Cross-study and cross-omics comparisons of three nephrotoxic compounds reveal mechanistic insights and new candidate biomarkers

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ABSTRACT

The European InnoMed–PredTox project was a collaborative effort between 15 pharmaceutical companies, 2 small and mid-sized enterprises, and 3 universities with the goal of delivering deeper insights into the molecular mechanisms of kidney and liver toxicity and to identify mechanism-linked diagnostic or prognostic safety biomarker candidates by combining conventional toxicological parameters with “omics” data. Mechanistic toxicity studies with 16 different compounds, 2 dose levels, and 3 time points were performed in male Crl: WI(Han) rats. Three of the 16 investigated compounds, Bi-3 (FP007SE), Gentamicin (FP009SF), and IMM125 (FP013NO), induced kidney proximal tubule damage (PTD). In addition to histopathology and clinical chemistry, transcriptomics microarray and proteomics 2D-DIGE analysis were performed. Data from the three PTD studies were combined for a cross-study and cross-omics meta-analysis of the target organ. The mechanistic interpretation of kidney PTD-associated deregulated transcripts revealed, in addition to previously described kidney damage transcript biomarkers such as KIM-1,CLU and TIMP-1, a number of additional deregulated pathways congruent with histopathology observations on a single animal basis, including a specific effect on the complement system. The identification of new, more specific biomarker candidates for PTD was most successful when transcriptomics data were used. Combining transcriptomics data with proteomics data added extra value.

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Introduction

The development of a new therapeutic drug is a complex, lengthy, and expensive process, and the rate of failure of drug candidates is high. Therefore, ways to reduce the rate of attrition during the later phases of drug development are of highest interest. One key barrier is the limited predictivity of preclinical animal models for safety and efficacy in humans. Several consortia initiatives, like the International Life Sciences Institute/Health and Environmental Sciences Institute (ILSI/HESI) projects (Amin et al., 2004; Kramer et al., 2004), the Toxicogenomics Project in Japan (TGPJ) (Uehara et al., 2010), and the Predictive Safety Testing Consortium (PSTC) (Dieterle et al., 2010), and the Toxicogenomics Project in Japan (TGPJ) (Uehara et al., 2010), have addressed this challenge by testing and evaluating new concepts for the preclinical drug development process.

Improvements in the diagnosis and prediction of safety relevant findings would greatly influence the drug development process by helping to refine the safety testing program and to rank drug candidates in a more robust way. This can lead to an improved process, including the related regulatory framework, and is expected to contribute to the
decrease in the cost of drug development and speed up the delivery of innovative medicines to patients.

The InnoMed-PredTox consortium was initiated with this background in mind, in particular with the major objective of delivering a more informed decision making earlier in preclinical safety evaluation. A more specific aim was to get deeper insights into molecular mechanisms of kidney and liver toxicity using male CrI:WI(Han) rats as a model system. It was hypothesized that improved diagnosis and prediction of safety relevant findings can be obtained by combining conventional toxicological parameters and “omics” data. In the frame of this project, mechanistic toxicity studies with 16 different compounds were performed in male CrI:WI(Han) rats at three different time points (1 day, 3 days, or 14 days of dosing) and two dose levels using a harmonized study protocol across multiple sites. In contrast to other comparable projects, 14 of the 16 compounds selected within the PredTox project were stopped during drug development, in most cases due to kidney and/or liver toxicity. Two reference compounds were selected in addition to these compounds: gentamicin (FP009SF) and trolitazone (FP010SG).

Organ samples and body fluids were analyzed both using conventional toxicological endpoints, including histopathology and clinical chemistry parameters, as well as “omics” technologies, including transcriptomics, proteomics, and metabolomics. An overview of the PredTox project is given by Suter et al. (2010).

Among the 16 studies performed within the PredTox project, BI-3 (FP007SE), Gentamicin (FP009SF), and IMM125 (FP013NO) showed kidney proximal tubule damage (PTD) and were selected for further joint analysis. They are characterized as follows:

FP007SE ((3S,5S)-5-[4’-Methoxycarbonylamidino-4-biphenyl]-oxyethyl)-3-{[(methoxycarbonyl)methyl]-2-pyrrolidinone) is an orally active non-peptide platelet fibrinogen receptor antagonist intended for the treatment of thromboembolic diseases. Target organs of toxicity in rat are liver and kidney. Data from 4- and 26-week toxicity studies in rats revealed that at 1000 mg/kg, time-related, nephrotic lesions were induced comprising tubular epithelial necrosis, intratubular accumulation of granulocytes, segmental tubulocystis, and tubular atrophy. Mechanistic investigations with the aim of understanding the pathogenesis of the observed histopathological effects on kidneys were not performed.

FP009SF, an aminoglycoside antibiotic that inhibits bacterial protein synthesis by binding to the 30S subunit of the ribosome, is a well-known reference compound for nephrotoxicity in human and rats. FP009SF is reabsorbed by proximal tubule cells in the S1 and S2 segment and mitochondrial Ca2+ sequestration and oxidative stress (Sundin et al., 2008). Brieferly, impairment of cellular energy production in the mitochondria and mitochondrial Ca2+ sequestration and oxidative stress (Sundin et al., 2001; Sassen et al., 2006; Banday et al., 2008).

FP013NO, the O-hydroxyethyl-l-α-(Ser)(8)-derivative of cyclosporine A (CsA) and is almost equipotent to CsA with regard to its immunosuppressive activity. Like CsA, FP013NO belongs to a class of immunosuppressive agents called calcineurin inhibitors used for the prevention of alloraft rejection in solid organ transplantation (Kahan, 1993). FP013NO nephrotoxicity is characterized by tubular atrophy and interstitial fibrosis in rats. Based on clinical experience with CsA, it is believed that FP013NO nephrotoxicity is a consequence of renal vasoconstriction and endothelial injury, as well as a direct toxic effect on the tubular epithelium (Donatsch et al., 1992; Hiestand et al., 1993).

Both the immunosuppressive and nephrotoxic effects of cyclosporines seem to be closely related to the inhibition of calcineurin.

A meta-analysis across the studies FP007SE, FP009SF, and FP013NO studies was performed together with an across “omics” platform analysis with the aim of generating a better understanding of the pathological mechanism behind PTD and the identification of promising mechanism-linked diagnostic or prognostic biomarker candidates for this pathology.

It was hypothesized that compounds causing the same kind of renal damage target the same or related mechanisms and molecular pathways. Molecular signatures that are common and specific for a group of phenotypically related rats across studies can be expected to reflect the observed PTD because relevant gene and protein sets are likely to be deregulated in a comparable manner. Therefore, histopathological anchoring of data was the basis for this analysis. Specificity of relevant mRNA and protein signatures was evaluated by comparing them to the findings of the 13 other non-kidney-toxicant studies of the InnoMed-PredTox Project (for more details of these studies, please refer to Suter et al., 2010; Boitier et al., in press; Ellinger-Ziegelbauer et al., 2010). Clinical pathology results (e.g., serum creatinine and blood urea nitrogen (BUN)), as well as analysis results from known urinary kidney toxicity biomarker candidates for all three PTD studies, are described in detail in Hoffmann et al. (2010).

Specific protein or gene expression profiles, the combination of both, as well as single gene or protein parameters generated from such short-term rat studies, could refine/improve interpretation of classical parameter measurements and could help to predict the potential for development of PTD earlier in the preclinical safety evaluation process. Since the latter is a major objective of the PredTox project, proteins or genes identified during mechanistic omics analysis were evaluated concerning their potential to serve as predictive biomarker candidates for PTD.

Materials and methods

In addition to the study design and methods described below, a more detailed description, the compound selection criteria, as well as a methodological description of transcriptomics (RNA extraction and expression microarray experiments) and proteomics 2D-DIGE analysis can be found in the parallel overview paper (Suter et al., 2010).

The full “omics” data set has been submitted to the EBI (European Bioinformatics Institute: www.ebi.ac.uk/), curated via the ISA infrastructure (Rocca-Serra et al., 2010) and will be accessible via the multi-omics BioInvestigation Index database (http://www.ebi.ac.uk/ bioinvidx/browse_studies.seam).

In the text, the term “deregulation” refers to both up- and down-regulation of the abundance of a transcript or protein. Furthermore, the terms gene expression and transcript abundance are used synonymously.

Study design. The treatment schedule of animals within the PredTox project was reported previously in detail by Mulrane et al. (2008). Briefly, all studies were performed with male CrI:WI(Han) rats using a harmonized protocol. Male CrI:WI(Han) rats (8–10 weeks old, weighing approximately 170–200 g) were distributed into three dose groups (5 rats per group and time point) comprising two dose levels (low dose and high dose) and a time-matched vehicle control. FP009SF and FP007SE were administered daily for 1, 3, or 14 days (n = 45 rats per study), whereas FP013NO was administered for 1, 3, 14, or 28 days (n = 60 rats). Dosages were selected according to prior knowledge of the test items and the animal test system. The selected doses were 25 and 75 mg/kg/day subcutaneously for FP009SF, 100 and 1000 mg/kg/day oral by gavage for FP007SE, and 30 and 100 mg/kg/day oral by gavage for FP013NO. Necropsy was performed after over night fasting at day 2, day 4, day 15, and day 29, and liver and kidney sections were aliquoted and fixed in formalin or snap frozen in liquid nitrogen.

Controlled histopathology vocabulary and histopathology scoring. A controlled histopathology vocabulary was defined within the PredTox consortium, which was used in all test sites by local pathologists. In addition, a peer review was conducted by one pathologist, and
discrepancies were discussed until agreement about all findings was achieved.

The histopathology scoring system was based on a severity grading (1–5) for each type of lesion given by the original pathologist reading the study and normalized across studies by the peer-review pathologist. Thus for tubular necrosis, tubular basophilia, tubular degeneration, and tubular regeneration, the increasing score reflected both the number of regions as well as the extent of cell involvement in each region. The increasing scoring for interstitial cell infiltration equally reflected the number of regions involved and the magnitude of cells involved.

An overview of single animal histopathology scores for PTD of all 16 PredTox study high-dose animals are shown in the upper parts of Figs. 1, 2, and 3.

Transcriptomics data analysis. Expression microarray data were obtained using the Affymetrix GeneChip platform according to the manufacturer’s (Affymetrix) instructions. All analyses were based on expression values generated by using the Robust Multi-array Analysis (RMA) condensing method. In order to normalize the data and to render them comparable across all studies, all condensed probe set expression values of the treated animal samples of a given study were divided by the median of the corresponding time-matched vehicle group. The data from individual vehicle samples were also normalized this way.

Median intensities and standard deviation were computed per replicate group. Ratios of the medians of treated sample groups (low, high) vs. their time-matched vehicle control groups were derived. For each study group, t-test statistics were computed on the basis of logarithmized and normalized expression values, t-test p-values and corresponding Benjamini-Hochberg q-values were calculated for all genes for each comparison between time-matched treated sample groups (low, high) and corresponding vehicle control group. Two-way analyses of variance (ANOVA) tests were performed using time and dosage as (fixed) factors. The p-values, as well as the Benjamini-Hochberg q-values, were reported for the two main factors and the time–dosage interaction effect.

To visualize gene expression profiles, heatmaps were generated from vehicle-normalized gene expression data, which were derived per gene either by dividing the absolute gene intensities of single replicates from the treated samples through the median intensity of all time-matched vehicle control samples or by dividing the median intensity of the treated samples of a treatment group through the median intensity of all time-matched vehicle control samples. The genes represent various pre-selected gene groups and are either in a predetermined order or were previously clustered using Euclidean as distance metric and average linkage as linkage parameter.

Kidney Protein Preparation and Proteomics (2D DIGE) data analysis. Proteomics data were obtained by 2D-DIGE analysis of the kidney tissue from high-dose treatment and vehicle groups following 14-day treatment.

Kidney tissue was homogenized in 6 volumes of a lysis buffer containing 10 mM Tris, 0.4% Triton-X-100, 106 U/ml DNase, 12.8 U/ml RNase A, 1 mM Pefabloc, 7 M urea, 2 M thiourea, and 4% CHAPS. The lysate was then centrifuged at 100,000×g for 45 min at 20 °C to remove cellular debris. The supernatants from the kidney extracts were aliquoted and stored at −80 °C. The protein concentration of the extracts was determined by the Bradford method (Protein Assay Dye Reagent, Bio-Rad) using BSA for the standard curve.

2D-DIGE experiments using pH 5.5–6.7 Immobiline DryStrips and 12.7% SDS-PAGE gels and deregulated protein identification (1.3-fold change, t-test p-value < 0.01) were performed according to a standardized protocol (Com et al., 2003, 2011; Guerreiro et al., 2008; Suter et al., 2010 (manuscript submitted in parallel)). Integration of proteomics and transcriptomics data (cross-omics analysis). For biological interpretations, both pre-selected gene and protein lists were subjected to pathway and network analysis employing the Ingenuity Pathway Analysis (IPA) tool (Ingenuity Systems). In addition, Entrez databases (http://www.ncbi.nlm.nih.gov/sites/entrez) including PubMed and Gene were queried for detailed gene-specific information. Using all this information, the
most strongly deregulated genes and proteins were categorized with respect to their biological function and relation to organ toxicity. For the combined evaluation of kidney transcriptomics and proteomics data, only the high-dose and latest time point (day 15 for FP007SE and FP009SF, days 15 and 29 for FP013NO) data were considered. For the integration of proteomics and transcriptomics data, proteins found significantly deregulated by 2D-DIGE analysis in the kidneys of the FP007SE, FP009SF, and FP013NO studies and PTD genes selected with a fold change cut-off at 1.5-fold and a t-test q-value < 0.01 were mapped by using IPA. Genes or proteins that were differentially regulated in the PTD list (defined above) as well as in one of the 3 PTD studies in the 2D-DIGE analysis were functionally...
The observed dose- and time-dependent increase of basophilic tubule of FP007SE and FP013NO can be considered as an indication of a regenerative process. Therefore, animals with basophilic tubules in the studies FP007SE and FP013NO were grouped together with animals showing “Degeneration/regeneration: renal tubule” in the FP009SF study. In order to perform an in-depth mechanistic cross-study analysis, a summary term of the three individual histopathological effects was created. The observations “Basophilic tubule: proximal,” “Degeneration/regeneration: renal tubule,” and “Necrosis: renal tubule” were grouped as “Proximal tubule damage” (PTD), as all of these processes are likely to represent a common toxicological response.

“Infiltrates: Mononuclear cell” (IMC) was observed in studies FP007SE, FP009SF, and FP013NO and could be a reaction to the observed cell damage in the proximal tubule of those animals but may also be an independent pathologic effect. Therefore, animals with infiltrates of mononuclear cells in the kidneys were analyzed separately in the cross-study analysis.

In general, for both histopathology groups, PTD and IMC, the histopathological grading was the highest in high-dose animals at day 15 or day 29 (for FP013NO).

The histopathology related gene expression lists for PTD and IMC were created by defining a positive group consisting of individual treated animal samples that showed a histopathology score of two or larger for the summary term PTD in both kidneys. For the group IMC, the positive group was defined as treated animals that showed a histopathology score of at least 2 for the summary term IMC in at least one kidney. Seven of these samples are also members of the positive group for the summary term PTD. For FP007SE and FP013NO, the score of PTD was computed as the maximum of the scores of the three individual terms. For FP009SF, the maximum was taken from the two terms “Degeneration/regeneration: renal tubule” and “Necrosis: proximal tubule”.

Mechanistic analysis of kidney gene expression in animals with PTD

The gene list (please refer to Supplemental material) for the mechanistic analyses were calculated by including all 16 relevant animals of FP007SE, FP009SF, and FP013NO. All animals showed a histopathology score of $\geq 2$ for the positive sample group in one of the three studies and were only chosen when the effect was observed in both kidneys. The cut-off criteria for the analysis were $-1.7 \leq$ median ratios or median ratios $\geq 1.7$ and t-test q-value $\leq 0.01$ between the positive and negative samples groups, the latter comprising 37 vehicle animal samples of studies FP007SE, FP009SF, and FP013NO. 604 Probe sets fulfilled these criteria.

For the mechanistic interpretation of the observed gene expression changes, the probe sets were classified according to the biological function of the genes and grouped into biological and toxicological pathways of their gene products. For each of the identified gene groups, specificity for kidney toxicity was tested by comparing gene deregulation in the kidneys of the three kidney toxicity studies with those of the 13 remaining non-kidney-toxicant PredTox studies. This was visualized via a heatmap (Fig. 1) for genes involved in the complement system. Specificity of deregulated genes was considered as an important factor for the identification of mechanisms and biomarker candidates, which are related to the pathology PTD.

The functional analysis of the 604 modulated probe sets (please refer to Supplemental material) revealed a deregulation of renal damage genes, e.g., clusterin (CLU), hepatitis A virus cellular receptor 1 (HAVCR1; synonym: KIM1), lipocalin 2 (LCN2), secreted phospho-protein 1 (SPP1 also known as Osteopontin), and tissue inhibitor of metalloproteinase 1 (TIMP1) (Dieteler et al., 2008; Han et al., 2008) (Fig. 2), of cell death and oxidative stress genes such as BCL2-related protein A1 (BCL2A1), ceruloplasmin (CP), and heme oxygenase (decycling) 1 (HMOX1) (Cory and Adams, 2002; Bradbury and Hoover, 1989; Srisook et al., 2005) and a deregulation of genes encoding cytokine receptors, proteins, e.g., laminin, gamma 2 (LAMC2), myosin binding protein C, fast type (MYBPC2), coronin, and actin binding protein 1A (CORO1A) (Zuk and Matlin, 2002; Dogra et al., 2007; Xavier et al., 2008). Furthermore, canonical pathway mapping analysis using IPA revealed a prominent deregulation of pathways involved in immune response including deregulation of genes such as the toll-like receptors TLR2, TLR4 (Chen et al., 2007). The strongest deregulation was identified for the complement system pathway where 11 genes were up-regulated (Table 2 and Fig. 1).

Table 1
Overview of histopathology observations across the three studies FP007SE, FP009SF, and FP013NO. Strong effects are marked with “+”, minimal effects with “(+)”. Effects where considered as minimal if the observation was made only for one animal in the high-dose group at day 15 or if the effect was very slight with a histopathology score of 1 but which was dose- and time-dependent. Histopathology terms that were used for the creation of combined term PTD (proximal tubule damage), comprising terms “Basophilic tubule: proximal”, “Degeneration/regeneration: renal tubule” and “Necrosis: proximal tubule” and for term IMC (infiltrate: mononuclear cell) are in bold.

<table>
<thead>
<tr>
<th>Histopathology term kidney</th>
<th>FP007SE</th>
<th>FP009SF</th>
<th>FP013NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basophilic tubule: proximal</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Degeneration/regeneration: renal tubule</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Necrosis: proximal tubule</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Infiltrate: mononuclear cell</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dilatation: renal tubule</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mineralization: renal tubule</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hypertrophy: renal tubule</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cast renal tubule: hyaline</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cast renal tubule: cellular</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hyaline droplet: renal tubule</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vascularization: proximal tubule</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Vacuolation: distal tubule</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dilatation: pelvis</td>
<td>(+)</td>
<td>+</td>
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</tr>
</tbody>
</table>
Deregulation of the PTD selected genes in studies not associated with kidney damage

During evaluation of the transcriptomics data for specificity with respect to kidney damage, it was obvious that some of the genes identified as mechanistically relevant for kidney toxicity were also deregulated in some of the studies without any observed kidney toxicity (by histopathology assessment). Striking similarities of gene expression patterns between the three PTD studies and studies to toxicology (by histopathology assessment). Striking similarities of gene deregulated in some of the studies without any observed kidney damage (Table 3).

Table 3
Background information concerning nephrotoxicity data of compounds FP005ME, FP008AL, FP012SV, and FP014SC. Gene expression patterns of high-dose animals from these studies showed similarities to the gene expression patterns identified as mechanistically relevant for kidney damage in a cross-study transcriptomics analysis approach with three PTD studies.

<table>
<thead>
<tr>
<th>Study number</th>
<th>Nephrotoxicity observed in the innomed-PredTox study</th>
<th>Nephrotoxicity observed as class effect of this compound</th>
<th>Nephrotoxicity observed in former studies with the same compound</th>
<th>Availability of data from studies longer than 2 to 4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP005ME</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>FP008AL</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>FP012SV</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>FP014SC</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
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</tbody>
</table>

Deregulation of genes identified with pathology-associated at earlier time points and/or lower doses

All samples included in the positive group (PTD histopathology score ≥ 2) for selection of deregulated genes belong to the high-dose group sacrificed at day 15 or day 29 (for the FP013NO study). One aim of the PredTox project was the identification of predictive markers, which may indicate toxicity before histopathological effects become visible. The deregulation of relevant genes at earlier time points and/or lower dose levels was therefore investigated. Only gene groups that were identified as mechanistically relevant in the high-dose animals (described above) were analyzed. During the inspection of these genes, time points and doses were analyzed using the geometric mean of animals for each time point and dose. Genes were only chosen when the deregulation was seen after treatment with at least two of the three compounds causing PTD and when the fold change was greater than 1.5 or smaller than −1.5. Table 4 summarizes the 21 genes identified by this analysis.

Several genes that were found to be deregulated at an earlier time point and/or lower dose showed at the same time some minimal histopathological effects in both or one kidney in some of the analyzed animals (Table 4, arrows). In contrast, several animals showed also a gene expression change without any histopathological effect. These may be considered as early diagnostic/predictive candidate biomarkers (Table 4, arrows and asterisks). This corresponds to a number of 10 high-dose/early time point animals and 11 low-dose/early time point animals in the three PTD studies. All show histopathology scores = 0 for PTD and IMC in both kidneys. Low-dose/late time point animals were excluded from the analysis as only 3 animals of one study (FP007SE) showed no histopathological effect in both kidneys, and one study was considered as not sufficient for the analysis.

As a result of this analysis, five genes were deregulated in the high-dose/early time point animals (ATF3, SPP1, CALB1—which is confirmed with two probe sets, proteosome (prosome, macropain) subunit, beta type 9 (PSMB9) and CP) and two genes (TIMP1 and CALB1) were identified to be altered at an early time point (day 2 and/or day 4) in low-dose animals (arrows marked with asterisks in Table 4). Not all of the analyzed animals with histopathology score = 0 showed a deregulation of these specific genes.

Cross-study proteomics analysis using 2D-DIGE data

For cross-study analysis of deregulated protein expression 2D-DIGE data from kidneys of high-dosed animals (day 15) of all three PTD studies were used and compared manually by selecting proteins, which were deregulated in at least two of the three PTD compounds. In addition, protein expression data from the three PTD studies were compared to the 2D-DIGE data of the studies FP002BI and FP006J. Both FP002BI and FP006J showed histopathological effects in the kidneys (hyaline droplets, vacuolation in proximal tubule), which were not observed in any of the three PTD studies. Deregulation of proteins in these studies could therefore be explained by these minimal effects. By comparing the proteomic data from the three PTD studies with studies FP002BI and FP006J, the specificity of deregulated proteins specific to the PTD endpoint can be judged to a certain extent. For each study, the respective proteins were annotated and a mechanistic evaluation was performed.

For 12 proteins, deregulation was observed in at least two of the three PTD studies (Table 5). In most cases, the proteins were deregulated in the same direction for these three studies. Only one protein, glycine amidotransferase (l-arginine:glycine amidotransferase) (GATM; synonym: AGAT) showed specific down-regulation in all three PTD studies. Deregulation of AGAT was not detected in studies FP002BI and FP006J. All other proteins were deregulated in only two PTD studies and/or were additionally deregulated in studies showing no damage to proximal tubule, i.e., FP002BI and FP006J. Please cite this article as: Mathis, K.A., et al., Cross-study and cross-omics comparisons of three nephrotoxic compounds reveal mechanistic insights and new candidate biomarkers, Toxicol. Appl. Pharmacol. (2011), doi:10.1016/j.taap.2010.11.006
were marked with arrows and asterisks. Direction of arrows indicates if genes are up- or down-regulated. HD: high dose; LD: low dose.

with small effects in histopathology were marked with arrows, candidate genes that showed deregulation in animals with histopathology grade=0 for PTD and IMC in both kidneys (AKR7A3) and D-amino-acid oxidase (DAO) and fl (MSRA), heat shock transcription factor 1 (HSF1) and n-myc conjugate-beta lyase (CCBL1), methionine sulfoxide reductase A proteins, including the antioxidant enzymes and repair enzymes in three different processes (Table 5) and comprises oxidative stress showed a deregulation of proteins (most down-regulated) involved among these proteins, only seven were consistently and phenotype-deregulated across the three PTD studies and were either not deregulated or deregulated in the opposite direction in the studies FP002BI and FP006JJ.

Table 4

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene symbol</th>
<th>Gene description</th>
<th>Affymetrix probe set ID</th>
<th>Early time point (ID)</th>
<th>Late time point (ID)</th>
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<th>Late time point (HD)</th>
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<td>Tissue inhibitor of metalloproteinase 1</td>
<td>1367712_at</td>
<td>↑*</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>SPPI</td>
<td>Secreted phospholipase 1 (Osteopontin)</td>
<td>1367581_a_at; 1373021_at</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td></td>
<td>ICAM1</td>
<td>Intercellular adhesion molecule 1</td>
<td>1387202_at</td>
<td>↑*</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td></td>
<td>CALB1</td>
<td>Calbindin 1</td>
<td>1370201_at</td>
<td>↑*</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td></td>
<td>HAVCR1</td>
<td>Kidney injury molecule 1</td>
<td>1387965_at</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td></td>
<td>CLU</td>
<td>Clusterin</td>
<td>1367784_a_at</td>
<td>↑</td>
<td>↑</td>
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<td>↑</td>
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<tr>
<td>Cytoskeletal protein</td>
<td>NEF3</td>
<td>Neurofilament 3, medium</td>
<td>1367845_at</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td></td>
<td>LCN2</td>
<td>Lipocalin 2</td>
<td>1387801_at</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td></td>
<td>MYO 1G</td>
<td>Myosin Ig (predicted)</td>
<td>1393049_at</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td>LAMC2</td>
<td>Lammin, gamma 2</td>
<td>1379340_at</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Immune response</td>
<td>CCL2</td>
<td>Chemokine (C-C motif) ligand 2</td>
<td>1367973_at</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td></td>
<td>PSMB8</td>
<td>Proteosome (prosome, macropain)</td>
<td>1367785_at</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td></td>
<td>PSMB9</td>
<td>Proteosome (prosome, macropain)</td>
<td>1370186_at</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td></td>
<td>RT1_CE5</td>
<td>RT1 class I, CE5</td>
<td>1371209_at; 1388255_x_at</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>oxidative stress</td>
<td>CP</td>
<td>Ceruloplasmin</td>
<td>1368420_at; 1368418_a_at;1368419_at</td>
<td>↑*</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
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</tbody>
</table>

The results of the cross-study 2D-DIGE proteomics analysis showed a deregulation of proteins (most down-regulated) involved in three different processes (Table 5) and comprises oxidative stress proteins, including the antioxidant enzymes and repair enzymes catalase (CAT), glutathione peroxidase 1 (GPX1), cytoplasmic cytostatic conjugate-beta lyase (CCL1), methionine sulfoxide reductase A (MSRA), heat shock transcription factor 1 (HSF1) and n-myc downstream regulated 1 (NDRG1), detoxification proteins aldo-keto reductase family 7, member A3 (afatoxin aldehyde reductase) (AKR7A3) and ε-amino-acid oxidase (DAO) and finally proteins with functions related to energy metabolism (agmatine ureohydrolase (agmatinase) (fragment) (AGMAT), aminoacylase-1A (ACY1), AGAT and phosphoenolpyruvate carboxykinase 1 (soluble) (PCK1)). Among these proteins, only seven were consistently and phenotype-specifically deregulated across the three PTD studies and were either not deregulated or deregulated in the opposite direction in the studies FP002BI and FP006JJ.

Cross-omics analysis of transcriptomics and proteomics data

For the integration of proteomics and transcriptomics data, proteins identified as significantly deregulated by 2D-DIGE analysis in the kidneys of the FP007SE, FP009SF, and FP013NO studies and PTD genes were mapped using IPA. The mapping resulted in a list of 12.

Table 5

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Function</th>
<th>UniProt accession no.</th>
<th>PTD studies [Fold change]</th>
</tr>
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<tr>
<td>CAT</td>
<td>Catalase</td>
<td>Oxidative stress</td>
<td>P04762</td>
<td>-2.13</td>
</tr>
<tr>
<td>GPX1</td>
<td>Glutathione peroxidase 1</td>
<td></td>
<td>P04041</td>
<td>-1.57</td>
</tr>
<tr>
<td>CCL1</td>
<td>Cysteine conjugate-beta lyase, cytoplasmic</td>
<td></td>
<td>Q068415</td>
<td>-1.49</td>
</tr>
<tr>
<td>MSRA</td>
<td>Methionine sulfoxide reductase A</td>
<td></td>
<td>Q123FM1</td>
<td>-1.35</td>
</tr>
<tr>
<td>HSF1</td>
<td>Heat shock transcription factor 1</td>
<td></td>
<td>Q03717</td>
<td>-1.36</td>
</tr>
<tr>
<td>NDRG1</td>
<td>N-myc downstream regulated 1</td>
<td></td>
<td>Q06381</td>
<td>2.88</td>
</tr>
<tr>
<td>AKR7A3</td>
<td>Aldo-keto reductase family 7, member A3 (afatoxin aldehyde reductase)</td>
<td>Dihydroxyacetone reductase</td>
<td>Q43918</td>
<td>-1.42</td>
</tr>
<tr>
<td>DAO</td>
<td>ε-amino-acid oxidase</td>
<td></td>
<td>Q35078</td>
<td>-2.32</td>
</tr>
<tr>
<td>AGMAT</td>
<td>Agmatine ureohydrolase (agmatinase) [Fragment]</td>
<td>Energy metabolism</td>
<td>Q589285</td>
<td>-1.86</td>
</tr>
<tr>
<td>ACY1</td>
<td>Aminoacylase-1A</td>
<td></td>
<td>Q6AY57</td>
<td>-1.72</td>
</tr>
<tr>
<td>GATM (AGAT)</td>
<td>Glycine amidinotransferase (-arginine-glycine amidinotransferase)</td>
<td>Phosphoenolpyruvate carboxykinase 1 (soluble)</td>
<td>P07379</td>
<td>-2.92</td>
</tr>
</tbody>
</table>

genes deregulated at both the transcriptomic and proteomic levels (Table 6).

In order to investigate if these genes/proteins can be considered as potential biomarkers for PTD, additional analyses were performed. First, deregulated genes were compared with kidney transcriptomics data from in vivo PredTox studies, which showed no histopathological kidney lesions. As shown in the heatmap in Fig. 3, some of the identified genes, such as CORO1A and capping protein (actin filament), gelsolin-like (CAPG), seem to be specifically deregulated in the 3 PTD studies. Only study FP008AL showed deregulation of CAPG in addition to the PTD studies. Further genes, cysglobin (CYGB), retinol binding protein 4, plasma (RBP4), acyl-CoA synthetase medium-chain family member 3 (ACSM3) or acyl-Coenzyme A dehydrogenase, short/branched chain (ACADSB), appeared to be non-specifically deregulated in studies with or without kidney effects.

Discussion and conclusion

A combined analysis of histopathological observations and "omics" data (i.e., transcriptomics and proteomics 2D-DIGE) allowed a better understanding of the molecular events associated with drug induced PTD. The approach was also able to identify potential biomarkers with the ability to reflect and in some cases predict the development of PTD. For this purpose, three compounds (FP007SE, FP009SF, and FP013NO) showing PTD after repeated dosing in rats were compared to each other and to compounds not inducing this pathology.

New hypotheses of molecular mechanisms leading to proximal tubule damage: a central role of the complement system?

Analysis of the kidney PTD-transcriptomics gene lists revealed a deregulation of several gene groups reflecting different biological processes. In most cases, these groups showed a specific time and dose response for the selected PTD studies compared to studies without visible proximal tubule damage. Deregulation of these pathways most likely reflects the molecular sequence that occurs within the cells of the kidney proximal tubule, starting with cell death and oxidative stress accompanied by inflammatory and immune response, tissue reorganization (as indicated by the deregulation of cytoskeleton and extracellular matrix components and regeneration processes).

Among the identified gene sets, some direct evidence for cell injury was shown by the up-regulation of genes involved in necrosis or apoptosis (e.g., Bcl2a1) (Cory and Adams, 2002) and genes involved in oxidative stress mechanisms (e.g., CP and Hmox1) (Broadley and Hoover, 1989; Sriskook et al., 2005). Upon initiation of injury, the morphology of the cells and tissue changes dramatically. Reorganization, regeneration, and repair mechanisms are initiated as a normal reaction of cells to damage. Therefore, the deregulation of genes involved in cytoskeletal reorganization or structure (e.g., LAMC2, MYBP2, CORO1A) is considered to be a reflection of those reactions. Several genes identified here were at the same time also described previously as markers for renal injury, including some genes involved in tissue reorganization (e.g., SPP1, KIM1, TIMP1, LCN2, and Icam1) or cell death and oxidative stress (e.g., Clu, Atf3, Hmox1) (Thukral et al., 2005; Perco et al., 2006; Dieterle et al., 2008; Han et al., 2008)). As expected most of these genes, like KIM1, LCN2 (also known as NGAL), Clu, Spp1, Hmox1, Atf3, and Icam1 showed relatively strong and specific deregulation at the gene expression level in animals of the PTD group when compared to control samples without any histopathological effect on the kidneys. Deregulation of some of the known nephrotoxicity markers described above has been confirmed using qRT-PCR, immunohistochemistry, and ELISA. Samples from these same studies showed that Clu and KIM1 could be early and sensitive non-invasive markers of renal injury (Hoffmann et al., 2010). In contrast, Calbi1 turned out to be not specific for the three PTD studies, at least at the mRNA level.

Among the strongest PTD-specific deregulated genes (same range as KIM1) was glycoprotein (transmembrane) nmb (GPNNM) (also known as Osteoactivin), which has been described to be increased in association with kidney damage after unilateral ureter obstruction (Nakamura et al., 2007). The results of the present analysis support the hypothesis of a possible link between kidney damage and up-regulation of Osteoactivin. The high and specific up-regulation of Osteoactivin mRNA makes it a very interesting potential candidate biomarker for kidney injury.

Activation of the immune system is the normal reaction of organisms to cell damage. The observed strong up-regulation of immune response genes might therefore reflect the normal reaction of animal to the observed tissue damage. Immune responses can be mediated by membrane receptors such as toll-like receptors TLR2 and TLR4.
TLR4 (Chen et al., 2007), which were strongly up-regulated in the three PTD studies. Importantly, the most prominent finding of the cross compound analysis of transcriptomics data was the strong up-regulation of genes involved in both the classical and alternative pathways of the complement system. The complement system effectively identifies and clears invasive pathogens, as well as injured host cells. An up-regulation could therefore be interpreted as a reaction to the damage occurring in kidney proximal tubule cells. In addition, a more direct association of kidney injury or kidney diseases with complement system activation has been reported previously (de Vries et al., 2003). Several studies have demonstrated that the filtration of complement proteins into the renal tubules, as occurs during proteinuric renal disease, causes tubular inflammation and injury (Lenderink et al., 2007). Targeting complement inhibitory molecules to the proximal tubules in a rat model of proteinuric kidney disease protects against renal dysfunction (He et al., 2005). Complement component 3 (C3), which is strongly and specifically up-regulated in all three PTD studies, was shown to be up-regulated in proximal tubule cells after Cyclosporin A-induced nephrotoxicity and was furthermore shown to be important in mediating drug induced renal injury (Kim et al., 2007; Turnberg et al., 2006). In conclusion, the observed strong up-regulation of complement system genes can be interpreted as an uncontrolled reaction of cells to a direct toxic attack, which at the same time contributes to tissue injury and might therefore play a central role in the continued development of the proximal tubule damage.

Proteomics assessment

The proteomics cross-study analysis revealed that deregulated proteins were involved in antioxidant defense, detoxification, and energy metabolism (Table 5). Among the identified “oxidative stress” proteins, three were deregulated specifically in at least two of the three PTD studies, namely CAT, HSF1, and NDRG1 (Cederbaum, 2009; Trougakos and Gonos, 2006; Han et al., 2008), which could reflect a specific response to PTD. In this context, down-regulation of the antioxidant enzymes CAT and HSF1 was not expected. Production of free radicals during oxidation reactions can start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. Therefore, up-regulation of antioxidant enzymes would be the predicted primary reaction of cells to oxidative stress. However, the lower level of antioxidants in the FP007SE and FP009SF studies compared to control might reflect a depletion of those enzymes following a long-term oxidative stress process during 14 application days. Protein up-regulation of the p53 target gene NDRG1 in this protein group is described as a response that occurs after oxidative stress in different species, which could also be a reaction to the damage to proximal tubule cells (Han et al., 2008).

Among the deregulated proteins involved in detoxification processes, AKR7A3 protein expression was phenotype-specific but weakly down-regulated in two of the PTD studies (FP007SE and FP009SF) and up-regulated or not detected in the two studies, which showed no PTD phenotype. It belongs to a family of proteins that are described to be involved in detoxification of aldehydes and ketones (Knight et al., 1999).

Two proteins, ACY1 and GATM (AGAT), associated with energy metabolism (Giardina et al., 2000; Wyss and Kaddurah-Daouk, 2000), were down-regulated in at least two of the three PTD studies. They were not detected or up-regulated in the two reference studies FP0028 and FP0067. Down-regulation of energy metabolism proteins can be generally interpreted as a stress reaction of damaged cells. During the project, the down-regulation of AGAT protein expression was confirmed by Western blot analysis (data not shown). An association of changes in AGAT protein with damage to the proximal tubule is also supported by the fact that AGAT protein is expressed exclusively in the S1 and S2 portions of the proximal tubule in rats (Takeda et al., 1992). Interestingly, a decrease of AGAT activity was previously described in relation to nephrotoxicity after PF009SF treatment and was accompanied by a decrease in the urinary concentration of guanidinoacetic acid (Kiyatake et al., 2004). Guanidinoacetic acid, a precursor of creatinine, is synthesized from arginine in the presence of glycine by AGAT in the proximal renal tubules (Magri et al., 1975). This suggests that urinary guanidinoacetic acid could be a promising new diagnostic biomarker candidate for PTD.

Biomarker identification using a cross-omics analyses approach

A limiting factor for the comparison of proteins and genes found significantly deregulated in this study is the fact that the number of deregulated proteins identified with 2D-DIGE was limited to 40–60 proteins, whereas lists of significantly altered genes selected in association with the PTD summary term comprised several hundred genes. Moreover, mainly high abundant proteins from pl 5.5 to 6.7 and MW 20 to 150 kDa are detected by this proteomics method. This excludes the possibility to identify proteins corresponding to PTD-deregulated genes such as KIM1, GPNMB (Osteoactivin), LCN2, CLU, or SPP1 (Osteopontin) whose pl are 9.00, 7.00, 7.70, 5.46, and 4.33, respectively. Nevertheless, we identified 12 proteins whose corresponding mRNAs were also significantly deregulated in kidney tissue. Ten were deregulated in the same direction and 2 in opposite directions. These 12 genes were investigated for their specificity to kidney damage by comparing gene expression deregulations in the kidney of the 3 studies associated with PTD to the remaining 13 PredTox studies without this pathology. CAPG and CORO1A showed the highest specificity among the identified cross-omics genes/proteins, but most of the identified genes showed only minimal specificity. CORO1A plays a role in early phagosome/lysosome fusion and was described to be a regulator of peripheral lymphocyte survival through activation of Ca2+ release from intracellular stores (Mueller et al., 2008). It is also associated with actin filaments of the cytoskeleton (Xavier et al., 2008). Also CAPG is also associated with actin filaments and contributes to the control of actin-based motility and has been detected in mouse renal medulla (Arai and Kwiatkowski, 1999). Deregulation of these cytoskeleton associated genes/proteins might reflect tissue reorganization in correlation of the CORO1A immune response processes following tissue damage (Xavier et al., 2008).

In summary, a combination of transcriptomics and proteomics data leads to the identification of two biomarker candidates, which were deregulated at both mRNA and protein levels. The detected proteins have a high abundance in damaged kidney tissue and have therefore an increased likelihood to appear in blood or urine of these animals upon kidney damage. The observation that CORO1A and CAPG are induced in response to tissue damage in the proximal tubule has to be confirmed with further experiments and with additional studies with other nephrotoxic compounds. Nevertheless, the specificity of the identified proteins CORO1A and CAPG, as well as the increased chance that these proteins could be detected in blood or urine, makes them very interesting new biomarker candidates for PTD.

The potential of gene expression patterns as more sensitive indicators for PTD than histopathology

In order to identify biomarker candidates earlier than histopathological observations, several functionally related gene groups identified with cross-study transcriptomics analysis were analyzed with respect to deregulation of genes at earlier time points and lower doses. Gene sets were analyzed within the list of significantly deregulated genes in conjunction with PTD-positive samples, for example, the complement system genes or kidney damage genes identified as important components for the pathomechanism of PTD.
In the original study outline of Innomed-PredTox, it was requested that the low dose should lie between the NOAEL (No Observed Adverse Effect Level) and the LOAEL (Lowest Observed Adverse Effect Level). Therefore, it was not unexpected that low-dose animals showed some affects on their kidneys. Candidate genes identified at earlier time points or at lower dose levels with small effects in histopathology were therefore detected (Table 4) as diagnostic biomarker candidates, whereas genes that showed deregulation in animals with histopathology score = 0 for PTD and IMC in both kidneys are considered as prognostic biomarker candidates (Table 4). Genes were only selected for this category when they showed deregulation in at least two animals of two studies without histopathological effects.

Five candidate marker genes were altered significantly at earlier time points (day 2 and/or day 4) after high-dose and two genes were deregulated after low-dose treatment at day 2 without any histopathological correlate (histopathology score = 0 for PTD and IMC in both kidneys). These were ATF3, SPP1, TIMP1, and CALB1 as well as PSMB9, which is involved in the immune response (also described as LMP2, large multifunctional peptidase 2) (Gaczynski et al., 1996) and as a scavenger of superoxide CP (Broadley and Hoover, 1989). CP was described as an antioxidant because of its ability to inhibit the oxidation of lipids (Halliwell and Gutteridge, 1990), as well as for its ability to scavenge superoxide anions and sequestering of free copper ions (Goldstein et al., 1979) and is therefore involved in the oxidative stress response. In contrast, up-regulated genes like KIM1, CLU, and all complement system genes only correlated with histopathological observations. The only gene that was consistently down-regulated in the high-dose groups at early and late time points, as well as at the low dose at day 2, without histopathological effects was CALB1.

It cannot be excluded that some focal histopathological effects were overlooked due to the fact that only one cutting plane of the fixed kidney was investigated. Keeping that in mind, some of the identified early/low-dose genes could be a reflection of a non-detected histopathological effect in the kidneys. Nevertheless, the fact that several animals showed early deregulation of genes supports the hypothesis that the identified candidate markers could be more sensitive with respect to time than histopathology and could therefore be considered as early diagnostic maker candidates. Validation of these identified marker candidates in terms of specificity, which showed in most cases only a small deregulation is essential. Based on the present study design and the limited number of studies and animals for each time/dose group, further work is required to better understand the prognostic value of these sensitive nephrotoxicity gene expression biomarker candidates.

Several of the identified genes showed high specificity for the PTD studies but a number of genes, including known kidney injury markers, e.g., activating transcription factor 3 (ATF3), calbindin 1 (CALB1), CLU, HAVCR1 (KIM1), HMOX1, intercellular adherence molecule 1 (ICAM1), LCN2, SPP1, and TIMP1, were also found to be deregulated in the non-kidney-toxicant PredTox studies (FP005ME, FP008AL, FP012SV, and FP014SC; Fig. 2).

Indeed two of these compounds, FP008AL and FP014SC, showed nephrotoxicity in former studies or were expected to show effects on the kidneys due to a class effect (Table 3).

This implies that not only single marker genes but also gene expression patterns could be sensitive and may be even good predictive markers for nephrotoxicity in animal studies.

Conclusions

This project has led to a deeper mechanistic understanding of the processes involved in the development of PTD. Histopathology-anchored classification of samples turned out to be an appropriate method for the analysis of transcriptomics and proteomics data. Different pathways could be identified as significantly deregulated and thus linked to the onset of proximal tubular injury. These included the complement system, pathways involved in cell death, oxidative stress and the accompanying inflammatory and immune responses, as well as tissue reorganization and regeneration. In addition, it was possible to identify a number of PTD-specific mRNA and protein biomarker candidates. Among them were candidate markers at the mRNA level, like the highly up-regulated and PTD-specific Osteoactivin and AGAT, which showed strong down-regulation on the protein level. A decrease in urinary Guanidinoacetic acid, associated with a renal AGAT protein expression decrease, may be a clinically useful indicator of PTD (Kiyatake et al., 2004). CORO1A, specifically up-regulated both transcriptionally and/or at the protein level, needs to be further characterized and qualified in preclinical studies. In order to bridge novel nephrotoxicity biomarker candidates into clinical applications, the detection of these markers in blood or urine is essential and has to be tested and qualified in further pre-clinical studies.

The application of single marker genes or proteins, as well as the analysis of the identified PTD-specific molecular patterns (e.g., complement system genes), in preclinical studies in combination with histopathology should lead to a more sensitive readout and better understanding of the lesion. This could then trigger a closer monitoring of kidney safety in subsequent animal studies and clinical studies during development of a new drug candidate, which has potential PTD liabilities.

Supplementary materials related to this article can be found online at doi:10.1016/j.taap.2010.11.006.

Conflict of interest statement

The PredTox project was evaluated by independent experts on request of the European Commission. There are no conflicts of interest of authors or consortium members since the project was funded by public (EU) funds. The PredTox project is dedicated to basic research, and there are no commercial interests.

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References


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