCLS viability using the ATP assay. Minor damage was defined as a < 20% decrease in ATP concentration compared to control and moderate damage as a 20–50% decrease compared to control. For APAP and AMAP, dose–response studies were performed previously (Hadi *et al.*, 2013), from which we selected 1 mM APAP and 3 mM AMAP for mouse, 5 mM APAP and AMAP for rat and 3 mM APAP and AMAP for human PCLS. For DF, dose–response studies are represented in Supplementary Fig. S1 and doses of 200 μM for mouse, 350 μM for rat and 500 μM for human PCLS were selected. The dose of 20 000 EU ml^{-1} LPS was based on the study of (Hadi *et al.*, 2012) to induce minimal toxicity, but to elicit an inflammatory response as assessed by the release of cytokines. The medium was collected, snap frozen in liquid nitrogen and stored at – 80 °C until further use.

Adenosine Triphosphate Content of Precision-cut Liver Slices

Viability of PCLS was determined after incubation by measuring the ATP content of the PCLS according to the method described earlier (de Graaf *et al.*, 2010). In brief, at the end of incubation, three replicate PCLS were collected individually in 1 ml 70% ethanol (v/v) containing 2 mM EDTA (pH 10.9) and snap-frozen in liquid nitrogen and stored at – 80 °C until analysis. The samples were homogenized using a Mini-BeadBeater-8 (BioSpec, Bartlesville, OK, USA) and centrifuged for 3 min at 13 000 rpm (16 000 g) and 4 °C. The supernatant was diluted 10 times with 0.1 M Tris–HCl containing 2 mM EDTA (pH 7.8) to reduce the ethanol concentration. The ATP content of the supernatant was measured using the ATP Bioluminescence Assay kit CLS II (Roche, Mannheim, Germany) in a black 96-well plate Lucy1 luminometer (Anthos, Durham, NC, USA) using a standard ATP calibration curve.

The protein content of the PCLS was determined by dissolving the remaining pellet after centrifugation in 200 μl of 5 M NaOH for 30 min. After dilution with water to a concentration of 1 M NaOH, the protein content of the samples was determined using the Bio-Rad DC Protein Assay (Bio-Rad, Munich, Germany) using bovine serum albumin for the calibration curve.

Mouse Urine Samples

For validation of the PCLS model, urine samples obtained from a previously performed mouse study (van Swelm *et al.*, 2012b) were used. In short, male FVB mice were treated with a single i.p. dose of 0–350 mg kg^{-1} APAP and placed in a metabolic cage for 24 h to collect urine. After 24 h, liver tissue was collected and homogenized using a Mikro dismembrator U (Sartorius Stedim, Nieuwegein, the Netherlands).

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Protein Profiling

Proteins were isolated from urine, liver homogenates or PCLS medium using Magnetic Beads based Hydrophobic Interaction Chromatography 8 beads (C8; Bruker Daltonics GmbH, Bremen, Germany) that bind hydrophobic proteins (Fiedler *et al*., 2007). Synthetic hepcidin-24 (Peptide International Inc., Louisville, KY, USA) was used as internal standard (IS) to enable comparison between samples. For protein profiling, MALDI-TOF MS (Microflex LT with software flexControl Version 3.0, Bruker Daltonics) was used. Of the prepared sample, 1 μl was applied to a MSP 96 polished steel MALDI target plate under nitrogen flow, followed by 1 μl of energy absorbing matrix, 5 mg α-Cyano-4-hydroxy-cinnamic acid in 1 ml 50% acetonitrile and 0.5% trifluoroacetic acid. Mass-to-charge (m/z) spectra were generated using MALDI-TOF MS in positive, linear ion mode and 350 laser shots. Initial laser power; 50% for 1–20 kDa and 60% for 10–160 kDa measurements, laser attenuator; offset 25% and range 20%. Pulsed ion extraction was set to 250 ns. Samples were measured in the 1–20 kDa mass range and 10–160 kDa mass range. Calibration was performed using protein calibration standard I for 1–20 kDa measurements and protein calibration standard II (both Bruker Daltonics) for 10–160 kDa measurements. Spectra were analyzed by means of the ClinProTools software (Bruker). Relative peak intensities were calculated by dividing mass peak intensity by the peak intensity of the IS.

Protein Identification

Protein identification was performed at the Nijmegen Proteomics Facility (Nijmegen, the Netherlands) using an electrospray ionization mass spectrometer (ESI LTQ; Thermo Fisher Scientific) with a liquid chromatography column placed in front of the ESI probe. Peptide and protein identifications were extracted from the ESI data by means of the search program Mascot using a *Mus musculus* RefSeq36 database. Finally, the peptides and proteins found by Mascot were validated with the in-house designed script PROTON, as described elsewhere (Wessels *et al*., 2011).

Hepcidin Determination

The concentration of hepcidin in PCLS medium was determined using MALDI-TOF MS analysis as previously described (Tjalsma *et al*., 2011). Briefly, hepcidin peptides in 100 μl PCLS medium were enriched using C8 beads, after which 2 nM synthetic hepcidin-24 was added as IS. The enriched hepcidin peptide fraction was analyzed with MALDI-TOF MS as described above. Spectra were analyzed using ClinProTools software and hepcidin concentration was calculated by means of the IS.

Western Blot

PCLS medium samples were normalized to protein concentration and loaded with Laemmli buffer on a 15% SDS gel. Gels were blotted on nitrocellulose using the iBlot protein transfer system (Invitrogen, Breda, the Netherlands) for carbonic anhydrase 3 (CA3) and superoxide dismutase 1 (SOD1) and traditional blot procedures using the Mini Protein 3 system (Bio-Rad Laboratories) with transfer buffer containing 2 mM $CaCl_2$ for calmodulin (CaM). Antibodies against SOD1 (1 : 2000) and CaM (1 : 1000) were purchased from Abcam (Cambridge, UK) and the antibody against CA3 (1 : 200) was obtained from Santa Cruz (Heidelberg, Germany). The secondary antibody used to detect SOD1 was a goat-antirabbit Alexa 680 antibody (Invitrogen; 1 : 10 000) and for CA3, we used a donkey-antigoat IRDye 800 antibody (Bio-Connect, Huissen, the Netherlands; 1 : 10 000). Signals for CA3 and SOD1 were visualized using an Odyssey scanner (LI-COR, Leusden, the Netherlands). CaM was detected using a goat-anti-rabbit horseradish peroxidase antibody (Abcam; 1 : 3000) in combination with an enhanced chemiluminescent scanner (LAS 3000; Fujifilm, Dusseldorf, Germany).

Statistical Analysis

All experiments were performed in three slices of five (mouse and human) or four (rat) individual livers. Statistics were performed using GraphPad Prism 5.02 (La Jolla, USA), unless indicated otherwise. $P < 0.05$ was considered statistically significant. Data were compared among groups using one-way ANOVA with a Dunnett's *post hoc* multiple comparisons test.

Results

Protein Profiles of Precision-cut Liver Slice Medium Resemble *in vivo* Mouse Urinary Profiles of Acetaminophen-induced Liver injury

The proteins in mouse PCLS medium were profiled after APAP treatment and compared to the mouse urinary protein profile that was previously demonstrated to be associated with APAP-induced liver injury (van Swelm *et al*., 2012b). Figure 1 demonstrates that a similar protein profile was observed in mouse PCLS medium after APAP treatment compared to the urinary protein profile after APAP intoxication. The toxicity profile is characterized by an increased number of protein peaks compared to the control. The protein profiles of homogenized control mouse liver and mouse liver after APAP treatment show that the proteins observed in PCLS medium and urine after APAP toxicity are normally present in liver. Moreover, there is no difference in protein composition of mouse liver after APAP administration compared to control, suggesting that the proteins detected in urine and PCLS medium are not specifically produced in liver upon APAP toxicity. Protein identification with ESI LTQ revealed that the same key proteins that differentiated between control and APAP-induced liver injury in mouse urine samples differed between control and APAP-treated PCLS medium (Table 1).

Similar Toxicity Profiles in Mouse, Rat and Human Precision-cut Liver Slice After Acetaminophen Treatment

To compare toxicity between species, we profiled mouse and rat PCLS medium samples incubated with APAP or AMAP for 24 h and human PCLS medium incubated with APAP or AMAP for 48 h. Treatment with APAP resulted in comparable toxicity profiles in PCLS medium of all species. However, incubation with AMAP did not lead to a protein profile in mouse PCLS medium, whereas in rat and human PCLS medium, AMAP incubation resulted in the same protein profiles as with APAP incubation (Fig. 2A).

One potential marker associated with DILI is the iron-regulating hormone peptide hepcidin, for which it was demonstrated that hepatic and plasma levels were downregulated in mice with APAP-induced liver injury because of oxidative stress (van Swelm *et al*., 2012a). To investigate whether acute APAP-induced hepatotoxicity in PCLS is mediated by a similar mechanism as observed

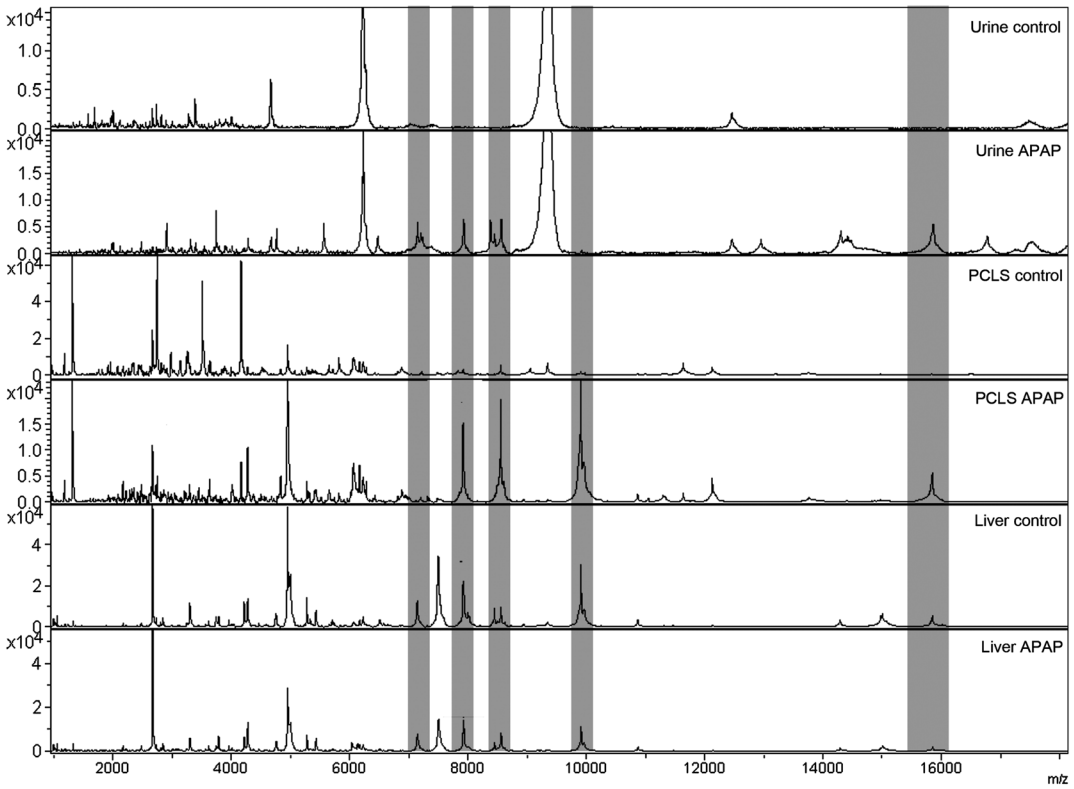

Figure 1. Protein profiles of mouse urine and liver homogenate, and mouse PCLS medium after APAP treatment. Protein profiles of protein masses (m/z in Da) versus peak intensity (arbitrary units) of mouse urine, PCLS medium and liver homogenate of control and APAP treatment. Shaded areas indicate proteins that differ between control and APAP treatment. APAP, acetaminophen; PCLS, precision-cut liver slices.

Table 1. Proteins identified in mouse urine and mouse PCLS medium after APAP administration

Protein	Reference	Protein mass (kDa)	EmPAI (APAP/control)	
			Mouse urine	Mouse PCLS medium
Fatty acid binding protein 1, liver	gi|8393343|ref|NP_059095.1	14.2	APAP only	4.83
Peroxiredoxin 5 precursor	gi|6755114|ref|NP_036151.1	21.9	48.65	APAP only
D-Dopachrome tautomerase	gi|6753618|ref|NP_034157.1	13.1	APAP only	APAP only
Carbonic anhydrase III	gi|31982861|ref|NP_031632.2	29.4	APAP only	3.27
Regucalcin	gi|6677739|ref|NP_033086.1	33.4	APAP only	2.08
Parkinson disease protein 7	gi|55741460|ref|NP_065594.2	20.0	APAP only	APAP only
Peptidylprolyl isomerase A	gi|6679439|ref|NP_032933.1	18.0	APAP only	APAP only
Triosephosphate isomerase 1	gi|226958349|ref|NP_033441.2	32.2	APAP only	APAP only
Quinoid dihydropteridine reductase	gi|21312520|ref|NP_077198.1	25.6	APAP only	APAP only
Peptidylprolyl isomerase C	gi|6679441|ref|NP_032934.1	22.8	APAP only	APAP only
Abhydrolase domain containing 14b	gi|171460960|ref|NP_083907.3	22.5	APAP only	APAP only
Hemoglobin, beta adult minor chain	gi|17647499|ref|NP_058652.1	15.9	APAP only	APAP only
Hemoglobin, beta adult major chain	gi|31982300|ref|NP_032246.2	15.7	APAP only	APAP only
Arginase 1	gi|7106255|ref|NP_031508.1	34.8	APAP only	5.90
Calmodulin-like 3	gi|13386230|ref|NP_081692.1	16.7	2.33	APAP only
Aldolase B, fructose bisphosphate	gi|21450291|ref|NP_659152.1	39.8	APAP only	APAP only
SEC14-like 2	gi|21362309|ref|NP_653103.1	46.3	APAP only	APAP only
Fumarylacetoacetate hydrolase	gi|240120112|ref|NP_034306.2	46.2	APAP only	APAP only

AMAP, 3-acetamidophenol; APAP, acetaminophen; PCLS, precision-cut liver slices.
EmPAI is a measure for protein abundance.
APAP only indicates that the protein was only present after APAP treatment and not present in control sample, thus no EmPAI ratio could be determined.

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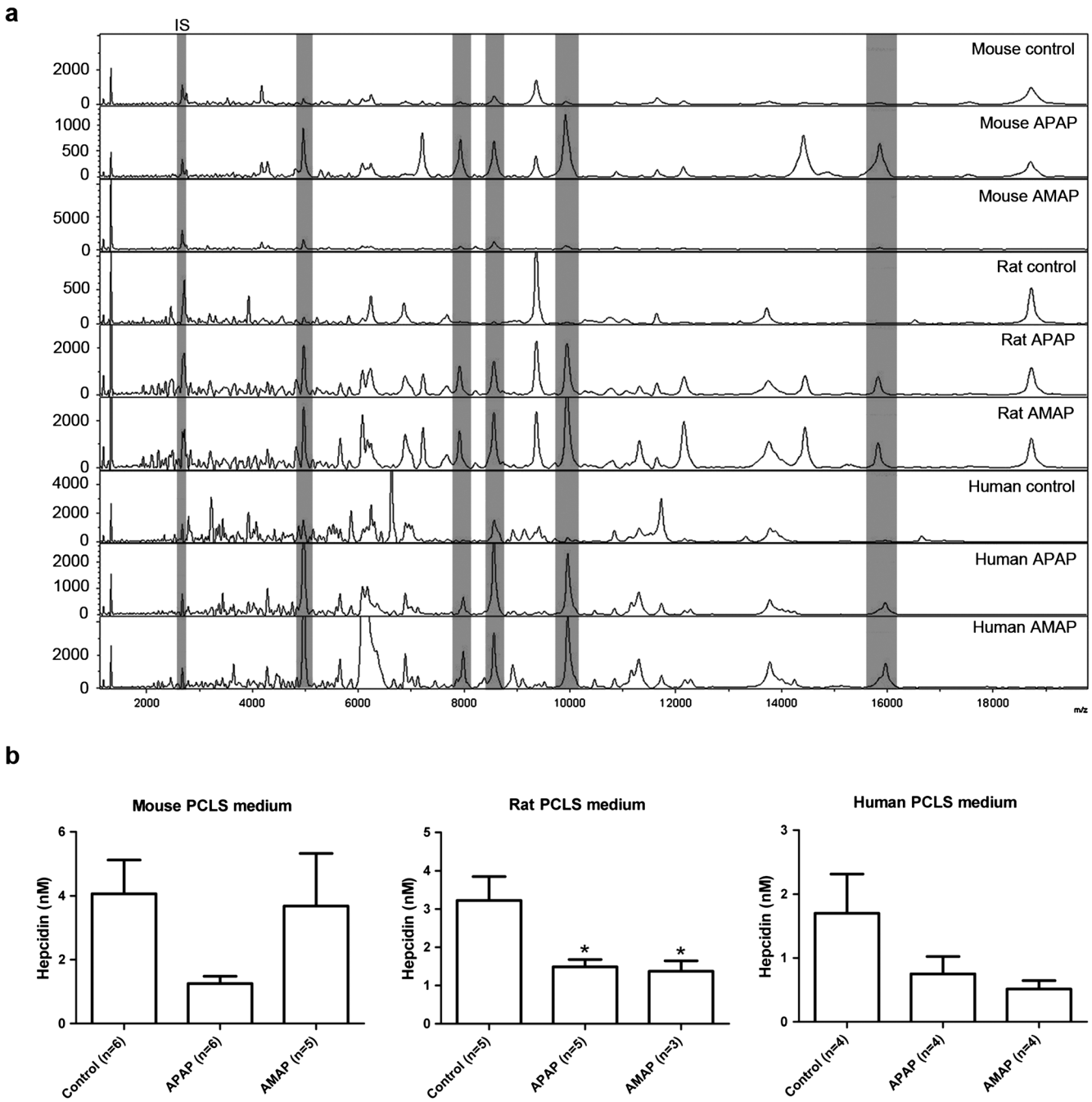

Figure 2. Protein profiles and hepcidin concentration of mouse, rat and human PCLS medium after APAP or AMAP treatment. Protein profiles of protein masses (m/z in Da) versus peak intensity (arbitrary units) of medium from mouse, rat and human PCLS of control, APAP or AMAP treatment (a). Shaded areas indicate proteins that differ between control and APAP/AMAP treatments. Hepcidin concentrations in medium of control PCLS and after APAP or AMAP treatment for all species (b). Every line represents the hepcidin concentrations for a single PCLS in control situation and after APAP and AMAP treatment. *$P < 0.05$ compared to control. AMAP, 3-acetamidophenol; APAP, acetaminophen; PCLS, precision-cut liver slices.

in vivo, we measured the concentration of hepcidin in PCLS medium. LPS-treated PCLS were considered as a positive control in these experiments by showing increased hepatic hepcidin excretion by the PCLS, which is the physiological response to LPS (Tjalsma *et al*., 2011). In concordance with the mouse *in vivo* data, hepcidin concentration was decreased in PCLS medium of all species after APAP treatment (Fig. 2B). AMAP treatment did not affect hepcidin concentration in mouse PCLS medium, but hepcidin concentration was decreased in rat and human PCLS after AMAP treatment. Hepcidin concentrations are known to vary substantially between individual patients (Galesloot *et al*., 2011), which hampers the assessment of statistically significant changes.

Diclofenac Treatment Resembles the Toxicity Profile of Acetaminophen

Incubation of mouse PCLS with DF resulted in protein profiles of PCLS medium that resembled the toxicity profile of APAP, albeit with lower relative peak intensities as compared to the internal standard (Fig. 3). LPS had minimal effects on the protein profiles.

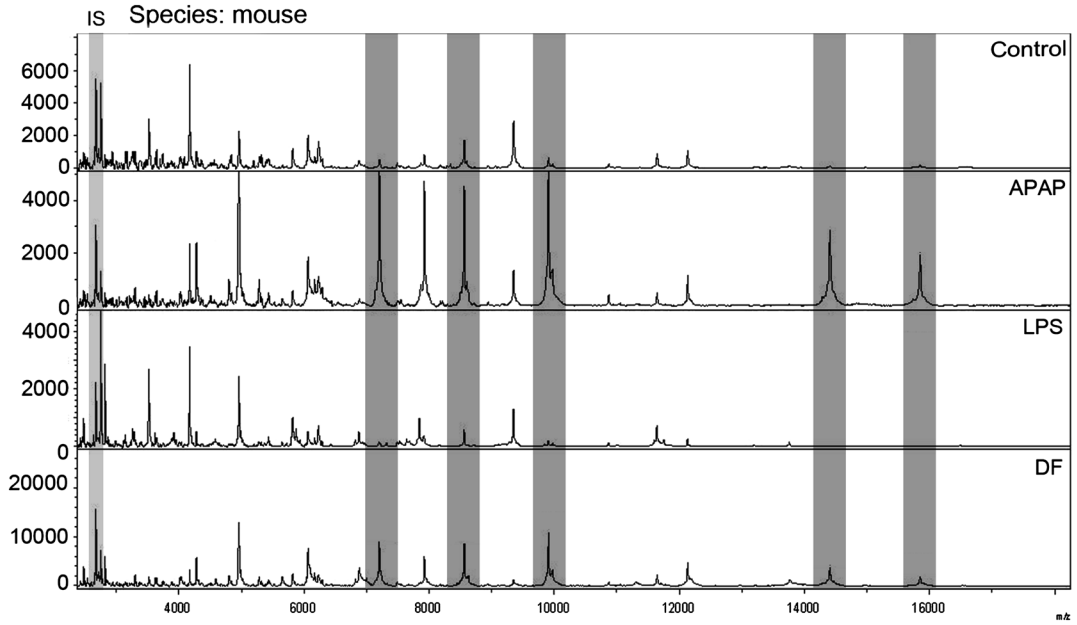

Figure 3. Comparison of PCLS medium protein profiles after incubation of APAP, DF or LPS. Profiles of protein masses (m/z in Da) versus peak intensities (arbitrary units) of medium from mouse PCLS of control and treatment with APAP, LPS and DF. Shaded areas indicate proteins that differ between control and APAP or DF treatment. The mass peak of the internal standard (hepcidin-24) is indicated as IS, which was used to compare relative peak intensities between samples. APAP, acetaminophen; DF, diclofenac; LPS, lipopolysaccharide; PCLS, precision-cut liver slices.

The medium samples of rat and human PCLS treated with DF proved to be difficult to profile using MALDI-TOF MS and because the protein profiles were of insufficient quality, as they showed high background signals and low peak intensities. Therefore, we could not compare the protein profiles of rat and human PCLS medium samples after DF incubation with those of mouse PCLS. Instead, we compared the presence of some key proteins identified after APAP treatment in mouse PCLS medium with medium of all species after all treatments by Western blot. Figure 4 shows that SOD1, CA3 and CaM are increased in mouse PCLS medium after APAP treatment compared to control, which confirmed the protein identification performed by ESI LTQ. Increased signals for these proteins were also observed for APAP and AMAP in rat and human PCLS, except for CA3 in human PCLS medium. In fact, we were not able to detect any CA3 in human PCLS medium. In rat PCLS, AMAP treatment resulted in a higher protein signal on Western blot than APAP treatment, suggesting AMAP to be more toxic than APAP. In concordance with the protein profiles AMAP treatment did not lead to increased protein expression of CA3 and CaM in mouse PCLS, although SOD1 protein expression after AMAP treatment was slightly higher compared to control. Incubation with DF demonstrated the same proteins present in PCLS medium of all species, but to a lesser extent than after APAP and/or AMAP incubation. Surprisingly, LPS incubation did not result in a protein profile that suggested toxicity, although Western blot analysis showed increased concentrations of SOD1 and CA3 in mouse PCLS medium and an increase of CaM in rat PCLS. In addition, hepcidin concentrations in PCLS medium samples were determined (Fig. 5). Similar to the findings in mouse plasma and PCLS medium after APAP-induced hepatotoxicity, incubation with DF resulted in decreased hepcidin concentrations in the medium samples of mouse, rat and human PCLS. The effect of DF was not statistically significant in rat and human PCLS due to the high variation, but DF treatment decreased the hepcidin concentrations in each sample. Incubation with LPS increased

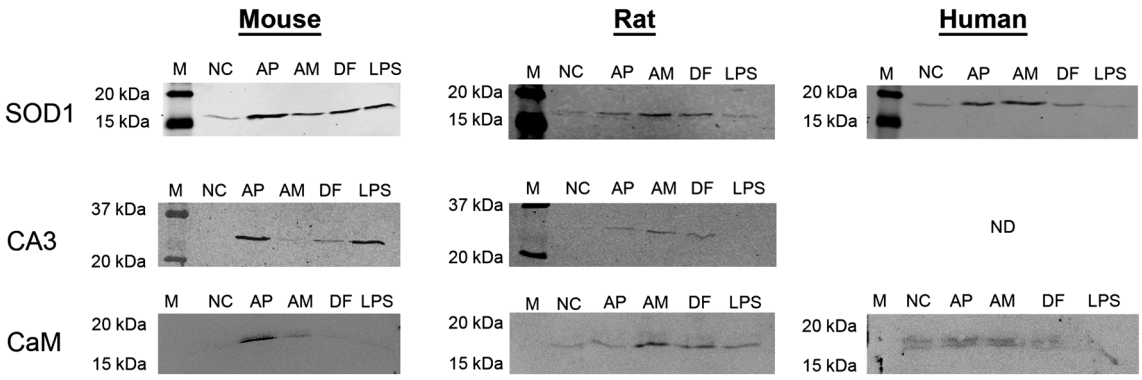

Figure 4. Presence of SOD1, CA3 and CaM in PCLS medium. The presence of SOD1, CA3 and CaM in PCLS medium of all species was demonstrated by Western blotting of NC and after incubation with AP, AM, DF and LPS. CA3 was not detected in human PCLS medium, indicated as ND. AM, 3-acetamidophenol; AP, acetaminophen; DF, diclofenac; LPS, lipopolysaccharide; NC, control medium; PCLS, precision-cut liver slices.

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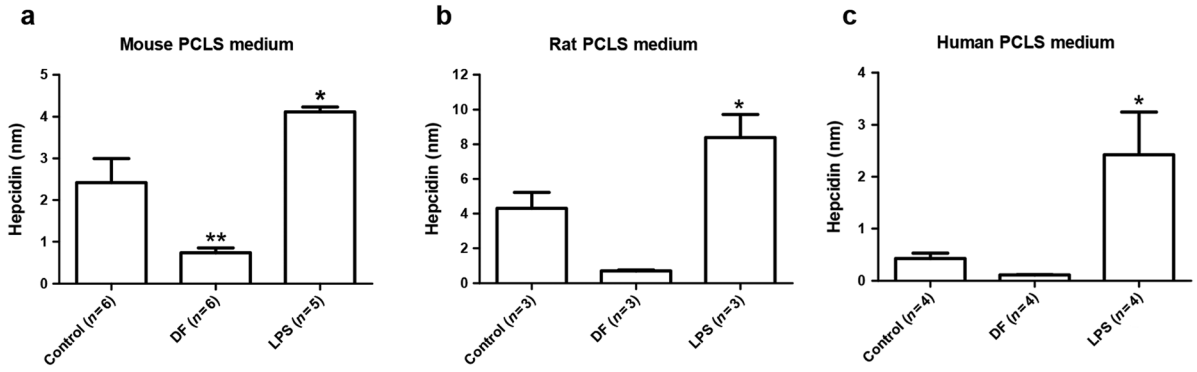

Figure 5. Hepcidin concentration in PCLS medium after DF or LPS treatment. Hepcidin concentration in medium of mouse (a), rat (b) and human (c) PCLS after treatment with DF or LPS. $^{*}P < 0.05$; $^{**}P < 0.01$ compared to control. DF, diclofenac; LPS, lipopolysaccharide; PCLS, precision-cut liver slices.

the hepcidin concentration in all species, which is in agreement with a previously described effect of the endotoxin (Tjalsma *et al*., 2011). These findings confirm that PCLS have maintained their physiological regulation of hepcidin.

Discussion

DILI remains a major problem in drug development, but also is a dangerous complication of drug treatment in patients. Model systems to assess the potential of a drug to cause DILI, and to further investigate the underlying mechanisms, are needed to reduce the attrition rate in drug development and the number of adverse events. We demonstrated that the response of mouse PCLS to APAP treatment is comparable to what we have observed *in vivo*, with respect to the proteins released upon toxicity and the decrease in hepcidin concentration. These results could be further extended to rat and human PCLS, but also to another hepatotoxic drug, DF, underlining the translational aspects of the PCLS system as a model for DILI.

It has to be mentioned that the strain and gender of the mice used for the *in vivo* study are different from those used to generate the mouse PCLS. This was the result of the two procedures being performed by different labs at different moments and later compared to each other. The strain and gender differences might explain that some of the proteins present in mouse urine were not found in mouse PCLS medium and vice versa, but most likely they are due to the presence of extrahepatic proteins in urine. However, the key proteins that distinguished urinary profiles of mice with APAP-induced liver injury from control were present in mouse PCLS medium. Besides similarities in effect on protein composition, the effect of APAP on hepcidin concentration was also comparable between the *in vivo* and PCLS experiments, which demonstrate that these parameters are robust enough to overcome variation introduced by strain and gender differences. This is especially of importance for the proteins that could be developed further as potential biomarkers.

The advantage of the PCLS system is that liver slices of multiple species can be used and compared, enabling investigation of species differences. The proteins associated with APAP-induced liver injury in mouse PCLS and urine could also be found in rat and human PCLS incubated with APAP. The difference in toxicity caused by AMAP between the species, as demonstrated by the protein profiles, Western blot data and hepcidin measurements was in line with the previous observation, with other viability parameters such as ATP and morphology, that AMAP causes species-specific toxicity (Hadi *et al*., 2013). In addition, from the protein profiles and Western blot data, it appeared that the same concentration of AMAP might be more toxic than APAP in rat PCLS, but not in human PCLS. Furthermore, we observed a species difference in response to LPS with Western blot analysis for SOD1 and CA3. Incubation of mouse PCLS with LPS resulted in increased medium concentrations of SOD1 and CA3 compared to control slices, whereas there was little or no effect of LPS on these protein concentrations in rat and human PCLS medium. This difference in response also demonstrates the necessity of an additional technique to confirm proteomics data, because the profiles of LPS treatment in mouse PCLS did not indicate any changes compared to control, whereas the Western blot data did. However, as LPS was used to demonstrate hepcidin regulation in the PCLS, we did not investigate this finding further.

Although CA3 could be detected in mouse and rat PCLS after APAP treatment, we were not able to detect CA3 in APAP-treated human PCLS medium by Western blot analysis, while it was shown previously that CA3 is present in human urine after severe APAP intoxication (van Swelm *et al*., 2012b). An explanation for this observation could be that the concentration of CA3 was below the detection limit of the Western blot technique or that the degree of APAP-induced injury we found in human PCLS was not severe enough for CA3 release.

Because we applied LC-MS/MS for protein identification, we were not able directly to assign a protein identity to the mass peaks that differed from the control profiles. The masses of most proteins that differed between control and APAP treatment were larger than the mass peaks observed in the profiles (Table 1), suggesting that they were present as protein fragments. Based solely on protein mass, the mass peak at 14.2 kDa could correspond to fatty acid binding protein 1, and the mass peak observed just before the 16 kDa mark could correspond to hemoglobin, but also to SOD1, as both proteins have a molecular mass of 15.9 kDa (Table 1). To evaluate the predictive potential of the observed protein profiles and the individual proteins as biomarkers in more detail, additional studies using advanced proteomics techniques, such as ESI-Q-TOF, should pinpoint the proteins to each mass peak. Nevertheless, our study demonstrates that the PCLS system can be used to detect protein profiles related to DILI, using proteomics techniques for biomarker assessment.

The proteins detected in mouse urine and PCLS medium after APAP treatment all play a role in processes known to be involved or affected in acute liver injury, such as oxidative stress, disruption of calcium homeostasis and mitochondrial dysfunction

(Abbas *et al.*, 2008; Abdelmegeed *et al.*, 2010; Delgado-Coello *et al.*, 2006; Handa *et al.*, 2009; Hiyoshi *et al.*, 2009; Raisanen *et al.*, 1999). These processes appear to play important roles in both APAP and DF-induced liver injury (Boelsterli, 2003; Hinson *et al.*, 2010; Jaeschke *et al.*, 2011); however, the exact molecular mechanisms involved have not been elucidated yet. Nevertheless, for some of the proteins identified in this study, potential roles can be postulated based on what is known in the literature. Reduction in SOD1 activity and increased SOD1 nitration have been reported to be involved in APAP-induced liver injury; however, their role in hepatotoxicity remains controversial, as both damaging and protective effects have been described (Abdelmegeed *et al.*, 2010). Fatty acid binding protein 1 may protect the hepatocyte against lipid peroxidation by disposal of fatty acids (Gyamfi *et al.*, 2008a,2008b), but could also be upregulated due to hepatic regeneration (Wang *et al.*, 2004). D-Dopachrome tautomerase is involved in melanin synthesis and may protect against oxidative stress by enhanced melanin production (Hiyoshi *et al.*, 2009). CA3 can act as a scavenger for reactive oxygen species and has been shown to be upregulated in hepatic tissue of rats treated with APAP (Kharbanda *et al.*, 2009; Raisanen *et al.*, 1999; Vullo *et al.*, 2008; Yamamoto *et al.*, 2006), whereas calmodulin has been shown to be involved in necrotic cell death (Ray *et al.*, 1993). Peroxiredoxin could protect against oxidative stress by detoxification of intracellular peroxide and peroxynitrate (Abbas *et al.*, 2008; Graves *et al.*, 2009). Finally, regucalcin is a calcium-binding protein involved in cell signaling, protein synthesis and degradation, DNA fragmentation and calcium homeostasis, and has been demonstrated to decrease the production of reactive oxygen species in HepG2 cells (Handa *et al.*, 2009; Yamaguchi, 2011). The proteins identified in this study may offer insight into the mechanisms that underlie DILI caused by APAP and DF, for which detailed information is currently lacking.

Overall, the kidney is the dominant organ for urine protein composition and therefore could be responsible for the changes seen in urine after APAP treatment (Thongboonkerd, 2008). The proteins identified in the *in vivo* mouse studies with APAP are not liver specific; however, we did not observe any sign of kidney injury (van Swelm *et al.*, 2012b). This was assessed by kidney histology and the determination in urine of kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin. As the liver was the only organ affected by APAP in this study, it was hypothesized that the proteins found in urine were released by the injured liver into the blood, similar to alanine aminotransferase. The proteins found in urine were small enough to be readily filtered by the glomerulus. The results of this study further strengthened this hypothesis as the proteins observed in mouse urine were released by the liver tissue, not only in mouse, but also by rat and human PCLS. This demonstrates that the PCLS system can contribute to studying the dynamics of potential protein biomarkers for DILI. Future studies will be directed to investigate the applicability of these biomarkers of human DILI and further validation in clinical studies.

Besides APAP, which is often used as model compound to study acute liver injury, we were able to analyze the toxicity-induced protein profile of DF, which has been associated with idiosyncratic DILI, but also acute hepatotoxicity (Boelsterli, 2003; Yano *et al.*, 2012). Because of the rare incidence of DF-induced liver injury (de Abajo *et al.*, 2004), it has been difficult to study this adverse reaction in humans. With the PCLS system, we were able to show the hepatotoxic properties of DF in human liver tissue. In line with previous studies that demonstrate a direct hepatotoxic effect of DF (Yano *et al.*, 2012), we showed a protein profile in PCLS medium of all species treated with DF that is similar to that after APAP incubation. The presence of the same key proteins in PCLS medium was demonstrated by Western blotting. Furthermore, we also observed a decrease in hepcidin concentrations. This implies that, like APAP, DF-induced acute hepatotoxicity may be accompanied by oxidative stress. Many of the proteins identified in APAP-treated mouse PCLS medium are related to processes of oxidative stress, including SOD1 and CA3, which were also present after DF incubation (Graves *et al.*, 2009; Gyamfi *et al.*, 2008a,2008b; Handa *et al.*, 2009; Hiyoshi *et al.*, 2009; Raisanen *et al.*, 1999). Furthermore, it has been demonstrated *in vivo* that the decreased concentration of hepcidin after APAP-induced hepatotoxicity is mediated via oxidative stress (van Swelm *et al.*, 2012a). Besides acute liver injury, the PCLS system has been used in studying other pathologies of liver injury, whether or not caused by drugs, such as hepatic fibrosis and cholestasis (Clouzeau-Girard *et al.*, 2006; van de Bovenkamp *et al.*, 2008; Van de Bovenkamp *et al.*, 2007; Westra *et al.*, 2013) and idiosyncratic DILI (Hadi *et al.*, 2012). This suggests that drug-induced changes in protein profiles in PCLS medium could be useful for studying the pathological mechanisms involved in DILI.

In conclusion, this is the first proteomics study on DILI in human, mouse and rat PCLS medium (*ex vivo*), demonstrating that similar toxicity-related protein profiles were found as in mouse urine (*in vivo*). Hence, the PCLS system proves to be a promising, translational model for the identification of potential protein biomarkers, which could be developed as urinary biomarkers for human DILI, and provide insight into the mechanisms of these drug-induced adverse reactions.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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