Metabolomics to find toxicity mechanisms-based biomarkers

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Introduction

Metabolomics is defined as global analysis of all endogenous metabolites in biological samples. With the importance of metabolites as main effectors of a particular phenotype induced by alteration in genome, transcriptome or proteome, metabolomics has become an indispensable part of the molecular profiling technologies in the area of systems biology. Since the changes in concentrations of metabolites reflect pathological alterations in a biological system of interest, and the technology measures the fluctuations in hundreds of endogenous metabolites simultaneously in non-invasive (ex. urine) or minimum invasive (ex. blood) samples, pharmaceutical society has great expectations to identify novel biomakers to monitor disease status, drug efficacy or side effects.

In this symposium, we would like to share our experience to evaluate the benefit of metabolomics using NMR or mass spectrometry (MS)-based methods when applied to biomarker research of drug-induced toxicity such as nephrotoxicity and phospholipidosis.

Metabolomics analysis on nephrotoxicity by 1HNMR

To evaluate the power of metabolomics to find biomarkers of toxicity, the technology was applied to nephrotoxicity. Male Crj;CD(SD)IGS rats received single dose of puromycin aminonucleoside (PAN), a glomerular toxicant, or citrinin, a renal tubular toxicant. Urine were chronologically collected for 24 hours over dry ice and

subjected to 1HNMR analysis. The urine was pH adjusted with phosphate buffer, centrifuged and applied to 1HNMR (Bruker AVANCE 500 NMR spectrometer). The data were processed using XWINNMR Ver. 3.5 and AMIX Ver. 3.2.4 (Bruker). The identity of metabolites was confirmed through 1HNMR measurement of authentic standards.

PAN (100 mg/kg, iv) decreased urinary excretion of endogenous metabolites such as citrate, dimethylglycine, TMAO or betaine on 4 days after dose (Fig.1). At that time, urinary protein and serum urea nitrogen was increased with no histopathological lesions in the kidney. Citrinin (60 mg/kg, ip) caused renal tubular necrosis on 3 days after dose, and increased urinary glucose excretion. Metabolomics revealed variety of perturbations in urinary metabolites including increased excretion of amino acids, ketone bodies or lactate, and decrease of TCA cycle metabolites (Fig.2). Citrinin-treated animals showed inter-animal variation in urinary glucose excretion, a hallmark of renal tubular dysfunction. For example, animal No. 00307 or 00311in Fig.3 had comparable urinary glucose levels with control animals (i.e., 00108 in Fig.3) despite exhibiting severe renal tubular lesions. These animals, however, showed disturbances in urinary metabolites, including increase of lactate or acetate and decrease of succinate, 2-oxoglutarate or citrate (Fig.3), indicating the compound injured renal cells to suppress energy production in mitochondria.

These results demonstrate several merits of metabolomics analysis. First, it can discriminate site of lesion, such as glomerular versus tubular, in the kidney, facilitating non-invasive diagnosis. Second, by integrating the metabolome data into biological context, one can estimate functional alterations in a target organ. For example, increased amino acids in urine could be translated to retarded re-absorption of the metabolites from proximal tubular epithelium. Third, global metabolite profiling could offer the most sensitive metabolite(s) that reflects biochemical alteration in a system, hence provides potential biomarker(s). The decrease of TCA cycle metabolites accompanied by increased production of alternative energy-related metabolites could

be a better biomarker of citrinin-induced nephrotoxicity, which compound is known to injure mitochondrial respiration following incorporation into kidney cells.

Biomarker search of drug-induced phospholipidosis by CE-TOF-MS

Despite its relative easiness to measure metabolome and usefulness to assess nephrotoxicity, we consider 1HNMR metabolomics had several disadvantages, compared with MS-based methods, such as sensitivity, resolution or metabolite identification. To evaluate another platform that overcomes these difficulties, we tried a metabolomics analysis using capillary electrophoresis time-of-flight mass spectrometry (CE-TOF-MS) in biomarker(s) search for drug-induced phospholipidosis (PLD).

Male Crj;CD(SD)IGS rats received oral doses of vehicle, amiodarone or MPC1 (a Mitsubishi Pharma Compound) for two weeks. PLD was evaluated by histopathology and lung phospholipids (PL) contents. Urine and plasma were obtained at the end of the treatment, and subjected to CE-TOF-MS analysis. Plasma was deproteinized and ultrafiltrated before analysis. Urine was centrifuged and applied to CE-TOF-MS.

Amiodarone (150mg/kg) induced microscopically visible PLD in the lungs with 2.1-fold increase in PL content in the organ. On the other hand, MPC1 had no effect on the histopathology with 1.4-fold increase in the PL contents, demonstrating MPC1 induced milder PLD than amiodarone. Through the CE-TOF-MS metabolomic analysis, thousands of peaks were detected and aligned from sample to sample to facilitate differential analysis between groups. The peaks were reduced by statistical analysis as well as correlation analysis with lung PL content to extract promising biomarker candidates. Tandem mass spectrometry with CE-Q-TOFMS system was employed to clarify the identities of these peaks by confirmation of the exact molecular weight, migration time and MS/MS fragmentation pattern.

CE-TOF-MS metabolomics identified numbers of metabolites whose concentrations in the urine or plasma were changes along with the severity of PLD.

Metabolites identified included phenylacetylglycine (PAG), a benchmark metabolite reported to be altered by PLD, which were detected by 1HNMR or LC-MS metabolomics. Comparison with PAG showed that many of the metabolites newly discovered had superior property as biomarkers of PLD, especially in detection of milder PLD in terms of statistical significance and magnitude of changes (Fig.4). The data demonstrated the advantage of the MS-based metabolomics approach to find novel biomarkers that otherwise would remain undetected.

Conclusion

We recognize that metabolomics, together with toxicogenomics and proteomics, will become an indispensable technology for pharmaceutical companies in biomarker search as well as mechanistic study to solve toxicological problems arose from research and development programs. Despite the usefulness of metabolomics in biomarker research as such shown above, however, we consider the current technology needs more improvement to fully facilitate the R&D process. For example, the improvement of detection sensitivity, accompanied by development of peak identification methodology is expected. Strategic selection of platforms is needed because metabolome consists of various types of substances that differ in their physicochemical feature. Until the achievement of these innovations, global metabolomics should be followed by targeted metabolomics, a focused but intensive analysis on particular types of metabolites, to yield best biomarkers.
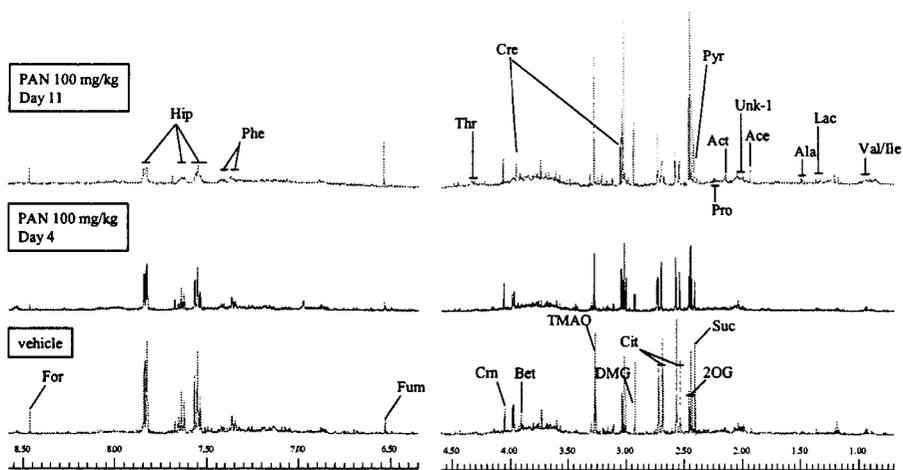
Fig.1 1HNMR spectrum of rat urine following dose of puromycin aminonucleoside (100 mg/kg)
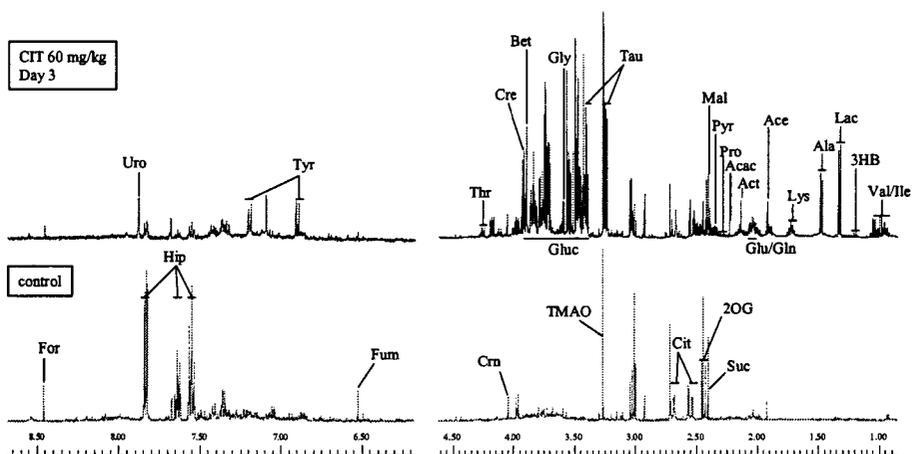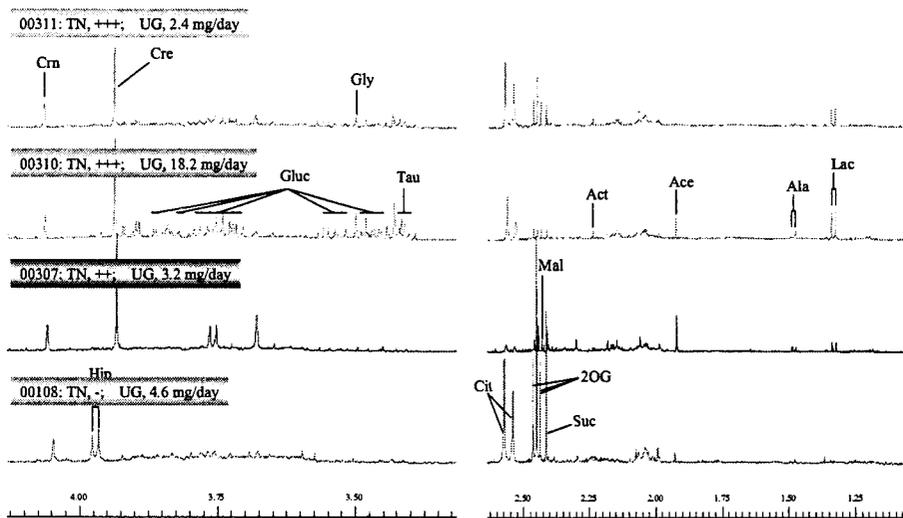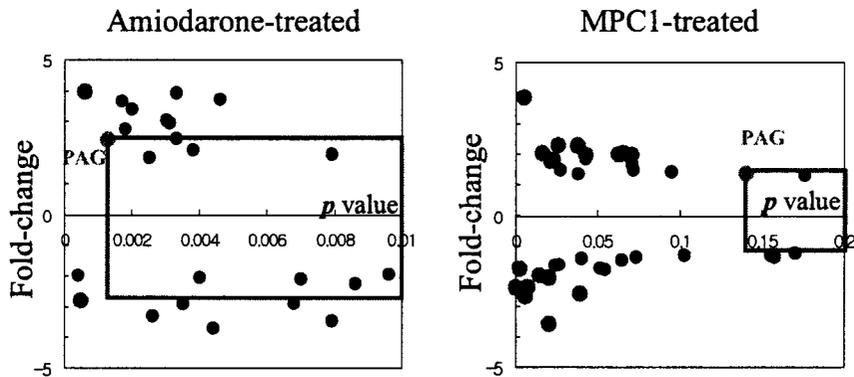

Fig.2 1HNMR spectrum of rat urine following dose of citrinin (60 mg/kg)

Fig.3 Metabolomics detected citrinin-induced nephrotoxicity which were not accompanied by increase in urinary glucose

TN, Tubular necrosis; UG, Urinary glucose excretion

Fig. 4 Comparison of the metabolites/peaks detected by CE-TOFMS with PAG to detect PLD. Each dot represents metabolite/peak. Pink dots mean the metabolites/peaks are superior to PAG in detecting PLD in terms of the magnitude of changes and p value. PAG is shown in green. Y axis: Fold change (mean intensity of peak in drug treated animals/that in the vehicle-treated animals). X axis: p value of each metabolite/peak, compared with the vehicle control.