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In vitro hepatotoxicity testing in the early phase of drug discovery

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Abstract
With remarkable advancement both in drug discovery, R&D activities and in concomitant technical supports, drug discovery strategies have become focused on drug safety screening in their early stages. The phenomenon suggests the necessity of evaluating toxicity of various compounds with small amounts of their samples as quickly as possible. This series of toxicological strategies is now commonly called "High-Throughput Toxicology", an essential part of toxicological study in the early phase of drug discovery, and particularly hepatotoxicity is one of key issues for drug safety evaluation to select the candidate compounds for new drug. In-vitro evaluation system as a screening system plays an important role in the early phase of pharmaceutical development. They are also important to clarify the mechanisms of toxicity observed during development. Toxicopanomics technologies (a collective designation for the "-omics" such as toxicogenomics, toxicoproteomics and metabonomics) is expected to be applicable to predictive toxicology and mechanism-based risk assessment. Currently, toxicopanomics technology is being applied to the development of new in-vitro safety evaluation systems. In this paper, we will introduce in-vitro hepatotoxicity screening tests, and show the correlations between in-vitro and in-vivo systems and its application of toxicopanomics technologies to in-vitro safety evaluation systems including toxicologically responsible biomarkers.

Keywords: high-throughput toxicology, hepatotoxicity, toxicopanomics, biomarker, *in vitro* evaluation

Introduction

In order to make a safety assessment in the early stage of drug discovery, there is a hurdle to jump over the existing traditional toxicological studies. That is high-throughput toxicological study. Namely, applied compounds to be tested in discovery phase are small amounts and many types that are pharmacologically targeted one. In this high-throughput approach, project strategies and directions as clarifying the toxicity generation, ranking of the candidates and risk assessment (critical or manageable?) are made. Then, paradigm-shift for high-throughput toxicology like in-vitro toxicological approach is needed. Generally, toxicological assessment with cell culture system, particularly hepatotoxicity would be applied in the early toxicological screening with molecular toxicological approach. These data also would be usable for further risk and safety assessment. In terms of introduction of in-vitro screening tests for toxicity evaluation, the following consideration should be addressed.

1) Sufficiently designed in-vitro system provides useful information to clarify toxicity generation and its mechanism.
2) Since quick and efficient measurement of multiple compounds is simultaneously available under the same condition, in-vitro tests are useful for the screening or ranking of compounds.
3) Investigation of hepatotoxicity as a major indicator of the general screening system can trigger the safety evaluation in the early phase of drug discovery, because most compounds are metabolized in the liver.

On the other hand, cells never function independently in an organism. Instead, they form close and complicated networks with each other or with the matrix of the organism to realize their functions in a three-dimensional structure. Therefore, in-vitro test data should be interpreted in view of the existence form of cells in the organism and the surrounding environment, and the results obtained must be reviewed in an in-vivo system. Particularly,

how much the cells to be used in an in-vitro test maintain normal properties significantly affects the accuracy of extrapolation into the subsequent relevant in-vivo system. Although primary cells in culture and tissue slices maintain relatively normal properties, it is difficult to maintain their properties over a long period. Established cell lines keep properties stable for a long time, but it must be noted that they are inconsistent in many aspects with the properties of normal cells. As other consideration, it would be pointed how to integrate the in-vivo metabolic factors of compounds into the in-vitro system environment.

In in-vitro hepatotoxicity screening system of cell and tissue culture, cell line culture, monolayer cell culture, spheroid/reaggregate cell culture and organ/slice/tissue culture systems are used. For the cell culture condition, cell suspension, monolayer, gel matrix and spheroid are applied. They are applied case by case approach, based on the purpose of its safety assessment.

Examples as several approaches in in-vitro hepatotoxicity testing

1. Traditional and toxicoproteomics approach for the estimation of biomarker

In the hepatotoxicity evaluation of compounds on an in-vitro screening system, it is essential to set biomarkers well reflecting in-vivo toxicity. Examples of developing hepatotoxicity biomarkers by means of proteomics technologies are explained below.

Four compounds known as hepatotoxicity were investigated: Acetaminophen (APAP), Amiodarone (AMD), Tetracycline (TC) and Carbon tetrachloride (CTC). APAP, AMD, TC and CTC, toxicity in rat primary cultured hepatocytes was evaluated. In investigation, morphological changes, lactate dehydrogenase (LDH) release (as the indicator of cytotoxicity) and changes in WST-1 (as the indicator of effects on mitochondrial respiration) were evaluated. Furthermore, changes in protein expression were analyzed by toxicoproteomics. It was found that all the compounds induced morphological changes and caused a dose-dependent increase in LDH release and a decrease in WST-1. Changes in WST-1 occurred in shorter exposure time than those in LDH, suggesting that mitochondrial respiration ability would be a useful parameter for cytotoxicity in in-vitro hepatotoxicity screening. The analysis of changes in protein expression by proteomics in APAP, TC and AMD-exposed samples showed changes in the expression of oxidative stress-related proteins and mitochondrial regulation-related proteins. And three proteins whose expressions commonly changed in response to the exposure to the three compounds were identified (glutathione peroxidase, peroxiredoxin 1, and peroxiredoxin 2). These data suggested that the oxidative stress-related proteins and mitochondrial regulation-related proteins were useful as hepatotoxicity markers in in-vitro system (Kikkawa, 2005; Yamamoto, 2005).

In order to compare the correlation between in-vitro and in-vivo hepatotoxicity and investigate hepatotoxicity biomarkers in-vivo hepatotoxicity studies in rats using same 4 hepatotoxicants (APAP, AMD, TC and CTC) were conducted. Three hundreds or 1,000 mg/kg of APAP, 300 or 1,000 mg/kg of AMD, 600 or 2,000 mg/kg of TC and 0.3 or 1 mL/kg of CTC were orally administered once to rats, and changes in blood biochemical parameters as well as histopathological changes were investigated 6 and 24 hours after administration. For the livers of APAP-administered rats, changes in protein expression were investigated by toxicoproteomics. It was found 24 hours after administration that all the compounds had caused histopathological changes such as inflammatory ones. Six hours after administration, immunohistological examinations revealed the expression of oxidative stress-related proteins including hemoxigenase-1, manganese superoxide dismutase, heat shock protein 70, selenium-dependent cellular glutathione peroxidase and 8-hydroxyguanosine. The analysis of changes in protein expression by toxicoproteomics detected changes in the oxidative stress-related proteins and mitochondrial metabolism-related proteins. And eight proteins whose expression commonly changed in response to the administration of the four compounds were identified (2-oxoisovalerate dehydrogenase α subunit, 60k Da heat shock protein, adenylate kinase isoenzyme 4, carbonic anhydrase III, glutamate dehydrogenase 1, NADP-dependent

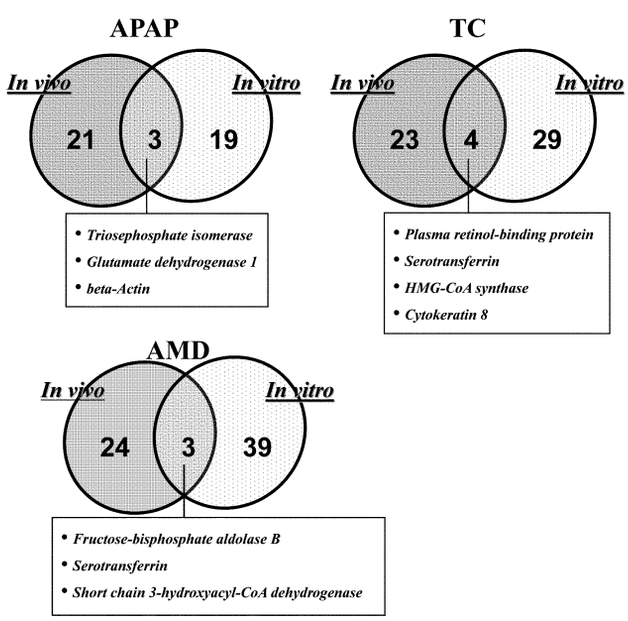


Fig. 1. Venn diagram showing the number of differentially expressed spots of the treatments with APAP, TC and AMD. This figure showed that several proteins were affected in both of in-vitro and in-vivo systems.

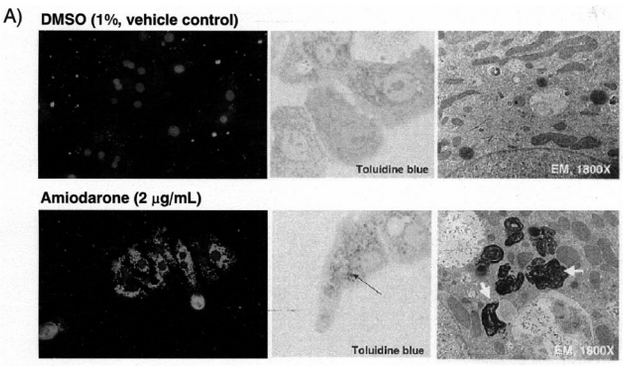
malic enzyme, NDRG1-related protein NDRG2b1, and serotransferrin). These results showed that the oxidative stress-related proteins and mitochondrial metabolism-related proteins are useful as hepatotoxicity evaluation markers in in-vivo systems (Kikkawa, 2006; Yamamoto, 2006).

In toxicoproteomics analysis, the expression of three proteins (triosephosphate isomerase, glutamate dehydrogenase 1, and beta-actin) in APAP, three proteins (fructose-bisphosphate aldolase B, serotransferrin, and short chain 3-hydroxyacyl-CoA dehydrogenase) in AMD and four proteins (plasma retinol-binding protein, serotransferrin, HMG-CoA synthase, and cytokeratin 8) in TC changed commonly between in-vivo and in-vitro. As reliable markers for in-vivo hepatotoxicity, these proteins may be used in in-vitro hepatotoxicity evaluation systems (Fig. 1).

2. Pospholipidosis estimation by fluorescence-probe in phospholipids

Phospholipidosis, a pathological condition with phospholipids excessively accumulated in cells, often impedes development in drug discovery. In general, drugs which induce phospholipidosis are known to contain both a hydrophobic domain and a hydrophilic cationic domain. These drugs are referred to as cationic amphiphilic drugs. Examples of predicting expression of phospholipidosis based on phospholipidosis evaluation on an in-vitro system are presented below.

In order to examine whether the employed in-vitro assay can estimate the phospholipidosis induction in-vivo, the validation studies were carried out with phospholipidosis-positive or negative compounds. The in-vitro experiment was performed based on



B)

		In vivo results	
		Positive	Negative
In vitro results	Positive	True Positive 10/10	False Positive 0/10
	Negative	False Negative 0/10	True Negative 6/6

Fig. 2.
A) Fluorescence microscopy images, toluidine blue staining and transmission electron microscopy (EM) images of primary rat liver cell cultures treated with DMSO (1%, vehicle control) and amiodarone (2 μg/ml). The fluorescence-labeled lipid appears as fluorescent droplets surrounding the nuclei. Accumulation of dense inclusion bodies were induced by amiodarone (Toluidine blue staining: black arrow). The inclusion bodies formed distinct lamellar structures (EM: white arrow). X1800.
B) Correlations between in-vitro and in-vivo evaluation systems for phospholipidosis.

a method by Gum et al. (2001) with modification (Tomizawa, 2006). Primary rat cells in culture were added with compounds and a fluorescent-labeled phospholipid analogue, and 24 hours later, intake of the fluorescent dye into the cells was observed under the microscope. Evaluation with 10 compounds known for their ability to induce phospholipidosis and 6 negative compounds yielded results correlating

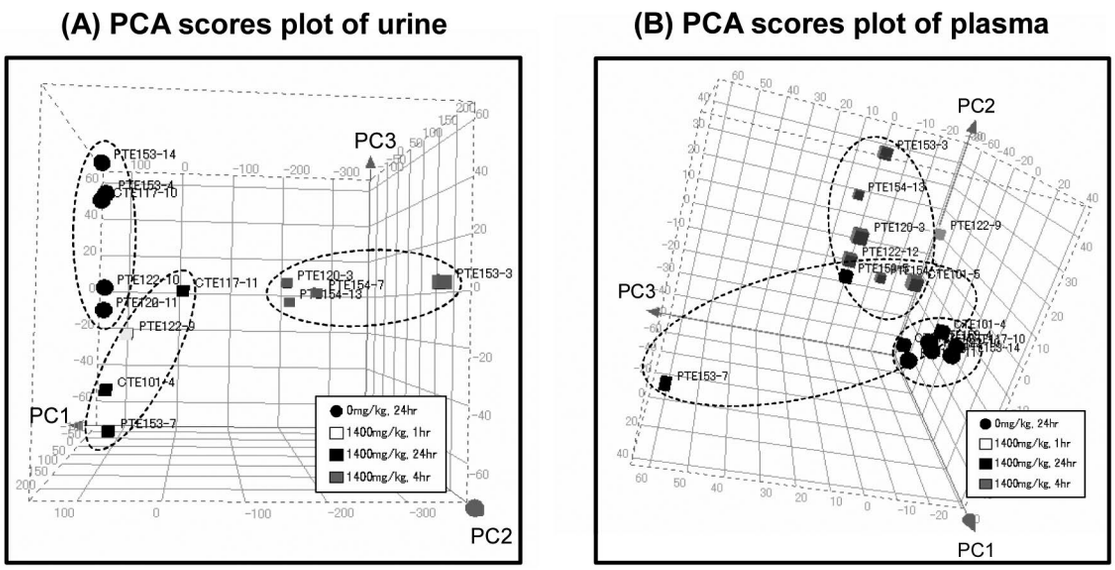


Fig. 3. This figure shows the results of metabonomic analysis using urinary and plasma samples. The figure on the left shows the PCA scores plot of urinary samples, and the figure on the right shows the PCA scores plot of plasma samples. As shown in the results of metabonomic analysis, the PCA scores plot indicated the clear separation among the treatment groups.

with their in-vivo induction (Fig. 2). From these results, this appraisal method was effective, and it was suggested the possibility as a phospholipidosis evaluation system at compound screening for safety evaluation.

3. Toxicopanomics approach for the evaluation of human hepatocyte transplanted chimeric mice

Human hepatocyte-chimeric mice were generated as previously descrived (Tateno, 2004). A uPA mouse with liver failure was crossed with a SCID mouse with immunodeficiency to produce uPA/SCID mouse, and human hepatocytes were injected to the spleen of the mouse to transplant human hepatocytes in the liver. In this experiment, mice with livers that were repopulated with human hepatocytes by ≥70% were used. To elucidate the usefulness of chimeric mice in toxicological studies, the validation study was carried out with acetaminophen (APAP). In this study, 1,400 mg/kg of APAP was orally administered once to chimeric mice, and intravesical urine and serum collected one, four and 24 hours later were used as samples. The effects on gene and protein expression profiles in liver of chimeric mice and endogenous metabolites secretion profiles into plasma and urine were investigated using Affymetrix GeneChip-based toxicogenomics, 2-dimentional electrophoresis-based toxicoproteomics and ^{1}H-nuclear magnetic resonance (NMR)-based metabonomics. As a change of toxicogenomics, adverse effects of APAP on detoxification process were characterized the gene expression in glutathione biosynthesis, CYP activation, sulfation, and glucronidation. In toxicoproteomics analysis, all protein expression changes were related to expressed protein-biomarker such as oxidative stress and energy metabolism. In metabonomics analysis, the PCA scores plot indicated the clear separation among the treatment groups in the urine and plasma (Fig. 3), and the energy metabolism related metabolites were defined as a biomarker (Yamamoto, 2007).

The results of toxicopanomics analysis were showed that the hypothetical mechanism of the effects of APAP on chimeric mice was related to oxidative stress, glycolysis/gluconeogenesis, lipid/fatty acid/amino acid metabolism. There is a possibility that the changes detected by toxicopanomics analysis may be used as markers for APAP-induced hepatotoxicity in humans because the animals used in the experiment were a human model.

Summary and perspectives

In safety assessment of compounds, accurate extrapolation in the test system and between species is an important and essential task. In many cases, there is a quantitative difference in the dose-response relation between humans and laboratory models, and in extreme cases, the biological response can be qualitatively different. Therefore, the importance of the development of "bridging biomarker" used for the association with the toxic reaction in the test system and between species has been recognized, and toxicopanomics is believed to be effective in the development of new bridging biomarkers. By using reliable bridging biomarkers, in-vitro screening system that can assess the toxicity in humans or animals accurately is expected to be developed.

As comprehensive analyses of genes or proteins became available, bioinformatics (information-processing technologies) has long been expected to grow to interpret floods of data created by those analyses. Bioinformatics is currently under energetic study and development as technologies to select and compile data characteristic of specific life phenomena. However, trends toward the next generation have already been found; i.e., system biology, a novel study area aimed to understand life phenomena as a system, is attracting attention. With the progress of development and implementation of new technologies represented by toxicopanomics, understanding of components constructing life such as genes and proteins has rapidly advanced. System biology is intended to comprehend such information in the dynamics of life phenomena (Kitano, 2002). Results from study on system biology will assume important roles in developing simulation models and analyzing life behaviors (including pathological conditions). The area of toxicology will also greatly benefit from system biology, which is expected to grow into "system toxicology."

While development of in-vitro safety evaluation systems is dramatically advancing, information has become batch-processed with the advent of high-throughput and comprehensive analysis systems. In the future, even in-silico systems for simulating life phenomena and automatically analyzing life behaviors are to be developed.

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