Ingenuity pathways analysis of urine metabonomics phenotypes toxicity of gentamicin in multiple organs†

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We introduce the use of Ingenuity Pathway Analysis to analyzing global metabonomics in order to characterize phenotypically biochemical perturbations and the potential mechanisms of the gentamicin-induced toxicity in multiple organs. A single dose of gentamicin was administered to Sprague Dawley rats (200 mg/kg, $n = 6$) and urine samples were collected at −24–0 h pre-dosage, 0–24, 24–48, 48–72 and 72–96 h post-dosage of gentamicin. The urine metabonomics analysis was performed by UPLC/MS, and the mass spectra signals of the detected metabolites were systematically deconvoluted and analyzed by pattern recognition analyses (Heatmap, PCA and PLS-DA), revealing a time-dependency of the biochemical perturbations induced by gentamicin toxicity. As result, the holistic metabolome change induced by gentamicin toxicity in the animal's organisms was characterized. Several metabolites involved in amino acid metabolism were identified in urine, and it was confirmed that gentamicin biochemical perturbations can be foreseen from these biomarkers. Notoriously, it was found that gentamicin induced toxicity in multiple organs system in the laboratory rats. The proof-of-knowledge based Ingenuity Pathway Analysis revealed gentamicin induced liver and heart toxicity, along with the previously known toxicity in kidney. The metabolites creatine, nicotinic acid, prostaglandin E2, and cholic acid were identified and validated as phenotypic biomarkers of gentamicin induced toxicity. Altogether, the significance of the use of metabonomics analyses in the assessment of drug toxicity is highlighted once more; furthermore, this work demonstrated the powerful predictive potential of the Ingenuity Pathway Analysis to study of drug toxicity and its valuable complementation for metabonomics based assessment of the drug toxicity.

1. Introduction

Drugs and medicaments are typically chemical substances not commonly present in foods, and most of them have some kind of side effect/toxicity in the organism. Drug toxicity usually occurs due to three major causes: over dosage of the medication, accumulation of the drug in the body over time and the inability of the body to eliminate the drug or its inherent toxic effect. Accordingly, liver and kidney are the primary targeted sites of drug toxicity.[1,2] It has been proved that more of 900 drugs in clinical use to cause the liver toxicity.[3] Moreover, the liver toxicity induced by drug is accounting for 5% of all hospital admissions and 50% of all acute liver failures.[4,5] Equally, the kidney toxicity is one of the most prevalent kidney issues and usually happens when the biological body is exposed to a drug or toxin.[6,7] Statistically, the development of approximate 30% of new drug candidates is halted due to toxicity and adverse effects in clinical studies.[8] The toxicity of liver and kidney is the major reason for the drugs to be withdrawn from the market or bankrupt during development of the drug candidates. Since the toxicity of liver and kidney are enormous and increasing problems for the pharmaceutical industry and in clinical practice, the earlier and more reliable detection of drug-induced toxicity in liver and kidney should improve clinical care of patients and benefit new drug development and discovery.[1,7,9] The measurement of small-molecule metabolites was introduced in clinical practices more than 100 years ago,[10] then called "clinical chemistry". This strategy provides a quick, inexpensive, quantitative and relatively noninvasive approach to monitoring and screening the drugs toxicity.[11–13] However, with increasing safety standards from worldwide regulatory agencies, an increased requirement for better safety biomarkers has been emphasized.[14] However, the sensitivity and specialty of the clinical chemistry analysis are quite varied and sometimes is considered sub-optimal, with changes generally occurring only after significant tissue injury occurred. Moreover, most current biomarkers are correlated to the targeted organ without clarifying the mechanism of toxicity.[14] A more effective understanding on the drug toxicity usually comes from a global analysis of system metabolites pool ("metabonomics"). Therefore, the toxicologists in preclinical development should soon replace routine clinical chemistry with metabonomics screenings in order to characterize the drug toxicity and identify clinical biomarkers for safety monitoring during

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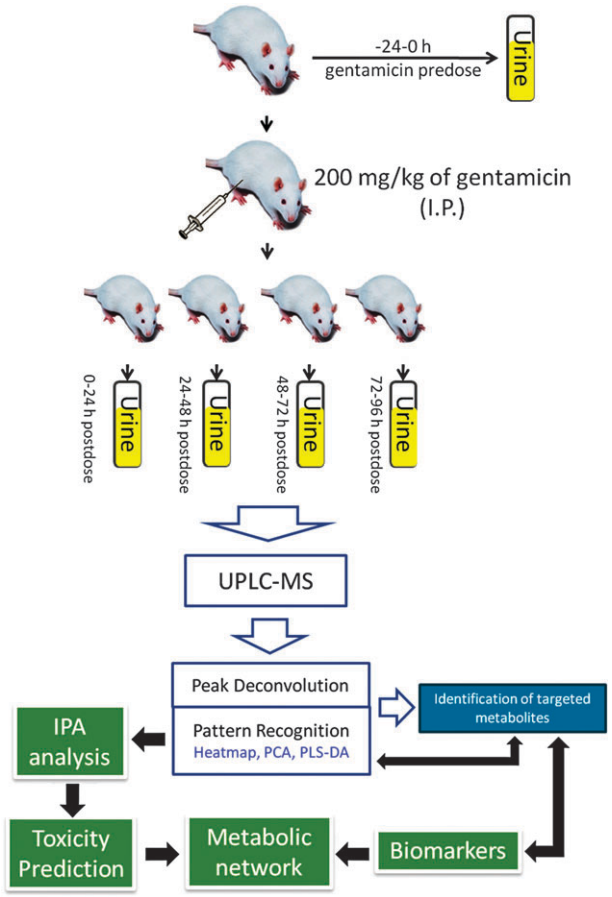

Fig. 1 Schematic representation of the workflow followed to evaluate the gentamicin system toxicity. The diagram globally summarizes the different steps and techniques utilized during the execution of this work. I.P.: intra-peritoneal injection; intra UPLC-MS: Ultra high Performance Liquid Chromatography combined with Mass Spectrometry detection; PCA: Principal Components Analysis; PLS-DA: Partial Least Squares-Discriminates Analysis; IPA: Ingenuity Pathway Analysis.

clinical practice.[14] Several studies had successfully applied the metabonomics technology to study drugs toxicity.[15–18] However, one big challenge of metabonomics study is processing and interpretation of raw analytical data. To characterize the metabonome, usually including pools of thousands of metabolites is not a simple and trivial matter, therefore diverse pattern recognition analyses have been employed to evaluate and fingerprint the metabolites in metabonomics studies, allowing to identify biomarkers increased or decreased as consequence of the toxicity of the drug.[19–22]

Gentamicin is a bactericidal aminoglycoside antibiotic that affects the bacterial growth by inhibiting protein synthesis in the susceptible bacteria. Its specific mode of action consists of binding the 30S subunit of the bacterial ribosome, and as consequence the protein synthesis is interrupted. Gentamicin is widely used for the treatment of bacterial infections, normally gram-negative bacteria including pseudomonas, proteus, serratia, and gram-positive staphylococcus.[23] However, it is known that gentamicin usually causes kidney toxicity due that it induces necrosis of cells in the proximal tubule, resulting in acute tubular necrosis, which can lead to acute renal failure.[24] Importantly, gentamicin is still recognized as an important antibiotic against life-threatening gram-negative bacterial infections, despite its known kidney toxicity.[25] Nevertheless, the understanding of the system toxicity of gentamicin is not clear, since most of the previous metabolomics studies have been limited to evaluate only the kidney toxicity of this drug.[2,26] Largely there have been overlook of latent threats of the gentamicin on other organs of patients treated with gentamicin. Consequently, it is necessary a systematic characterization of gentamicin toxicity by global metabonomics to screen its effects in entire biological organisms, which could enhance its efficiency in clinical uses.

The Ingenuity Pathways Analysis (IPA) IPA is a proof-of-knowledge based software that helps researchers to model, analyze, and understand the complex biological and chemical systems in life science research. IPA contents an extensive repository of biological and chemical knowledge offering to the researcher access to the most current findings available on genes, drugs, chemicals, protein families, normal cellular and disease processes, and signaling and metabolic pathways.

IPA extensive library of well-characterized signaling and metabolic pathways provides the researcher with a starting point for exploration and a bridge between novel discovery and known *in vivo* or *in vitro* experimental biological systems. The software allows to looking for information on genes and chemicals, their impact on diseases and cellular processes, and their role in signaling and metabolic pathways. In relation to drugs and metabolism, IPA permits to not only visualize time course and dose response effects, but it helps the researcher to analyze the data in the context of molecular mechanisms and to relate molecular changes to organismal physiology and pathophysiology.

In this study, we introduce the combination of urine metabonomics with the proof-of-knowledge based IPA to characterize the system toxicity of gentamicin in the entire the biological organism. The IPA represents a powerful tool to predict the potential and targeted pathway functions, which will be a beneficial complement for the rapid assessment metabonomics-based screening of the drug toxicity. The workflow employed for the assessment of gentamicin toxicity analyzed by metabolomics combined with IPA is schematized in Fig. 1.

2. Materials and methods

2.1 Chemicals and reagents

Acetonitrile and LC-MS grade water were purchased from Thermo Fisher Scientific Inc. (Pittsburgh, PA, USA). Leucine enkephalin, Indomethacin and LC-MS grade Formic Acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2 Animals treatments and sampling

The six rats used in this study, Sprague Dawley weighing 200 ± 20 g were purchased from Beijing Weitong Lihua Experimental Animal Co., Ltd (Beijing, Beijing, China). The animals were maintained in metabolic cages in a controlled environment at 22 ± 2 °C, with a twelve-hour light-dark cycle

and a rodent laboratory chow diet and water provided *ad libitum*. The animal studies were carried out in accordance with the Chinese National Research Council Guidelines and were approved by the Subcommittee of Animal Research Care and Laboratory for Animal Resources of the Chinese Academy of Medical Sciences. The animals were acclimatized for seven days to the indicated conditions prior to experiments, and then put in the metabolic cages during periods of urine collection below specified. The pre-dosage urine samples were collected in the morning at −24 to 0 h (C), then the rats were fasted overnight and orally dosed with gentamicin (200 mg/kg b.w.) by gavages.[2,27] Post-dosage urine samples were collected in the mornings of 0–24 h (D1), 24–48 h (D2), 48–72 h (D3) and 72–96 h (D4). The collected urine samples were centrifuged to 10 000 rpm at 4 °C for 5 min, and then frozen at −80 °C. The samples were thawed and filtered with 0.2 μm syringe filters prior LC/MS analysis.

2.3 UPLC-MS conditions

Chromatographic analysis was performed in a Waters Acquity UPLC system using an ACQUITY BEH C18 column (100 mm × 2. 1 mm, 1.7 μm particle size) (Waters Corp., Milford, MA, USA). A flow rate of 0.5 ml/min (back pressure: 8000 psi) and a 5 μl of injection volume were used. The column eluent was directed into the mass spectrometer without split. Chromatographic separations were performed by a gradient method, 0–30% B in 5 min, 30–80% B from 5 to 9 min and then 80–100% B from 9 to 10 min (A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile).

Mass spectrometry detection was performed using a Micromass Q-TOF (Waters MS Technologies, Manchester, UK) equipped with an ESI source operating simultaneously in positive and negative ionization modes. The desolvation gas rate was set at 700 l/h under at a temperature of 350 °C. The flow rate of the cone gas was set at 50 l/h and the temperature of the source was 120 °C. The capillary and cone voltages were set at 3000 V and 35 V for the positive ionization mode, and 2700 V and 35 V for the negative ionization mode. All the data was acquired using the lockspray to ensure accuracy and reproducibility. Leucine-enkephalin was used as the lock mass (*m*/*z* 556.2771 for positive ionization mode; *m*/*z* 554.2551 for negative ionization mode) to a concentration of 60 fmol/l and infusing it at a continuous flow rate of 25 μl/min. The raw data was collected using the centroid mode, from *m*/*z* 50 to *m*/*z* 1000, using a lockspray frequency of 0.50 s and data averaging over ten scans.

2.4 Metabolic profiling and pattern recognition analyses

The urine samples collected at different time-points (C, D1, D2, D3 and D4) were analyzed by UPLC-MS to obtain global metabolic profiling in both, positive and negative ionization modes. The mass spectra signals of the metabolic profiles were deconvoluted and integrated using MarkerLynx XS v4.1 (Waters MS Technologies, Manchester, UK) to generating multivariate data matrices.

The ion intensity of each mass signal detected was normalized to construct data matrices (retention time mass-to-charge normalized peak area). Using the data matrices, the unsupervised heatmap analysis, unsupervised principal components analysis (PCA), supervised partial least-squares discriminates analysis (PLS-DA) and supervised orthogonal partial least-squares discriminates analysis (OPLS-DA) were performed to identify and characterize metabolic perturbations signatures induced by gentamicin.[19–22] The biomarker metabolites primarily involved in metabolic perturbations signatures were identified by using combinational pattern recognition analyses.

2.5 Ingenuity pathway analysis

To predict latent toxicity of gentamicin the proof-of-knowledge based IPA was performed to characterize the biomarker metabolites confirmed by the pattern recognition analyses and to further evaluate the biomarkers based on their metabolic associations in biological function. The IPA analysis allows study the indomethacin by validating the phenotypic biomarkers and related to biological functions. Furthermore, phenotypic biomarkers of gentamicin toxicity were identified by IPA sub-toxicity analysis, and correlating them with proof-of-knowledge databases to discerning the mechanism of action.

2.6 Statistical analysis

The normalized quantitative data in all cases is expressed as Mean ± SEM. Statistical comparisons were made by one-way ANOVA followed by Tukey's test. Statistical differences are considered significant when the test *p* value is less than 0.05.

3. Results and discussion

3.1 Metabonomics analysis

To characterize toxicity of gentamicin in multiple organs, an UPLC-MS based metabonomics method was applied to investigate global metabolic changes of the urine from rat at −24 to 0 h (C) pre-dosage, then at 0–24 h(D1), 24–48 h (D2), 48–72 h (D3), 72–96 h (D4) post-dosage of gentamicin. An automatic threshold for peaks intensity was set up; about 700 peaks in positive ions (ESI+) and 1200 peaks in negative ions (ESI−) were detected by MarkerLynx software when acquiring data from 0.1 to 10 min of running time. The chromatographic data was deconvoluted and normalized using the MarkerLynx software, generating multivariate data matrices containing retention time, mass-to-charge ratio and abundance for each ion. Heatmap plots were generated using the ion signals of metabolites detected in both, positive (Fig. 2A) and negative (Fig. 2B) ionization modes from the samples collected ant different times before and after the drug administration (C, D1–D4). The results indicate a marked differentiation in the abundance of numerous metabolites after the administration of the drug, it obviously indicating that several biochemical metabolic pathways are significantly altered after just 24 h of the administration of gentamicin. The differences accentuate with the time, indicating a time-dependent patter during the entire time-course of sampling. Moreover, the HCA analysis by targeting observation shows C, D1, D2, D3 and D4 are validated clustered classified, and C indicates high similarity with D1 and D2 by comparing with D3 and D4 (Fig. 2),

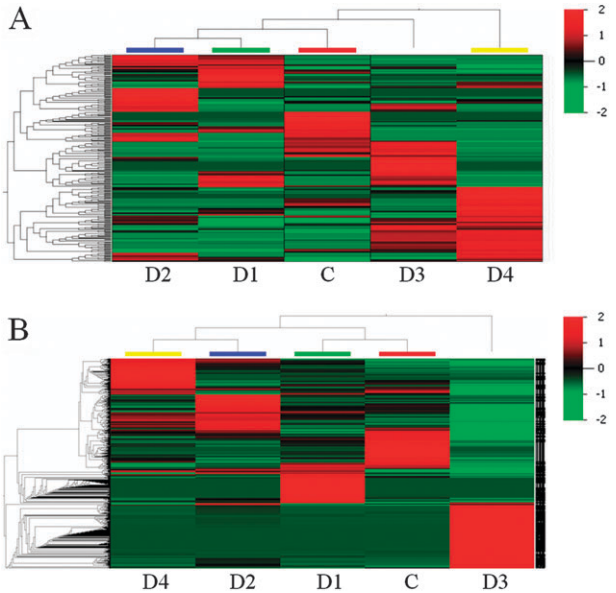

Fig. 2 Heatmap of the total ions intensities from the urine metabolomics analysis. The heat-map graphically represents individual ions changes of abundance. The ions intensities are represented in a log 2 scale. The columns represent the treatments (C: −24–0 h pre-dosage of gentamicin; D1: 0–24 h, D2: 24–48, D3: 48–72 h and D4: 72–96 h post-dosage of gentamicin). The columns order was established by the software R version 2.11.1, using a hierarchical clustering agglomeration according to the profiles' similarities. The rows represent individual ions observations, and as for the columns, their overall similarities were used to establish the position of the nodes between two given clusters of observations. A: heatmap of metabolites detected in positive ion mode; B: heatmap of metabolites detected in negative ion mode.

suggesting that gentamicin induced metabolic perturbation is a progress process or not action of fasting phase.

3.2 Pattern recognition analysis

In order to better visualize the subtle similarities and differences among these complex data sets, multiple pattern recognition methods were employed to phenotype urine metabonome of gentamicin induced toxicity in rats. The unsupervised PCA analysis shows differentiated of the control groups (−24–0 h pre-dosing of gentamicin) to the gentamicin treated groups (0–24 h (D1), 24–48 h (D2), 48–72 h (D3), 72–96 h (D4) post-dosing of gentamicin), the specific results are shown in Fig. 3A and 4A. Furthermore, the supervised PLS-DA analysis reveals a much bigger metabolic signature difference of the urine collected from gentamicin treated groups and control group, and this change was further enforced with increasing days e of the sampling (Fig. 3B and 4B). The mass spectrometry signals (ions) responsible for this differentiation of gentamicin treated groups to control group are characterized by variable importance plots from PLS-DA analysis (Fig. 3C and 4C). These ion signals of significant change at pre-dosing and post-dosing of gentamicin are thought to correspond to endogenous metabolites in the urine. Moreover, those signals of gentamicin metabolites were excluded during PCA and PLS-DA analysis, mostly characterized perturbations in a group of endogenous metabolites. There are total 35 of endogenous metabolites are found to show the most marked perturbations over the entire time-course of the sampling. These 35 of marked metabolites by characterizing variable importance plots are found to be proofed contributable to significant perturbations of biochemical metabolism induced by gentamicin, they are deduced identified as pyroglutamic acid, glycerol 3-phosphate, thymine, D-Ribose 5-phosphate, agmatine, 3-Methylglutaconic acid, histidylproline diketopiperazine, L-Serine, hydroxypyruvic acid, hippuric acid, prostaglandin E2, 2-Furoylglycine, 2-Methoxyestrone, androstanediol, lactosamine, 2-Methylhippuric acid, 2-Furoic acid, 3-Hydroxyhippuric acid, creatine, ophthalmic acid, 5-Aminoimidazole ribonucleotide, deoxyadenosine, hexanoylglycine, gluconic acid, methyl bisnorbiotinyl ketone, vanillactic acid, glycyl-L-leucine, cholic acid, biotin, N-a-Acetylcitrulline, spermine, proline betaine, nicotinic acid, propinol adenylate, urocanic acid by combinational analyses of reference compounds, databases searching and theoretical identification.

Here, the m/z 178 as an example to elucidate the biomarker identification process. Firstly, the molecular weight (MW) and proposed formula of the potential biomarker was to be determined, the extract mass was acquired, making sure the MW is 179 (Supplemental Fig. 1A), subsequently the accurate molecular weight was also measured through the Q-TOF micro MS system (179.23), and the proposed formula was determined by extract mass and element composition analyses ($C_9H_9NO_3$) (Supplemental Fig. 1B). Secondly, further information about fragmentation patterns of the marker was searched for in the mass spectrum, and the MS/MS scan was employed in this step (Supplemental Fig. 1C). Furthermore, the database of Pub Compound (pubchem.ncbi.nlm.nih.gov), human metabolome database (http://www.hmdb.ca/), chemspider (http://www.chemspider.com/), METLIN Metabolites Database (http://metlin.scripps.edu/), *et al.* was retrieved subjected to the information got from the above process, the metabolites was proposed as hippuric acid (Supplemental Fig. 1D). Finally, the collision induced dissection mechanisms confirmed this deduction (Supplemental Fig. 1E). As a result, the biomarker of 178 was identified as hippuric acid. The other metabolites were identified by according to the same protocol.

These metabolites are found to be significant variations in abundance levels between a control group and treated groups over the entire time-course of the sampling, and characterization with multiple variation patterns are determined by relative quantification (Fig. 5). It is found that most or some of them could phenotype or involve in the development of gentamicin toxicity.

For example, the hippuric acid is an acyl glycine formed by the conjugation of benzoic acid with glycine, the amount of hippuric acid in the urine is a useful index of liver function[28] and kidney function,[29] the kidney toxicity is characterized by significantly decreasing hippuric acid in the urine.[29] In the present study, gentamicin are found to decrease slightly hippuric acid in the urine at 0–24 h post-dosing before remarkable decreasing at 24–48 h, keeping equal level until 48–96 h (Fig. 5), suggesting that gentamicin by targeting some biochemical pathways to induce kidney toxicity. Additionally, the gentamicin is found to marked increase the abundance level of creatine in the urine

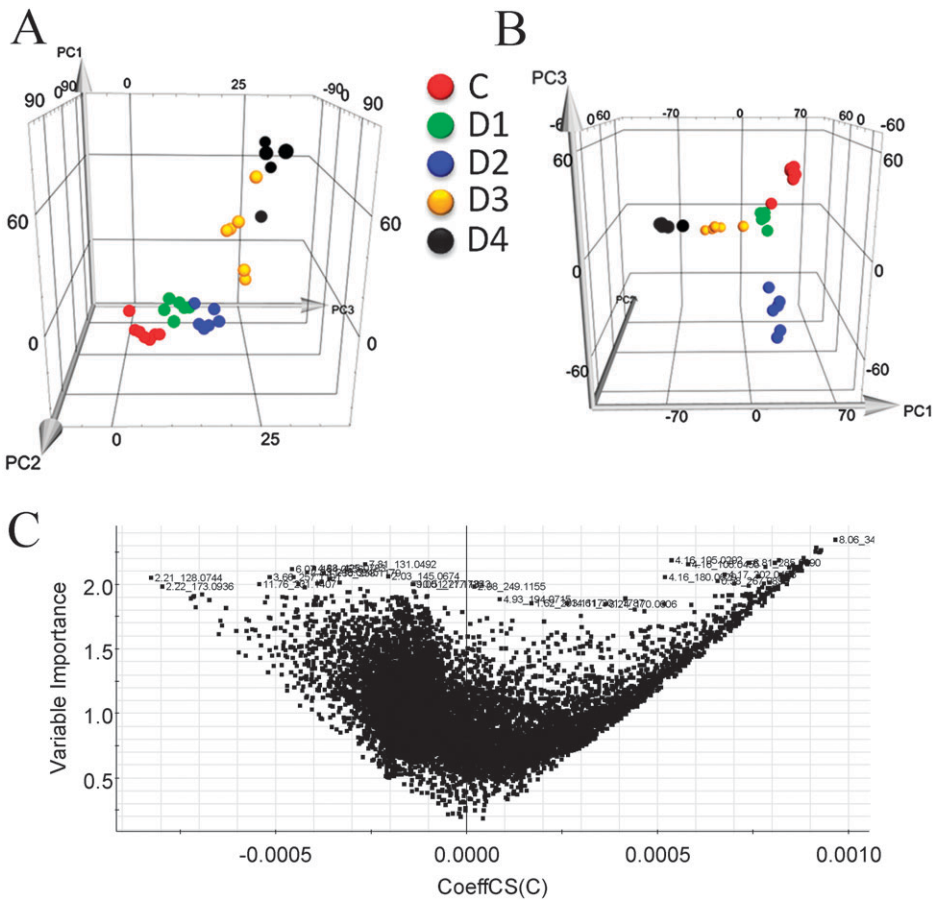

Fig. 3 Pattern recognition analyses of the positive ionization mode mass spectra metabolome from rats' urine. (A) PCA score plot from using the total ions intensities from the entire metabolome of 6 rat's urine samples screened by Q-TOF positive ionization mode. (B) Same data from Fig. 3A analyzed by PLS-DA. (C) scatter plot of the variable importance. The variables correspond to the different ions that contribute to the group's separation established in the PLS-DA analysis. Ions having a larger variable of importance coefficient imply a more significant contribution to the group's distinctive clusters formation, as consequence that the treatments (drug and time) promotes changes in the levels of these ions. C: −24–0 h pre-dosage of gentamicin; D1: 0–24 h, D2: 24–48, D3: 48–72 h and D4: 72–96 h post-dosage of gentamicin.

at 0–24 h post-dosing, keeping equal level at 24–96 h (Fig. 5). It is reported that the creatine is a well-known urinary biomarkers of liver injury.[30,31] This pattern of changes in creatine induced by gentamicin is an indicator of hepatic necrosis with glutathione depletion. The creatine is synthesized and metabolized in the liver and the observed increase in the urine suggests leakage from necrotic cells.[31] In short, these examples could support occurrence of the kidney toxicity and liver toxicity induced by the gentamicin.

Furthermore, the heatmap analysis for these 35 targeted metabolites is performed to validate PLS-DA analysis without noise interference (Fig. 6A), the result shows the same trends as showing in Fig. 4, the abundance level of these 35 targeted metabolites in the urine of rats at pre-dosing of gentamicin are total different to that at post-dosing of gentamicin. The marked time-dependent of abundance changes over the entire time-course is confirmed by the heatmap analysis (Fig. 6A). Moreover, the HCA plot shows the similarity between C and D1 is higher than D2 or between D3 and D4, being support that the gentamicin induce obvious biochemical perturbations of the endogenous metabolites. This pattern of change of targeted metabolites is characterized by the heatmap analysis to maximally prove biochemical perturbations induced by the gentamicin in rats. The validation of above pattern recognition analysis based global metabolites profiling is also confirmed by this analysis.

Subsequently, such 35 targeted metabolites are subjected to PCA and PLS-DA analysis in order to maximized phenotype the effect of gentamicin on biochemical metabolisms without interference of noise signals. As it showed in Fig. 6B and C, the metabolites level at −24–0 h pre-dosing of gentamicin are significantly different to that at 24–48 h, 48–72 h, and 72–96 h post-dosing of the gentamicin, consisting with Fig. 2 and 3. However, these pattern recognition analyses of targeted metabolites can more directly characterize the similarity and difference between groups when comparing with the analysis of global metabolites. By this analysis, the biggest differentiation distance in PCA and PLS-DA plots are found between −24–0 h pre-dosing and 24–48 h post-dosing of the gentamicin, suggesting the most significantly metabolic perturbation may appear at 24–48 post-dosing of the gentamicin. Fig. 6D demonstrates the coefficient between variables (targeted metabolites) and observations (groups), it can be seen that the coefficient of different metabolites at pre-dosing and

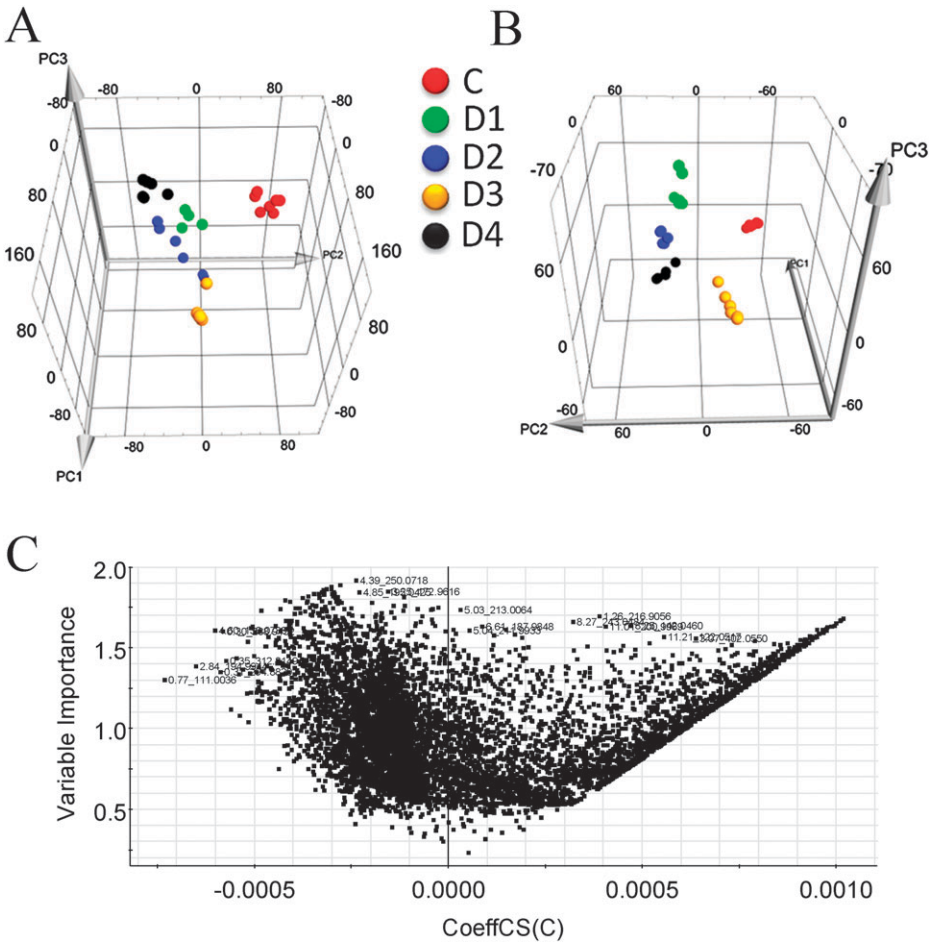

Fig. 4 Pattern recognition analyses of the negative ionization mode mass spectra metabolome from rats' urine. (A) PCA score plot from using the total ions intensities from the entire metabolome of 6 rat's urine samples screened by Q-TOF negative ionization mode. (B) Same data from Fig. 4A analyzed by PLS-DA. (C) scatter plot of the variable importance. The variables correspond to the different ions that contribute to the group's separation established in the PLS-DA analysis. Ions having a larger variable of importance coefficient imply a more significant contribution to the group's distinctive clusters formation, as consequence that the treatments (drug and time) promotes changes in the levels of these ions. C: −24–0 h pre-dosage of gentamicin; D1: 0–24 h, D2: 24–48, D3: 48–72 h and D4: 72–96 h post-dosage of gentamicin.

post-dosing groups of gentamicin are thoroughly different. These targeted metabolites are found to closely coefficient with single and keep lower coefficients with other groups over the entire time-course of the sampling, preliminarily suggesting these metabolites by targeting different functional pathways to contribute these perturbations of metabolic signature induced by gentamicin by revealing difference of changing pattern of time-dependent of these targeted metabolites.

In the present study, a sampling based different time points from −24 to 96 h is figured out to phenotype system metabolic perturbation induced by gentamicin. As results, the mean trajectory plot combined with PLS-DA plot at time-points post-dosing is shown in Fig. 7A, which highlights the changes of the metabolic trajectory of these endogenous metabolites of the time-dependent induced by gentamicin over the entire time course of the sampling, showing a number of marked changes of the urine metabolome from rats treated with the gentamicin. Subsequently, the variable importance plot orders the importance of these 35 metabolites in the contribution to metabolic perturbation induced by gentamicin(Fig. 7B), revealing contribution of other metabolites are almost the same excepts the metabolites of first top 5 with a little high contribution, but the difference is not significant. The coefficient plot of time-points and targeted metabolites is shown in Supplemental Fig. 2, it is found that these metabolites marked coefficient with the different time-point pre-dosing and post-dosing of the gentamicin are obvious different, but somewhat overlay of the coefficient metabolites within inter time-points is found, supporting again the different targeted metabolites by different changing patterns of action over the entire time course to contribute to metabolic perturbation induced by gentamicin.

3.4 Proof-of-knowledge based ingenuity pathway analysis

The metabolites are interconnected through metabolic reactions, generally grouped into metabolic pathways.[32] Classical metabolic maps provide a relational context to interpret metabonomics experiments and a wide range of tools have been developed to locate metabolites within metabolic pathways. However, the representation of metabolites within separate disconnected pathways overlooks most of the connectivity of the metabolome.[32] By definition, the reference pathways

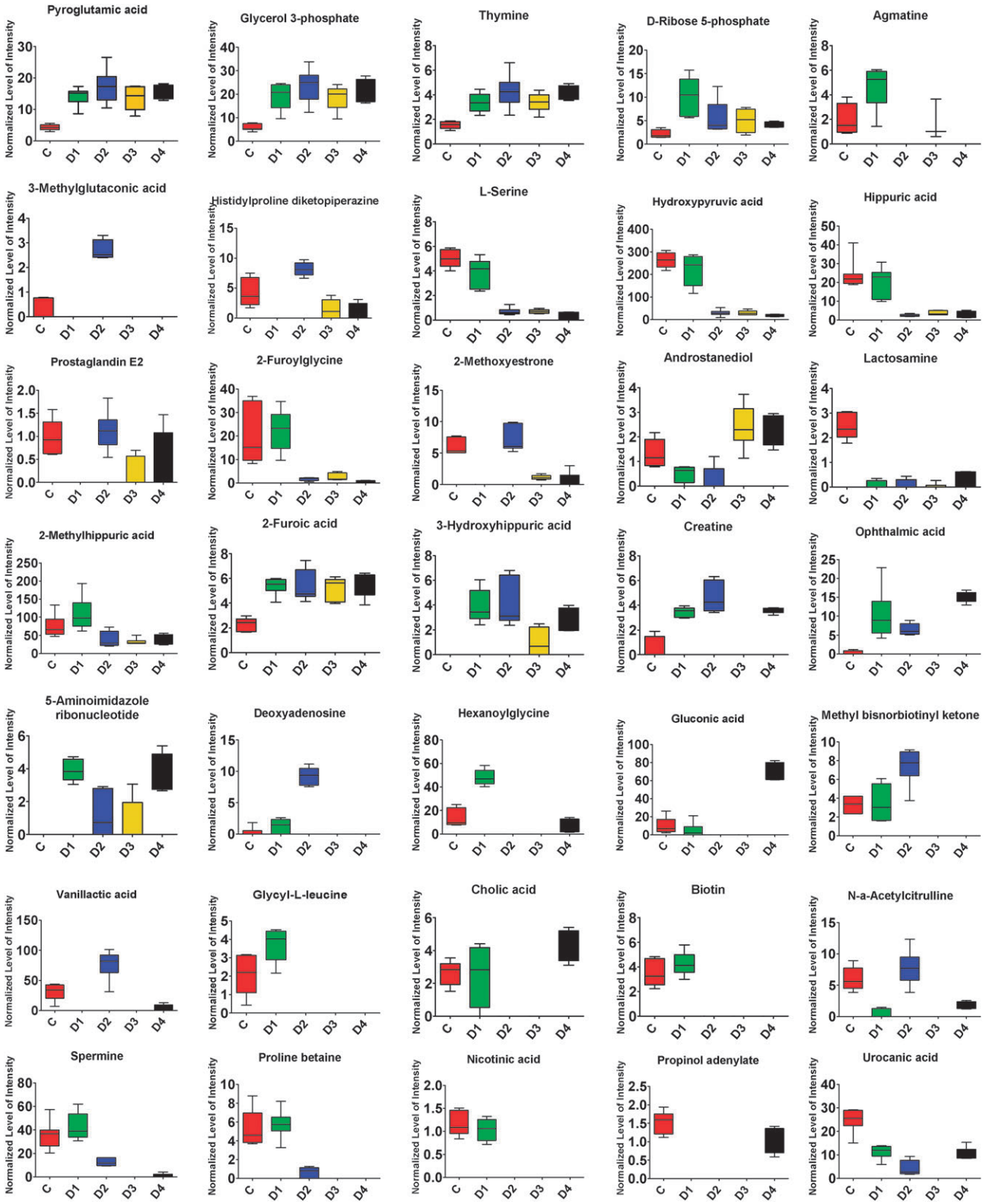

Fig. 5 Relative abundance of the 35 most affected metabolites by the administration of gentamicin. From the variable of importance plots (Fig. 3C and 4C), the metabolites exhibiting the most significant scores were selected, identified and their relative abundance levels are represented in the graphs. C: −24–0 h pre-dosage of gentamicin; D1: 0–24 h, D2: 24–48, D3: 48–72 h and D4: 72–96 h post-dosage of gentamicin; Bar: median; Box: 25–75% quartiles; Whiskers: min-to-max observation.

cannot integrate novel pathways nor show relationships between metabolites that may be linked by common neighbors without being considered as joint members of a classical biochemical pathway.[32]

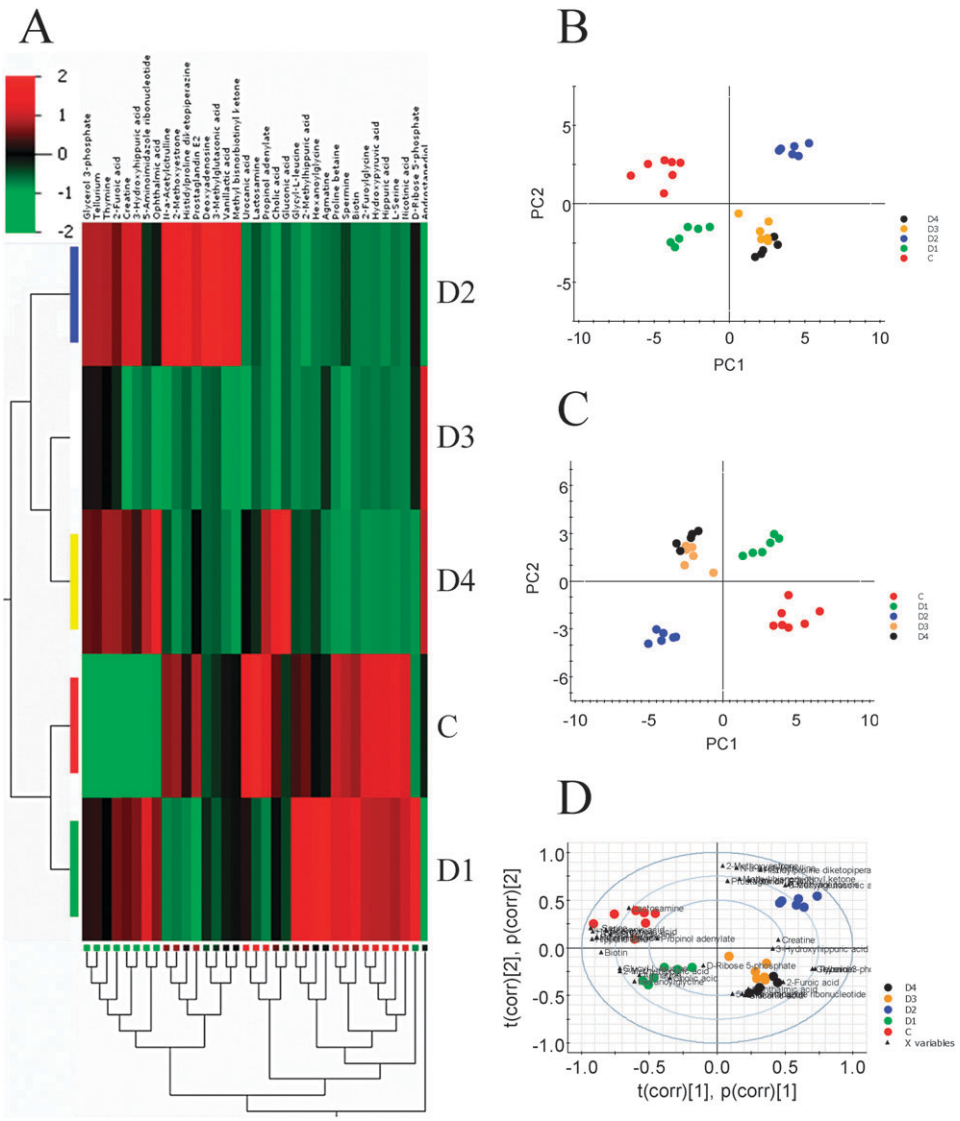

Fig. 6 Pattern recognition analyses of the 35 metabolites most relevantly changed by gentamicin. (A) Heat-map of the individual ions changes of abundance. The ions intensities are represented in a log 2 scale. The rows represent the treatments (C: −24–0 h pre-dosage of gentamicin; D1: 0–24 h, D2: 24–48, D3: 48–72 h and D4: 72–96 h post-dosage of gentamicin). The columns represent the metabolites ion intensities. (B) PCA score plot from using the ions intensities from the 35 most important metabolites changed in rat's urine samples by gentamicin. The metabolites were evaluated and identified by Q-TOF. (C) Same data from Fig. 6B analyzed by PLS-DA. (D) Bi-loading Scatter plot of the PLS-DA from the 35 most significant metabolites. Triangles: metabolite ID; Circles: Sample ID. This plot shows the correlation of the abundance from individual metabolites and the groups' distribution over the entire time-course of screening for gentamicin effect. Proximity of a given metabolite with a sample group indicates the existence high correlation coefficient.

The Ingenuity Pathway Analysis (IPA) is a proof of knowledge based comprehensive software of data analysis that can help researchers model, analyze, and understand the complex biological and chemical systems at the core of life science research.[33] By sub-metabolomics analysis in the IPA, the phenotypic data of the metabolites can be validated correlated the targeted metabolites with the potential metabolic pathways, and related to biochemical functions. Thus, the toxicity courses and target can be characterized *via* the toxicity analysis in IPA by correlating the biological function phenotype from the metabolomics analysis with the toxicological phenotype from the toxicity analysis.

Using the IPA Analysis, we characterize 19 endogenous metabolites from the 35 targeted metabolites to plot the marked change of biochemical metabolism induced by the gentamicin *via* the contribution screening. By the screening analysis of targeted toxicity, 12 of these 19 metabolites are determined to progressively contribute the toxicity induced by gentamicin (Supplemental Fig. 3), including 2-methoxyestrone, agmatine, biotin, cholic acid, creatine, hippuric acid, histidyl-proline diketopiperazine, hydroxypyruvic acid, L-serine, nicotinic acid, prostaglandin E2 and spermine. As it showed in Fig. S2, these proposed metabolites by targeting different classes of metabolic pathways to phenotype or involve in metabolic perturbation induced by the gentamicin toxicity, the amino acid metabolism, small molecule biochemistry, cellar development, growth, proliferation, and organismal injury and abnormalities,

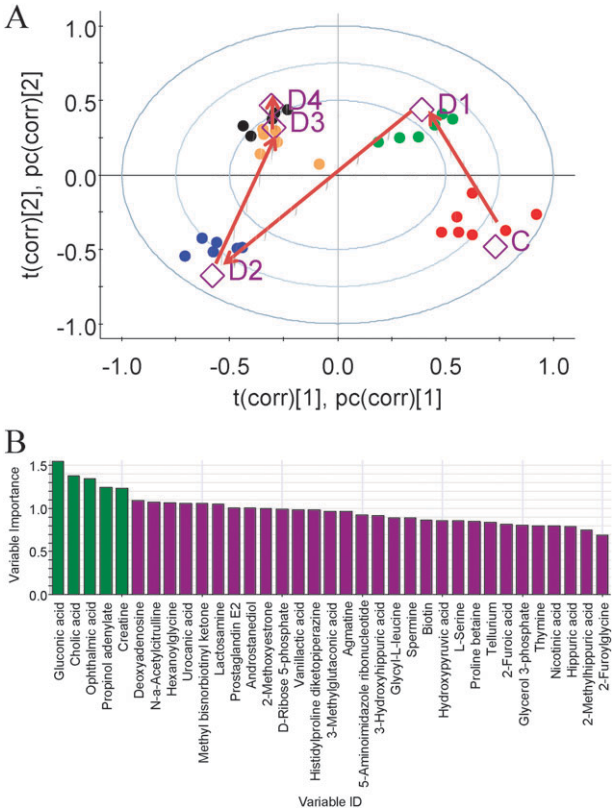

Fig. 7 Visualization of changes in the metabolic trajectories induced by the administration a single-dose of gentamicin. (A) Mapping positions established by the 35 most relevantly changed urine metabolites prior and after the administration of (C: −24–0 h pre-dosage; D1: 0–24 h, D2: 24–48, D3: 48–72 h and D4: 72–96 h post-dosage). The arrows indicate the average trajectory of the 35 metabolites most relevantly changing in the urine samples. (B) Variable importance bar plot shows the order of importance of main 35 metabolites contributing to the distinctive metabolic clustering. Bars in Green represent the five more significant metabolites; Bars in Purple represent metabolites contributing similarly but less than the previous five to the influencing the metabolic trajectories changes.

etc, majorly involve in the development course of gentamicin toxicity (Supplemental Fig. 4)

3.5 Toxicity prediction of multiple organs and phenotypic biomarkers

The IPA analysis is performed by targeting 12 of marked metabolites to phenotype system toxicity of the gentamicin on the rats. The gentamicin was found to induce the toxicity of multiple organs by targeting the liver, kidney and heart system. Especially, the toxicity of liver and kidney is predominant (Fig. 8). Four biomarkers are confirmed to significantly phenotype such system toxicity course of multiple organs of the gentamicin (Supplemental Fig. 5), including creatine, nicotinic acid, prostaglandin E2 and cholic acid. The IPA analysis prove that the creatine can be used to phenotype liver and kidney toxicities, the nicotinic acid can characterize functional abnormalities of heart systems and marked changes of prostaglandin E2 and cholic acid can reflect liver toxicity progress. This result is shown in Table 1 generated automatically from the IPA predictive analysis of the toxicity.

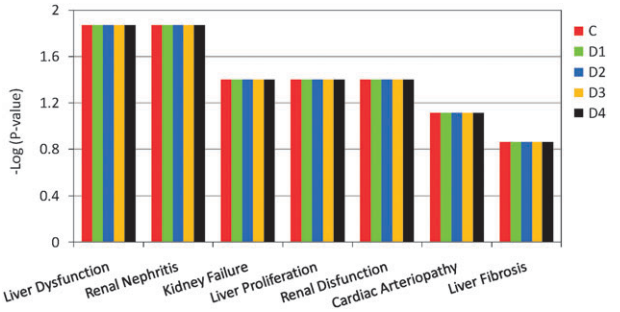

Fig. 8 Unsupervised toxicity predictions of gentamicin by the proof-of-knowledge based IPA analysis. A comprehensive analysis of the urine metabolome before and after the administration of gentamicin leaded to the identification and validation of specific biomarkers reflecting the toxic effects of this drug in liver, kidney and heart. The IPA toxicity model generated using the 35 most relevantly changed urine metabolites predicts a drug harmful effects primarily in liver and kidney, hover it is also predicted heart injure. The IPA prediction of the effects is based on the proof-of-knowledge association of changes in the phenotypic biomarkers with the most probabilistic metabolic pathways and organs. C: −24–0 h pre-dosage; D1: 0–24 h, D2: 24–48, D3: 48–72 h and D4: 72–96 h post-dosage.

Considering the correlation of creatine in urine with liver toxicity has been discussed in the former section, so here importantly highlighted that creatine phenotype kidney toxicity induced by the gentamicin, a group of data show that the creatine in urine is marked increased during development of kidney toxicity,[34,35] the gentamicin induces marked increase of creatine in urine (Fig. 5), suggesting the development of kidney toxicity at post-dosing of gentamicin, further supporting creatine is also a good biomarker to phenotype the kidney toxicity besides characterizing the liver toxicity.

Synthesis of the prostaglandin E2 is inhibited with the liver toxicity development,[36,37] thus, marked lower of prostaglandin E2 in the urine can phenotype liver toxicity occurrence. Because the prostaglandin E2 is known to protect against liver injury by enforcing host defense,[38] the remarked reduce prostaglandin E2 in urine suggests the function of host defense was degenerated, so that it was easy to be attacked by the toxicity, so as to damage synthesis of prostaglandin E2. In the present study, the prostaglandin E2 is found to be marked reduced at 0–24 h post-dosing of gentamicin, before recovering to control level at 24–48 h post-dosing of gentamicin, and significantly reduced again at 48–96 h post-dosing of gentamicin (Fig. 5), suggesting that this is a progressive course of gentamicin induced liver toxicity. Therefore, the reduction of prostaglandin E2 in urine is a changing pattern, and keeping remarked lower level at last, which characterizes the development of liver toxicity induced by gentamicin.

Moreover, the cholic acid was found to be marked increased in urine of patients with liver toxicity,[39] the increased cholic acid cannot only phenotype the development of liver toxicity but also deteriorate liver toxicity.[40] In this study, the cholic acid is increased marked at 72–96 post-dosing of gentamicin, and showing a bigger vibration in abundance with slight change at 0–24 h, and remarked reduced at 24–72 h post-dosing of gentamicin, suggesting again that this is a progressive course of the liver toxicity induced by gentamicin, and the

Table 1 IPA-Tox biomarker association of gentamicin toxicity in various organs

Category	Function	Function Annotation	*p*-value	Molecules
Liver Dysfunction	Dysfunction	Dysfunction of liver	1.34E-02	Creatine
Renal nephritis	Nephritis	Nephritis of humans	1.34E-02	Creatine
Liver proliferation	Proliferation	Proliferation of hepatocytes	3.96E-02	Prostaglandin E2
Renal dysfunction	Dysfunction	Dysfunction of kidney	3.96E-02	Creatine
Cardiac arteriopathy	Coronary artery disease	Coronary artery disease	7.77E-02	Nicotinic acid
Liver steatosis	Hepatic steatosis	Hepatic steatosis	1.38E-01	Cholic acid

marked increase of cholic acid at 72–96 h post-dosing of gentamicin can phenotype liver toxicity of gentamicin (Fig. 5).

At last, the nicotinic acid (niacin) is a well-established treatment for dyslipidaemia-an important cardiovascular disease (CVD) risk factor, It is still the most efficacious drug in terms of its ability to increase HDL cholesterol content accompanied by a decrease in all atherogenic lipoproteins as well as fatty acids and triglycerides.[41,42] Niacin has been used for decades in the treatment of patients with disturbed lipid and lipoprotein metabolism, this being the main cause of atherosclerotic changes in cardiovascular diseases.[43] There is data confirming the beneficial action of niacin against migraine and hyperphosphataemia associated with renal failure.[43] Therefore, it was deduced that marked decrease of nicotinic acid is a phenotype of cardiac disorders or toxicity, suggesting synthesis of nicotinic acid is inhibited or decomposition is accelerated. In the present study, nicotinic acid is decreased slightly at 0–24 h post-dosing of gentamicin before remarked reduce at 24–96 h post-dosing, the data show the nicotinic acid is in deficiency over the time-course(Fig. 5). This suggests an increase occurrence of cardiovascular disease development, the gentamicin by blocking metabolic course of nicotinic acid to increase level of atherogenic lipoproteins as well as fatty acids and triglycerides so that the cardiovascular disease development is triggered indirectly, with a type of heart toxicity.

Together, gentamicin-induced kidney toxicity is characterized by tubular necrosis mainly involving proximal tubules.[44] It is shown that oxidative stress was the main cause of gentamicin-induced kidney toxicity.[45] However, the limited data demonstrated the toxicity of gentamicin on other organs; this should overlook some latent threat of the gentamicin on other organs of patients. It is necessary to introduce a method to overall clarify system toxicity of the gentamicin. In the present study, we proposed a metabonomics method by combining with proof of knowledge IPA could phenotype toxicity of multiple organs of the gentamicin, the data validated characterize the toxicity of multiple organs of gentamicin and of the behavior time-dependent by pattern recognition analysis and IPA, four phenotypic biomarkers of the toxicity induced by gentamicin are identified as creatine, nicotinic acid, prostaglandin E2, and cholic acid, the proof of knowledge based the published data analysis validates these four biomarkers and strongly supports the prediction of gentamicin toxicity on the kidney, liver and heart system.

4. Conclusions

An importantly successful application for metabonomics has been in to diagnosing drugs-induced toxicities in kidney, liver and heart, it resulting to be a much better approach to determine toxicity than classical clinical chemistry analysis of specific chemicals. Here we validated the use of urine metabonomics screening combined with IPA analysis as an usable strategy to characterize the biochemical perturbation induced by gentamicin toxicity, revealing urine biomarkers for the toxic effect in multiple organs and the potential metabolic pathways targeted by the drug. Using this strategy, was confirmed the gentamicin toxicity in the kidney, liver and heart. Four phenotypic biomarkers, creatine, nicotinic acid, prostaglandin E2, and cholic acid were identified and validated to be involved in the development of gentamicin toxicity in these organs. Our work confirms and strengthens the feasibility of metabonomics to investigate the drug toxicity in multiple organs, and it highlights the strong potential of the Ingenuity Pathway Analysis application on metabonome data for the assessment of drugs and chemicals toxicity.

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