白い点は，各種の腫瘍病変のみならず非腫瘍病変全体がひとつの関数として動くことであり，それらの病変の発症にリンクするゲノムの不安定性の亢進といった共通因子の関与を示唆しているのであるが，現時点での知見は，現象論的事実にとどまる。図25に戻ってさらに興味深いことは，これに対して酸化的ストレス（ROS）の吸収を促進するチオレドキシン過剰発現マウスにベンゼンを曝露すると，図26のように平均寿命が大きく回復・延長するのみならず，最長寿命の延長傾向が見られる。

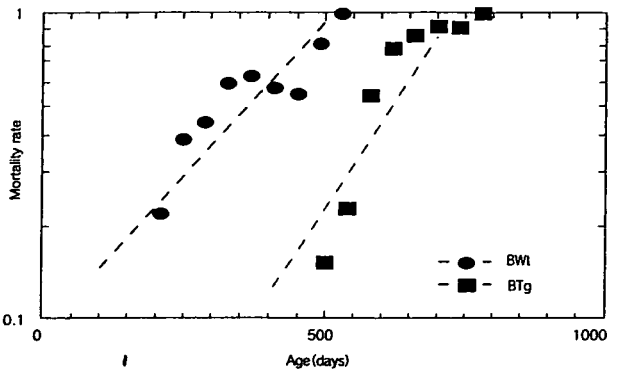

図26　ベンゼン曝露による寿命短縮とチオレドキシン過剰発現マウスにおけるベンゼン毒性の予防

6.4 生体の異物応答と種々の抗毒作用の寿命への影響

これらの毒性学的な寿命短縮要因とカロリー制限やチオレドキシン過剰発現による寿命延長（抗毒作用）の要因を図27に総合的にまとめた。ここに示すGompertz Curveは，実データ（real data）にもとづく寿命とトキシコロジーの相互関係である。これらの背景を構成する生体に対する異物の反応性を明らかにすることは，寿命に対する異物の要因の究極的なメカニズムを明らかにすることになるものと考えられる[50]。

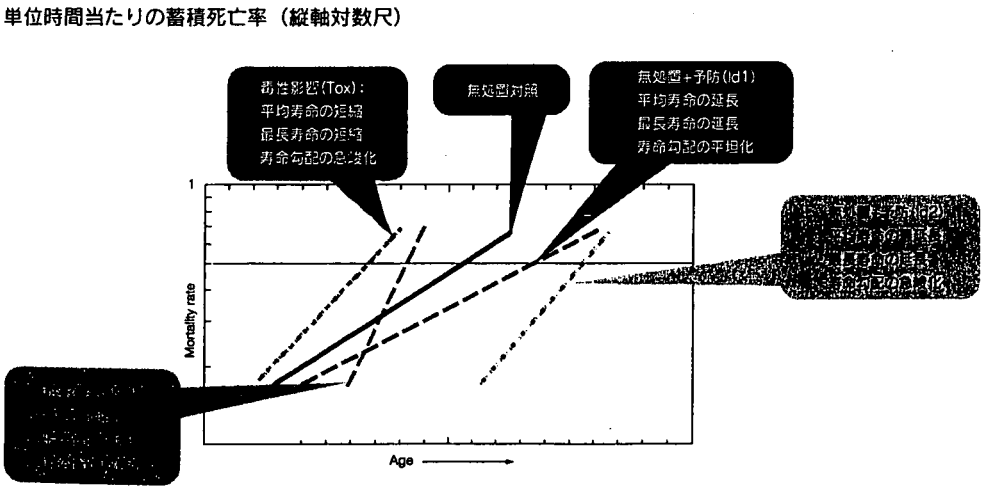

図27　寿命とトキシコロジー：まとめ

おわりに

本稿では，発生，成長，老化の三つに区切ってトキシコロジーの将来について筆者の考え方を述べた。それらのことがらは，いずれも，まだガイドラインの作られていない事柄である。ガイドラインのないこれらの事柄に注目するモチーフが何処にあったかというと，はじめに気づくことは，今回取り上げた周辺の事柄には分からないことが山積していて，現在進められている試験の解釈にしても，しばしばその辺のところを検討しないと，うまく説明がつかないということがあるということに起因している。そしてもっと大枠のところから考えてみると，私たちをとりまく社会一般のニーズがそれを求めるようになっている，ということではないかと思われる。

すなわち，少子高齢化社会は限りなく子供の安全に対する取り組みを求めており，WHOでも国連でも大きな問題になっているところである。思春期の問題も，胎生期に次ぐ第2の形態形成期にあって，不明の点がたくさんあり，この時期の過ごし方が一生を決めるところさえあるにも拘わらず，これまで，あまり顧みることがなかった。加えて，筆者の卒業後の研究生活は，まだ当時あたらしい分野であった老年学・老年病理学からはじまっている。いまや高齢者人口は，地域によっては25%にも及び，全国平均でも2割に達している状態にある。高齢者が自らの生活に対して，多くのものを云う人々になろうとしている。

私たちの携わる毒性学もそれらの変化に対応してゆく必要を求められている。もとよりキチンとした答えのある領域ではないが，本稿が議論のきっかけの一助とでもなれば，望外の喜びである。

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In vitro gene expression analysis of nephrotoxic drugs in rat primary renal cortical tubular cells

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ABSTRACT: **Rat primary renal cortical tubular cells were exposed to seven test substances, some with, and some without, known direct renal tubular cell toxicity. Cells were exposed to the substances at either one-third or one-tenth of the TC_{50} for cytotoxicity for 6 h or 24 h, so as not to induce cytotoxicity but to cause some transcriptional changes. Transcriptional profiles were investigated by using the Affymetrix Rat Toxicology U34 arrays, containing probes for more than 850 genes and ESTs. Four direct toxicants, cisplatin (CDDP), its less nephrotoxic analogue carboplatin (CBDCA), cephaloridine and gentamicin, were grouped together in a hierarchical clustering. In addition, the four direct toxicants affected more than 32 transcripts at their subcytotoxic concentrations at either 6 h or 24 h exposure. On the other hand, diclofenac, cyclosporine A and zinc, which are not considered to be directly toxic to tubules, affected less than 12 transcripts. Decreased Map3k12 and increased Hmox1 were commonly observed among the four direct toxicants, which appeared to be responses to cellular damage. Two platinum complexes, CDDP and CBDCA, induced similar changes, regardless of exposure duration or concentration. The types of transcriptional changes observed in this study were consistent with previously reported *in vivo* data, although there were some differences. These observations suggest that an *in vitro* gene expression analysis approach using GeneChip is feasible for screening for direct tubular toxicity of drugs and may help to clarify the underlying mechanisms of tubular toxicity. Copyright © 2008 John Wiley & Sons, Ltd.**

KEY WORDS: *in vitro*; primary culture; nephrotoxicity; kidney; microarray; gene expression

Introduction

The evaluation of nephrotoxicity is critical in the course of drug development, because it is one of the major toxicities of drugs. This study assessed the feasibility of an *in vitro* gene expression profiling approach, using microarray, as a nephrotoxicity testing tool. *In vitro* approaches to screen for drug-induced toxicity are important, especially in the early stage of drug development, where only a small amount of test item is available. In addition, *in vitro* methods are useful for evaluating direct toxicity of drugs against target cells. This study selected the renal tubular cell as a test cell because it is one of the critical targets in drug-induced nephrotoxicity. Although microarray technology is considered to be a prospective tool for drug screening, there are few published reports on expression analysis of nephrotoxicity using microarrays, and there are no published reports on *in vitro* expression analysis of nephrotoxicity comparing several drugs using microarrays. Recently, the MicroArray Quality Control (MAQC) consortium showed reproducibility across different microarray platforms, including the Affymetrix GeneChip, and revealed good correlation between TaqMan data and microarray data (Shi *et al.*, 2006). Their data indicated the reliability of expression analysis using GeneChip. This study used the Affymetrix GeneChip Rat Toxicology U34, which contains probes for more than 850 genes and expressed sequence tags (ESTs). The study first compared the expression profiles of two different culture conditions of primary rat renal cortical tubular cells without drug exposure, both of which are utilized in our laboratory, i.e. conventional culture on collagen I plates and culture on collagen I-gel coated plates. After optimization of the culture condition, expression analysis of *in vitro* nephrotoxicity was performed with seven test substances, using the collagen I-gel culture condition. Four of the substances, cisplatin (CDDP), the less nephrotoxic analogue carboplatin (CBDCA), cephaloridine (CR) and gentamicin (GM), have been reported to cause direct tubular necrosis (Borch and Pleasants, 1979; Luft *et al.*, 1975; Wachsmuth, 1981; Wolfgang *et al.*, 1994) (Table 1).

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Table 1. TC_{50} of test substances and test concentrations for the microarray experiment

Test substance	Cytotoxic conc.[a] TC_{50} (μM)	Test conc. for microarray exp. 1/10 TC_{50} (μM)	1/3 TC_{50} (μM)	Test substance information Category	Tubular toxicity Mechanisms	References
Directly toxic to the tubule						
CDDP	18[b]	18	60	Platinum complex (anti-cancer)	+ (direct tubular necrosis)	(Borch and Pleasants, 1979)
CBDCA	2520[b]	252	840	Platinum complex (anti-cancer)	+ (less nephrotoxic than CDDP)	(Wolfgang *et al.*, 1994)
CR	2310[b]	231	770	β-lactam (anti-bacterial)	+ (direct tubular necrosis)	(Wachsmuth, 1981)
GM	40	4	13	Aminoglycoside (anti-bacterial)	+ (direct tubular necrosis)	
Not directly toxic to the tubule						
DF	859[b]	86	286	Phenylacetic acid derivative (NSAID)	+ (hemodynamic, allergic)	(Murray and Brater, 1993; Perazella, 2005; Perazella, 2002)[c]
CsA	51	5	17	Polypeptide (immunosuppressant)	+ (hemodynamic)	(Busauschina *et al.*, 2004)
Zn	176	18	59	Trace element	– (not considered to be nephrotoxic in humans)	(Klaassen, 2004)
					– (low tox to cultured tubular cells)	(Rodilla *et al.*, 1998)

[a] Determined in a conventional monolayer culture condition based on the WST-1 colorimetric assay.
[b] Previous data from our group (Kawashima *et al.*, 2001).
[c] References for NSAIDs. + nephrotoxic. – not nephrotoxic.

The other three substances, a nonsteroidal anti-inflammatory drug (NSAID) diclofenac (DF), cyclosporine A (CsA) and zinc (Zn), are not considered to be directly toxic to tubules (Table 1), but DF and CsA are associated with tubular damage and therefore these substances were selected in order to confirm whether or not they would induce toxicological changes in the same setting as the direct toxicants. NSAID-induced acute tubular necrosis *in vivo* is attributed to an allergic reaction (Perazella, 2002) and to the disturbed blood flow in the kidneys which is caused by cyclooxygenase inhibition (Perazella, 2005; Murray and Brater, 1993). Also, CsA-induced tubular toxicity is attributed to disturbed blood flow in the kidney (Busauschina *et al.*, 2004). Zinc is not considered to be nephrotoxic in humans (Klaassen, 2004), or in cultured human proximal tubular cells (Rodilla *et al.*, 1998). Therefore, DF, CsA and Zn were chosen on the supposition that they would not be directly toxic to cultured tubular cells in the same setting as direct toxicants. Two test concentrations 1/10 and 1/3 of the cytotoxic concentration, TC_{50}, were used, in order to avoid cytotoxicity but to induce some transcriptional changes. The results were compared with previously reported studies.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (Nippon SLC Inc., Shizuoka, Japan), 6–8 weeks old, were used. Both kidneys were isolated under anesthesia with 60 mg kg^{-1} sodium pentobarbital administered intraperitoneally.

Primary Cell Preparation

Renal cortical tubules were isolated by a modified collagenase perfusion method described previously (Elliget and Trump, 1991; Jones *et al.*, 1979; Lash and Tokarz, 1989). Briefly, both kidneys were perfused *in situ* via the abdominal aorta first with 20 ml of Ca^{2+}/Mg^{2+} free Hank's balanced salt solution (HBSS–, GibcoBRL, NY, USA) containing 0.5 mM EGTA (Wako Pure Chemical, Osaka, Japan) and 10 mM HEPES (GibcoBRL) and then with HBSS with calcium and magnesium (HBSS+, GibcoBRL) containing 1 mg ml^{-1} collagenase (*Clostridium histolyticum* origin, Wako Pure Chemical), 0.17 mg ml^{-1} type I-S hyaluronidase (Sigma, St Louis, MO, USA), 1 mg ml^{-1} BSA Fr.V (Seikagaku Corp, Tokyo, Japan) and 10 mM HEPES. After isolation and decapsulation of the kidneys, the cortex was finely minced using razor blades, digested in the collagenase solution, and shaken at 66 rpm for 10–50 min at 37 °C. The dispersed cortex was filtered through 70 μm nylon meshes, layered onto a performed 45% (v/v) Percoll

(Amersham Biosciences, Piscataway, NJ, USA) gradient, centrifuged at 30 000 *g* for 10 min, and the tubule fractions were then harvested. Renal tubules were suspended in culture medium DMEM/Ham's F12 (GibcoBRL) supplemented with 15 mM HEPES, 100 U ml^{-1} penicillin (GibcoBRL), 100 μg ml^{-1} streptomycin (GibcoBRL), 100 ng ml^{-1} hydrocortisone (Sigma) and 10% (v/v) FCS (GibcoBRL) and seeded onto collagen I plates. After incubation for approx. 1.5 h at 37 °C, adherent cells, including fibroblasts, were discarded and the tubule suspension was transferred onto new collagen I plates for a 2-day pre-culture. At the end of the pre-culture, the plates were rinsed with Ca^{2+}/Mg^{2+} free Dulbecco's phosphate buffered saline (DPBS–, GibcoBRL) and then dispersed with HBSS– containing 0.3% trypsin (GibcoBRL) and 0.53 mM EDTA (GibcoBRL) at 37 °C for 22–27 min. After inactivating trypsin by adding FCS, the cells were rinsed with ice-cold DPBS– and suspended in the above-mentioned culture medium. Cells with a viability of 97% and above, based on trypan blue exclusion, were seeded onto new plates and used as primary cells for further experiments. Cultures were maintained at 37 °C in a water-saturated 5% CO_2–95% air incubator, and the culture medium was changed every day.

Culture Plate Preparation

For optimization of the culture condition, two different culture plates were used. For one type of culture plate, a solution of 400 μl of 2.3 mg ml^{-1} collagen I (3.25 mg ml^{-1} rat tail collagen I, BD Biosciences, Bedford, MA, USA) was poured onto each well of 12-well plastic plates (Iwaki, Tokyo, Japan) and the gel layer was formed according to the manufacturer's instructions. The liquid inside the gel was replaced by culture media three times before use. For the second culture plate type, conventional 12-well collagen I plates (Iwaki, Tokyo, Japan) were used.

For determination of the cytotoxic concentration, TC_{50}, of primary cells, conventional 96-well collagen I plates (Iwaki, Tokyo, Japan) were used.

For expression analysis of *in vitro* nephrotoxicity, 12-well collagen I-gel plates were used.

Culture Condition Optimization

The transcriptional profiles of rat primary renal cortical tubular cells were compared in two different culture conditions. The cells from the same animal were divided and seeded onto two different culture plates: 12-well collagen I plates (2.5×10^4 cells/cm^2) and 12-well collagen I-gel coated plates (5.1×10^4 cells/cm^2). A smaller number of cells were required to form a confluent culture in the conventional culture conditions, because cells in this culture condition became flattened and spread more compared with the cells on collagen I-gel. Total RNA was extracted from freshly isolated tubules and from primary cells after 48 h culture. Experiments were performed in duplicate using two animals, resulting in duplicate microarrays per condition.

In Vitro Nephrotoxicity Expression Analysis

Test Substances

CDDP, CBDCA, CR, GM, DF and Zn were obtained from Sigma. CsA (Sandimmune® injection) was obtained from Novartis Pharma K.K., Tokyo, Japan. The substances were dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical) and used as the stock solutions.

TC_{50} Determination

Every test culture was compared with the corresponding negative control of 1% DMSO which was prepared in each independent experiment. Cytotoxic concentration, TC_{50}, values for CDDP, CBDCA, CR and DF were taken from data previously published by our group (Kawashima *et al.*, 2001) (Table 1). The TC_{50} value for GM, CsA and Zn were determined according to the same method. Briefly, primary cells were seeded onto conventional 96-well collagen I plates (4.4×10^4 cells/cm^2). Twenty-four hours after seeding, the cells were exposed to serial dilutions of test substances or 1% DMSO for 24 h in triplicate, and TC_{50} values were determined based on the colorimetric assay with WST-1 (Roche Diagnostics, Tokyo, Japan). Each culture medium contained 1% DMSO.

Drug Exposure and RNA Extraction

In each independent experiment, a negative control culture was prepared for each exposure duration of 6 h or 24 h. The cells were seeded onto 12-well collagen I-gel plates (5.1×10^4 cells/cm^2). First, culture media of the 24 h exposure groups were exchanged for test culture media twice with an interval of 15 min in order to replace media inside collagen I-gel. At the same time, the culture media of the 6 h exposure groups were exchanged with new, test substance-free, culture media. Secondly, 18 h after the former culture exchange, culture media of the 6 h exposure groups were changed to test culture media twice, with an interval of 15 min. The test culture media contained a test substance at either 1/10 or 1/3 TC_{50} and 1% DMSO. Culture media for the negative control contained 1% DMSO. The cells were harvested for RNA extraction 24 h after the first culture media exchange. Cell experiments were performed with four replicates. After checking the quality of total RNA, equivalent amounts of total RNA from three or four cultures were

pooled into one sample, and then used for hybridization cocktail preparation. Each hybridization cocktail was hybridized onto one microarray per sample.

Hybridization-Cocktail Preparation

Total RNA was extracted with Sepasol I (Nacalai Tesque, Kyoto, Japan) and purified with an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Double-stranded cDNA was synthesized from 5 μg of total RNA with Superscript II Reverse Transcriptase system (Invitrogen Life Technologies, Carlsbad, CA, USA) with T7-$(dT)_{24}$ primer (5-'GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG(T)24-3') (Sigma Genosys Japan KK, Hokkaido, Japan). Biotin-labeled cRNA was synthesized from the cDNA template with a T7 MegaScript kit (Ambion, Austin, TX, USA) in the presence of biotin-labeled NTP and purified with an RNeasy Mini kit. The cRNA was fragmented at 95 °C for 35 min in a buffer containing 40 mM Tris-acetate, pH 8.1, 100 mM KOAc and 30 mM MgOAc. The final hybridization cocktail included 0.05 μg μl^{-1} cRNA, 0.1 mg ml^{-1} herring sperm DNA (Promega, Madison, WI, USA), 0.5 mg ml^{-1} acetylated BSA (Sigma), 0.05 nM biotinylated-control oligonucleotides and 0.1 M MES buffer (pH 6.7).

Hybridization, Staining and Scanning

Rat Toxicology U34 Arrays (Affymetrix, Santa Clara, CA, USA) which contain 25-mer probes for more than 850 genes and ESTs were used. Microarrays were pretreated with a solution containing 0.5 mg ml^{-1} acetylated BSA, 0.5 mg ml^{-1} herring sperm DNA and 0.1 M MES buffer at 40 °C for 15 min. Then the arrays were incubated with the hybridization cocktail at 45 °C for 15 h. The arrays were stained with a solution containing 10 μg ml^{-1} streptavidin R-phycoerythrin conjugate (Molecular Probes, Eugene, OR, USA) and scanned using a laser scanner system (Affymetrix). One sample was hybridized onto one microarray.

Data Analysis

Calculations of signal intensity, reliability of each probe and 'Detection Call' expressed as Present, Absent or Marginal, were performed with the Affymetrix Microarray Suite ver5.0 software (MAS 5.0). The average signal intensity of each array was scaled to one value of 150 in order to compare multiple arrays. Signal intensities of housekeeping genes were stable. Mean ± SD and CV% of signal intensity were as follows: 3586 ± 410 (11%) for ribosomal protein S29 (X59051) and 2368 ± 285 (12%) for GAPDH (M17701). Pair-wise comparison analyses were performed with Affymetrix MAS 5.0, where the Signal Log Ratio (log 2 ratio) and the 'Change Call' (Increase, Decrease or No-Change) were calculated. Hierarchical cluster analysis of Signal Log Ratio was performed with TIGR MeV ver3.1 software (Saeed *et al.*, 2003) using squared Pearson with complete linkage. Probes which satisfied all the following criteria in at least one pair-wise comparison were identified as differently expressed probes: (i) the signal intensity of 100 or more and 'Detection Call' is Present; (ii) 'Change Call' other than No-Change; (iii) Signal Log Ratio of 1 or more, or −1 or less (i.e. ≥ two-fold change criteria). In the culture condition optimization experiment, each condition consisted of duplicate arrays from two animals; pair-wise comparisons were performed between two different culture conditions of corresponding animals. In the *in vitro* nephrotoxicity expression analysis, each condition consisted of one array; pair-wise comparisons were performed between the drug exposure group and the corresponding negative controls which was set in each independent experiment. Signal intensities of the primary cells relative to the freshly isolated tubules were calculated in MS Excel.

Results and Discussion

Culture Condition Optimization

Expressions of 20 transcripts were identified as different depending on the two culture conditions (Table 2). There was a tendency for the expression levels of these transcripts in collagen I-gel cultures to fall between those in the freshly isolated tubules and conventional monolayer cultures. For example, decreased antioxidants (Mt1a, Gstm1) and induction of a cell cycle-associated gene (Ccng1) were suppressed in collagen I-gel cultures compared with conventional monolayer cultures.

In both culture conditions, increased apoptosis-inducible genes, Gadd45a and Hspb1, were observed compared with the freshly isolated tubules, suggesting some damage in the primary cultures. One reason may be a reduction of cell attachment in primary cultures, compared with cells *in vivo* which are surrounded by other cells. A previous report showed that loss of cell anchorage triggered apoptosis of hepatocytes (Smets *et al.*, 2002). This may also explain the reason why induction of Gadd45a and Hspb1 in the collagen I-gel culture was suppressed compared with the corresponding cells in the conventional monolayer culture (Table 2). Microscopically, the tubular cells in the conventional monolayer culture became flattened and spread more compared with the cells on collagen I-gel and thus the cell density was greater in the collagen I-gel culture. These results suggest possible differences, in terms of decreased cell anchorage (especially in the conventional monolayer culture) and different severities of stress, between the two culture conditions tested.

Based on these results, collagen I-gel culture was adopted for the expression analysis of *in vitro* nephrotoxicity.

Table 2. Transcripts that expressed differently between conventional monolayer culture and collagen I-gel culture (signal intensities of freshly isolated tubules are adjusted to a value '1' for every transcript)

			Signal intensities relative to freshly isolated tubules				
Probe ID	Symbol	Description	Freshly isolated tubules	mono-1	mono-2	gel-1	gel-2
M11794	Mt1a	Metallothionein 1a	1	0.1	0.1	0.3	0.6
X06769	Fos	c-fos	1	0.1	0.4	0.2	0.3
X04229	Gstm1	Glutathione S-transferase μ1	1	0.3	0.4	0.6	1.0
H32189	Gstm1	Glutathione S-transferase μ1	1	0.6	0.4	1.0	1.0
X63594	Nfkbia	NFkBα	1	1.3	1.6	2.9	2.8
AI014091	Cited2	Cbp/p300-interacting transactivator, with ED-rich tail2	1	2.2	1.2	0.9	1.0
X64401	Cyp3a3/3a1	Cytochrome P450 subfamily 3A	1	0.9	2.5	1.8	2.0
M58040	Tfrc	Transferrin receptor	1	2.1	2.0	0.5	1.2
AA899854	Top2a	Topoisomerase 2α	1	1.7	3.5	0.7	5.6
AI070295	Gadd45a	GADD45α	1	2.2	3.2	0.9	2.5
AI070295	Gadd45a	GADD45α	1	3.4	2.8	1.7	2.2
AB004096	Cyp51	CYP51 (Sterol 14 alpha-demethylase)	1	2.6	4.0	1.4	1.5
AA997614	Cyp51	CYP51 (Sterol 14 alpha-demethylase)	1	2.5	4.4	1.1	1.6
U17697	Cyp51	CYP51 (Sterol 14 alpha-demethylase)	1	3.2	5.4	1.7	2.5
X70871	Ccng1	Cyclin G1	1	2.6	7.4	2.5	3.0
AA875509	Mdm2_predicted	Transformed mouse 3T3 cell double minute 2	1	6.7	8.3	3.5	3.4
AI228738	Fkbp1a	FK506 binding protein 1a	1	12.0	14.6	5.7	8.1
AI176658	Hspb1	hsp27	1	9.2	12.3	4.5	6.6
M86389	Hspb1	hsp27	1	38.0	13.1	19.0	6.3
AA998683	Hspb1	hsp27	1	38.1	21.2	17.4	12.0

Average of two biological replicates. Mono: conventional monolayer culture. Gel: culture on collagen I-gel. Cells in mono-1/gel-1 cultures were derived from the same animal. Cells in mono-2/gel-2 cultures were derived from another animal.

Table 3. Direct tubule-targeting substances affected more transcripts compared to substances supposedly not directly toxic to the tubule (either 6 h or 24 h exposure of each substance)

Test substance	Total number[a]	Up-regulated[a]	Down-regulated[a]
Directly toxic to the tubule			
CDDP	61	29	34
CBDCA	64	16	48
CR	50	42	14
GM	32	21	11
Not directly toxic to the tubule			
DF	12	9	3
CsA	12	10	2
Zn	5	3	2

Some overlap with the up-regulated and the down-regulated depending on the time points.
[a] Transcripts with ≥2-fold change at either 1/10 or 1/3 of TC_{50}.

In Vitro Nephrotoxicity Expression Analysis

Overall Changes

Direct toxicants induced the most drastic transcriptional alterations *in vitro*, affecting over 32 transcripts; however, substances reportedly not directly toxic to the tubule affected less than 12 transcripts (Table 3). Decreased Map3k12 and increased Hmox1 were common changes among the four direct toxicants at 24 h, which were considered to be responses to cellular damage (Fig. 1B). This kind of commonality across the direct toxicants was not observed at 6 h (Fig. 1A). Similarly, two concentrations of each test substance formed tight clusters at 24 h

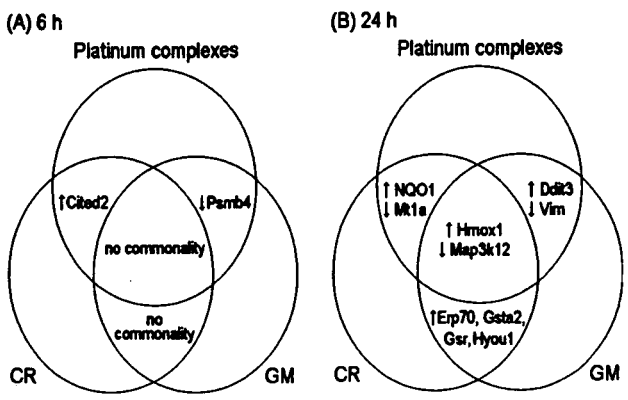

Figure 1. Transcripts similarly affected across the direct toxicants. ↑ increase, ↓ decrease

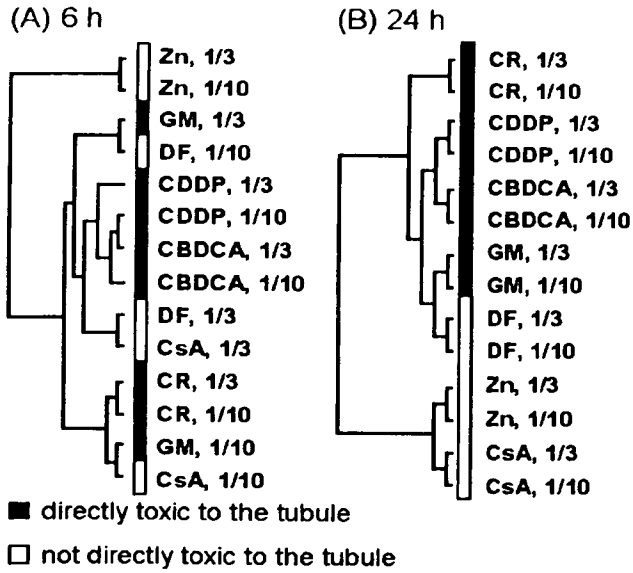

Figure 2. Hierarchical clustering of gene expression profiles across the concentrations of all the test substances. 1/3: one-third of TC_{50}, 1/10: one-tenth of TC_{50}

but not necessarily at 6 h in hierarchical cluster analysis (Fig. 2). In other words, 1/10 TC_{50} required a longer exposure duration to induce similar effects to 1/3 TC_{50} and therefore 6 h exposure may not be suitable to simply distinguish the type of drugs although a 6 h exposure should be useful to analyse the mechanism of toxicity. Additionally, the four direct toxicants grouped together at 24 h (Fig. 2). There were more commonalities at 24 h between CDDP and the less nephrotoxic analogue, CBDCA (Fig. 3), where they formed tight clusters regardless of the exposure duration (Fig. 2).

Considering these results together, the present test system appeared to be a useful tool for distinguishing substances with direct tubular toxicity from those without direct tubular toxicity. Also, this test system is sensitive enough to classify similar drugs, such as CDDP and CBDCA, even at a low concentration, 1/10 TC_{50}, even at an early time point of 6 h.

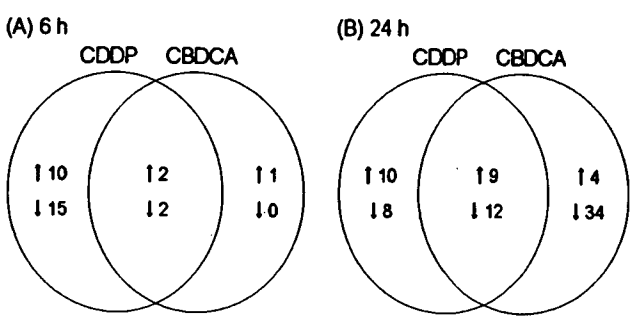

Figure 3. Similarity of CDDP and CBDCA. The number of transcripts either similarly regulated or differently regulated. ↑ increase, ↓ decrease

Drugs Directly Toxic to Tubules

CDDP and CBDCA

The platinum complexes induced Hmox1 and some p53-inducible genes such as Cdkn1a, Ccng1, Pgy1, Ddit3, Pcna, Hspb1, Hspa1 and Fos (Table 4), whereas the increases of Ccng1, Pgy1 and Fos were CDDP specific. Expression of p53 itself was not affected. Although most of these changes were observed at 24 h, some CDDP-induced changes, such as increases of Hspa1, Fos and Hmox1, were observed at 6 h unlike CBDCA. These differences between the two drugs might be related more to the toxicity of CDDP. An increase of Nqo1 at 24 h, which stabilizes p53 (Asher *et al.*, 2002), was observed as a related change to increased p53-inducible genes. These changes were consistent with the previously reported CDDP-induced nephrotoxicity *in vivo* that showed increases of antioxidants including Hmox1 (Thompson *et al.*, 2004) and p53-inducible genes such as Cdkn1a, Ccng1, Pgy1, Myc, Timp and Igfbp3 (Huang *et al.*, 2001; Thompson *et al.*, 2004), and Pcna and Ddit3 (Zhou *et al.*, 2004). A decreased Vim was also consistent with the previously reported *in vivo* CDDP-induced nephrotoxicity (Francescato *et al.*, 2004). On the other hand, no changes were observed that were reported in previous *in vivo* studies, including decreases in arginine metabolism-associated genes (Amin *et al.*, 2004; Thompson *et al.*, 2004) or organic anion transporters (Demeule *et al.*, 1999; Huang *et al.*, 2001). Decreases of growth factors such as Egf and Ngf (Huang *et al.*, 2001; Thompson *et al.*, 2004) were not confirmed in our study because the microarray used does not contain the corresponding probes. Although not previously reported, decreases in the following genes were seen; Atp1a1, Clu, Hsd17b4, Msh2, Map3k12, Map3k1, Mt1a, Scd2 and Stk39. On the other hand, the *in vitro* study by Huang and colleagues with the rat tubular cell line, NRK-52E, showed increased p53-inducible genes after CDDP exposure, but did not show any changes related to oxidative stress, arginine metabolism, Egf, or organic anion transporters. It can be concluded that some stress associated changes *in vivo* are reproducible *in vitro*, but this does not apply to all changes.

A previous study suggested that CDDP-induced tubular toxicity in mice is mediated by ERK activation both *in vivo* and *in vitro* (Arany *et al.*, 2004). They showed pretreatment of mouse proximal tubular cell TKPTS with an ERK inhibitor improved survival by inhibiting apoptosis after CDDP treatment. Considering their data, lower expression of Mapk6 (ERK3) in CBDCA-treated cells than in CDDP-treated cells in our study may reflect lower damage in the CBDCA-treated cells. Also, higher expressions of Cdkn1a and Hmox1 in CBDCA-treated cells than in CDDP-treated cells may reflect less cytotoxicity in the CBDCA-treated cells, consistent with

Table 4. Transcriptional alteration affected by CDDP and/or CBDCA (log2 changes compared with corresponding negative controls)

			CDDP				CBDCA			
			6 h		24 h		6 h		24 h	
Probe ID	Symbol	Description	(1/10) [a]	(1/3) [b]	(1/10)	(1/3)	(1/10)	(1/3)	(1/10)	(1/3)
AI176308	Cdc42	—	−0.4	−0.1	−0.1	0.1	−0.3	−0.3	−0.3	−1
AI013834	Hsd17b4	17β-HSD4 (17β-hydroxysteroid dehydrogenase 4)	−0.7	−0.6	−0.5	−0.7	0	−0.3	−0.7	−1.6
S83279	Hsd17b4	17β-HSD4 (17β-hydroxysteroid dehydrogenase 4)	0.1	0.4	−0.4	−0.8	0	0	−0.5	−1.2
S83279	Hsd17b4	17β-HSD4 (17β-hydroxysteroid dehydrogenase 4)	−0.3	−0.2	−0.7	−1.2	−0.4	−0.1	−0.9	−2.1
M60322	Aldr1	Aldehyde reductase 1	0.3	0.6	0.1	0.1	−0.1	0	0.3	1.1
M28647	Atp1a1	ATPase, Na+K+ transporting, α1	−0.2	−0.3	−0.4	−1.1	0.2	−0.2	−0.8	−1.5
D84450	Atp1b3	ATPase, Na+K+ transporting, β3	−0.5	−1.2	−0.2	−0.2	−0.3	−0.3	−0.4	−0.3
L15079	Abcb4	ATP-binding cassette, sub-family B (MDR/TAP), 4 (mdr2, Pgy3)	−0.1	−0.3	−0.2	−0.7	0.2	−0.2	−0.6	−1
U34963	Bcl2l1	Bcl2-like 1 (Bcl-XL)	0.5	0.3	0.1	−1.2	0.4	0	0	−0.6
U77933	Casp2	Caspase 2	0.1	−0.4	−0.3	−0.9	−0.1	−0.1	−0.6	−1.2
AA900476	Cited2	Cbp/p300-interacting transactivator, with ED-rich tail2	0.7	1	0.2	−0.7	0.7	0.9	1.2	1.2
AA900476	Cited2	Cbp/p300-interacting transactivator, with ED-rich tail2	0.4	1	0.4	−0.2	0.9	1	0.9	0.8
AI014091	Cited2	Cbp/p300-interacting transactivator, with ED-rich tail2	0.8	1.2	0.5	0.6	0.4	0.9	0.9	0.9
M61875	Cd44	CD44 antigen	0.1	−0.3	0.2	−0.6	0	−0.2	−1.1	−2.3
AA925473	Cdc42	cdc42 homolog(yeast)	0	−0.6	−0.4	−1	0	0	0	−0.1
L41275	Cdkn1a	cdk inhibitor 1A ($p21^{cip}$)	1.4	1.2	2.4	2	1.7	1.3	3.4	4.2
X06769	Fos	c-fos	0.5	1	−0.4	−0.8	0.1	0	0.1	−0.1
M64733	Clu	Clusterin/apolipoprotein J	−0.1	−0.6	−0.7	−1	−0.1	−0.5	−0.8	−1.4
D14014	Ccnd1	Cyclin D1	−0.3	−1.1	−0.1	0.1	0.3	0	−0.3	−0.7
X70871	Ccng1	Cyclin G1	0.1	0.5	1.5	1.9	−0.1	0	0.8	0.8
J01436	mt-Cytb	Cytochrome b, mitochondrial	0.5	0.6	−0.5	−1.2	0.1	0.2	−0.2	−0.8
AF007107	Cyb5	Cytochrome b5	−1.1	−0.7	0.1	0.7	−0.7	−0.7	−0.2	−0.1
D00636	Dia1	Diaphorase 1 (NADH cytochrome b reductase)	−0.2	−1.2	−0.3	−0.6	0.1	−0.1	−0.2	−0.6
X94185	Dusp6	Dual specificity phosphatase 6	0.3	0.5	−0.3	−2.2	0.2	0	0	−0.5
AA685903	—	EST, similar to glucose regulated protein, 94 kDa (GRP94)	0.2	0.4	−0.2	−0.9	−0.5	0.1	−0.4	−1.9
AI013297	—	EST, similar to NADH dehydrogenase (ubiquinone) Fe-S protein 4	−0.3	−1.4	−0.1	−0.2	−0.3	−0.3	−0.3	−0.5
AI233365	—	EST, similar to RIKEN mouse cDNA 0610006H08	−0.7	−1.3	−0.2	−0.5	−0.2	−0.4	−0.5	−1.3
U05014	Eif4ebp1	Translation initiation factor 4E binding protein 1	0.2	0.2	0.9	1.2	0.2	0.3	0.4	0.3
U30186	Ddit3	GADD153 (DNA-damage inducible transcript 3)	−0.2	0.9	1	1.5	0.1	−0.2	0.7	1.9
U62940	Grpel1	GrpE-like 1, mitochondrial	−0.2	0	0.7	1.1	0	0	0.3	0.8
Z75029	Hspa1a	Heat shock 70 kD protein 1A	0.7	1.6	0.3	0.6	0.3	0.4	0.4	1
AA818604	Hspa1b	Heat shock 70 kD protein 1B	0.9	2	0.2	0.2	0.5	0.6	0.6	1.4
L16764	Hspa1b	Heat shock 70 kD protein 1B	0.3	1.5	0.1	0.8	0.1	0.3	0.1	0.7
M14050	Hspa5	Heat shock 70 kD protein 5	0	−0.4	0.3	−0.1	−0.3	0	−0.4	−1
J02722	Hmox1	Heme oxygenase (decycling) 1	1.6	2.8	0.8	2	0.7	0.8	1.3	2.9
AA998683	Hspb1	hsp27	0	0.3	0.9	1.8	0.1	0.1	1.2	1.9
AI176658	Hspb1	hsp27	0.2	0.5	1.3	2.2	0.4	0.4	1.4	2.1
M86389	Hspb1	hsp27	0.6	0.2	1.3	1.9	0.6	0.7	1.5	1.9
U59809	Igf2r	Insulin-like growth factor 2 receptor	0.2	−0.4	−0.2	−0.6	−0.1	−0.1	−0.4	−1.2
AI171630	Mapk14	MAP kinase 14 (p38)	−0.7	−0.5	−0.2	0.1	0.2	0	−0.3	−1
U73142	Mapk14	MAP kinase 14 (p38)	−0.6	−0.7	−0.3	−0.1	−0.1	−0.5	−0.2	−1
M64301	Mapk6	MAP kinase 6	−0.3	−0.4	1	1.1	0	−0.3	−0.2	−1.1
M64301	Mapk6	MAP kinase 6	−0.2	−0.9	0.4	0.4	−0.2	−0.3	−0.2	−1.1
U48596	Map3k1	MAP kinase kinase kinase 1	−0.3	−0.7	−0.1	−3.8	0.3	−0.3	−0.8	−1.9
U48596	Map3k1	MAP kinase kinase kinase 1	−0.3	−1	−0.3	−2.8	0	−0.4	−0.9	−2.3
D49785	Map3k12	MAP kinase kinase kinase 12	−0.4	−1.4	−0.6	−2.3	−0.7	−0.2	−0.5	−1.8
M11794	Mt1a	Metallothionein	−0.1	−0.3	−1.3	−2.2	0.1	0	−1.7	−1.5

Table 4. *(Continued)*

Probe ID	Symbol	Description	CDDP 6 h (1/10)[a]	CDDP 6 h (1/3)[b]	CDDP 24 h (1/10)	CDDP 24 h (1/3)	CBDCA 6 h (1/10)	CBDCA 6 h (1/3)	CBDCA 24 h (1/10)	CBDCA 24 h (1/3)
X93591	Msh2	Mismatch repair protein	−0.4	−0.6	−0.6	−1.2	−0.1	−0.5	−0.5	−1.1
D87336	—	mRNA similar to bleomycin hydrolase	−0.4	−1	−0.2	−0.5	−0.1	−0.4	−0.3	−1
AA799499	—	mRNA similar to NADH dehydrogenase (ubiquinone) 1β3	−0.6	−1	−0.2	0.1	−0.3	−0.5	−0.2	−0.3
M13011	—	mRNA similar to transforming protein p21/H-Ras-1(c-H-ras)	−0.2	0.3	0.5	1	0.4	0.1	0.3	0.9
J02679	Nqo1	NAD(P)H:Quinone oxidoreductase-1 (NQO1)	0.2	0.5	0.6	1.1	0	−0.3	0.3	1.3
D84346	Nckap1	NCK-associated protein 1	−0.3	−1	−0.4	−0.6	−0.1	−0.4	−0.4	−1
X68394	Nras	Neuroblastoma RAS viral (v-ras) oncogene homolog	−0.2	−0.3	−0.3	−0.6	0	−0.2	−0.4	−1
L26267	Nfkb1	NFkB p105 subunit	−0.5	−1.6	−0.4	−0.9	−0.1	−0.3	−1.1	−2.3
X63594	Nfkbia	NFkBα	−0.2	−0.4	−0.5	−1.4	−0.1	−0.3	−0.2	−0.3
X63594	Nfkbia	NFkBα	−0.2	−0.1	−0.5	−1.4	0.1	−0.2	−0.3	0
M76704	Mgmt	O-6-methylguanine-DNA methyltransferase	−0.2	0	0.4	−0.3	0	−0.2	−0.4	−1.1
J04791	Odc1	Ornithine decarboxylase (ODC)	−0.2	0.3	0.4	1	0.1	0.1	0.2	0.5
M24604	Pcna	PCNA	−0.3	0.2	0.5	1.1	−0.4	−0.2	0.5	1.3
M81855	Pgy1	p-glycoprotein/multidrug resistance 1 (mdr1)	0.1	−0.9	1.2	0.6	0.1	0	0.3	−0.6
U05989	Pawr	PKC apoptosis WT1 regulator	−0.2	−1.1	−0.1	−0.5	−0.2	0	−0.5	−2.1
M86870	Erp70	Protein disulfide isomerase related protein (CaBP, intestinal-related)	0.1	0.2	0.6	−0.1	0.2	0	−0.4	−1.2
AA892532	P5	Protein disulfide isomerase-related protein	−0.3	−0.2	−0.4	−0.5	−0.2	−0.3	−0.5	−1.1
D21799	Psmb2	PSMB2 (proteasome (prosome, macropain) subunit, β2)	0	0.1	0.3	0.4	0.2	0.3	0.2	1.1
L17127	Psmb4	PSMB4 (proteasome (prosome, macropain) subunit β4)	−1.1	−0.6	0.3	1.1	−0.3	−1.1	0.4	0.7
L17127	Psmb4	PSMB4 (proteasome (prosome, macropain) subunit β4)	−1.7	−0.6	0.6	2	−0.6	−1.5	0.4	0.8
U12187	Rrad	ras-related associated with diabetes	−0.3	0	0.8	1.3	−0.3	−0.5	0.1	0.3
AI230406	Rab10	ras-related protein rab10	−0.3	−0.2	−0.4	−0.8	−0.1	−0.2	−0.6	−1.6
U15211	Rara	Retinoic acid receptor α	1	0.5	−0.2	−0.8	0.1	0.4	0	−0.4
D88190	Stk39	Serine threonine kinase 39 (yeast STE20/SPS1 homolog)	0.2	0	−0.9	−1.5	−0.1	0	−0.6	−1.6
K00750	—	Similar to cytochrome c, somatic (LOC365738), mRNA	0	1.1	−0.1	0	−0.1	0.2	−0.1	0.2
AA875509	—	Similar to mdm2 gene product (LOC314856), mRNA	0.6	1.1	1.5	1.5	0	0.5	1.2	1.5
M15114	Scd2	Stearoyl-Coenzyme A (CoA) desaturase 2	−0.1	−1.6	−0.6	−1.7	0.1	−0.2	−1.3	−3.2
D83796	Ugt1a1~8	UDP glycosyltransferase 1A	0.3	−0.6	0.4	−0.1	0.2	0.3	0.3	−1.2
J02612	Ugt1a1~8	UDP glycosyltransferase 1A	0.1	0.4	0.2	−0.4	0.3	0.4	0	−1.1
J05132	Ugt1a1~8	UDP glycosyltransferase 1A	−0.2	−0.7	−0.4	−0.6	0.1	−0.1	−0.3	−1.5
X62952	Vim	Vimentin	0.2	−0.2	−0.1	−1.1	0.1	0.1	−0.4	−1.4
AA945867	Jun	v-jun sarcoma virus 17 oncogene homolog (avian)	−0.5	−0.6	−0.2	−0.6	0.2	−0.1	−0.6	−1.2
AI175959	Jun	v-jun sarcoma virus 17 oncogene homolog (avian)	0.7	−0.1	−0.3	−1.2	0.8	0.6	0	−0.2
AI112516	Zfp36l1	Zinc finger protein 36, C3H type-like 1	−0.2	0.2	0.1	−0.4	−0.3	−0.1	−0.1	−1.2
AI136891	Zfp36l1	Zinc finger protein 36, C3H type-like 1	−0.1	0	−0.4	−0.6	−0.2	−0.1	−0.5	−1.3
AA799889	—	Transcribed locus	−0.2	−0.9	−0.4	−0.7	−0.2	−0.4	−0.7	−1.2
AA892248	—	—	0.5	0.9	−0.6	−0.9	0.6	1.3	0	0.5
AA892248	—	—	0.6	1.4	−0.6	−0.8	0.5	1.3	0	0.5
AI009141	—	Chromobox homolog 1 (Drosophila HP1 beta) (predicted)	0.4	−0.4	−0.3	−1.3	−0.1	0.2	−0.2	−0.8
AI010371	—	LUC7-like 2 (S. cerevisiae) (predicted)	−0.2	−0.4	−0.4	−1	0	−0.2	−0.6	−2
AI010725	Canx	Calnexin	0	0.1	−0.4	−0.9	0	−0.2	−0.7	−1.9
AI010725	Canx	Calnexin	0	−0.1	−0.4	−0.8	−0.1	−0.1	−0.8	−2.1
AI012051	—	Baz2b_predicted	−0.1	−0.4	−0.6	−0.8	0.1	0	−0.2	−1.9
AI102620	—	Transcribed locus	−0.4	−1.9	−0.6	−1.5	−0.2	−0.5	−1.4	−2.6
AI229291	—	Pura_predicted	−0.4	0.1	−0.4	−1.7	−0.4	−0.3	−0.5	−1.4

[a] One-tenth of TC_{50}.
[b] One-third of TC_{50}. — no symbol or no description.

previous studies that have reported that a high expression of Cdkn1a protected mouse kidney proximal tubular cells from CDDP-induced apoptosis in TKPTS cells (Price *et al.*, 2004) and *in vivo* (Megyesi *et al.*, 1996) and a heme oxygenase inducer protected the tubular cell line LLC-PK1 from CDDP-induced toxicity (Schaaf *et al.*, 2002).

CR

Although the effect of CR (Table 5A) on transcription in the kidney is not well investigated, increases of GSTs were consistent with a previous study *in vivo* and *in vitro* (Moritz *et al.*, 1995). The increases of GSTs, as well as other antioxidants (Gsr, Nqo1, Cyb5, Hmox1 and possibly NADH dehydrogenase 1α9-like mRNA), appeared to be consistent with the report that CR-induced nephrotoxicity is based on oxidative stress (Tune, 1997). These increases, together with increased stress-inducible chaperones (Calr, Canx and Erp70) and oxidoreduction-associated genes (Akr1d1, Aldr1 and Me1), were thought to be an adaptation to stress. Increases of other stress-associated genes (Bcl2l1, Gadd45a, Psmb4 and Hyou1) were also observed. Most of the stress-associated changes and the inductions of antioxidants were observed at 24 h; on the other hand, changes at 6 h included increases of transcription-associated genes (Cited2, Jun, Junb and Ptb), a growth factor receptor (Met), a translation factor (Eef2) and signal transduction-associated genes (Map3k1 and Dusp6).

Similarities to the other test substances were limited. Compared with the two platinum complexes, the increased Nqo1 and decreased Mt1a, both of which are related to oxidative stress, were commonly observed at 24 h (Fig. 1B). Compared with GM, increases of Gsr, Gsta2, Erp70 and Hyou1 were commonly observed at 24 h, all of which were stress associated changes (Fig. 1B).

GM

GM exposure resulted in increases of oxidative stress-inducible genes (Gsr, GSTα2 and Hmox1), stress-inducible chaperones (Calr, Canx, Erp70, Hspa1a and Hspa5) and an oxidoreduction enzyme (Akr1d1) at 24 h (Table 5B). Inductions of Cyp1a1 and Cyp1B1 may also be a consequence of the stress because oxidative stress has been reported to induce these enzymes (Husbeck and Powis, 2002) independently from the pathway through AhR. These stress-associated changes appeared to be consistent with the report that GM induces oxidative stress in the kidney *in vivo* (Walker *et al.*, 1999). Though the effect of GM on transcription in the kidney is not well investigated, *in vivo* changes in the whole kidney have been reported 1 week after a single injection (Amin *et al.*, 2004). An increase of Hmox1 was confirmed in this study, but there were some inconsistencies, such as increased clusterin seen in previous work but not observed in our study; increased AA957270 and osteopontin and decreased Ngf which had been seen in previous work could not be confirmed in this study because the microarray used did not include the relevant gene probes.

Similarities to the other test substances were also limited. Similarities to the two platinum complexes are increased Ddit3 and decreased Vim at 24 h (Fig. 1B).

Drugs Not Directly Toxic to Tubules

Although NSAID-induced tubular toxicity is attributed to disturbed renal blood flow due to cyclooxygenase inhibition (Murray and Brater, 1993; Perazella, 2002), some direct effects against tubular cells were observed mainly at 24 h exposure (Table 5C): oxidative stress associated changes (increases of Hmox1 and Nqo1) and other stress-associated changes (increases of Gadd45α and Ddit3. These changes might be related to the reported effects of DF in mice, where oxidative stress parameters in blood increased and DNA damage occurred in the whole kidney 24 h after administration (Hickey *et al.*, 2001). The data suggest that DF has some direct toxic effects on the renal tubule.

With regard to CsA, no apparent signs of toxic effects were observed, even though an increase of chaperones (Calr and Canx) and Gsr suggests an adaptation process to stress (Table 5D). Increases of Ugt1a and Cyp51 suggest some effects on the metabolic ability of the kidney. The lack of apparent toxic effects appeared to be consistent with the report that CsA-induced nephrotoxicity is attributed to a vasoconstriction reaction (Busauschina *et al.*, 2004).

No apparent signs of toxic effects from Zn were observed (Table 5E) except for an increased chaperone (Calr). A transient increase of Mt1a at 6 h appeared to be consistent with the known ability of Zn to induce metallothionein (Durnam *et al.*, 1984). The lack of apparent toxic effects is consistent with the description that Zn is not considered to be nephrotoxic in humans (Klaassen, 2004) as well as in cultured human proximal tubular cells (Rodilla *et al.*, 1998).

In summary, drugs with direct toxicity against the tubules induced more transcriptional alterations compared with the other drugs tested, and were grouped together in hierarchical clustering. Types of transcriptional changes observed in our study were consistent with previously reported *in vivo* data, although there were some differences. It was concluded that an *in vitro* gene expression analysis using microarrays is feasible for screening direct tubular toxicity and may help to clarify the underlying mechanisms of tubular toxicity.

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Table 5. Transcriptional alteration (log2 changes compared with corresponding negative controls)

Probe ID	Symbol	Description	6 h		24 h	
			(1/10)[a]	(1/3)[b]	(1/10)	(1/3)
(A) CR						
AA875327	—	mRNA similar to Wbscr1	1.9	1.9	0.0	0.2
X53363	Calr	Calreticulin	1.5	1.7	0.5	0.3
X74565	Ptbp1	Polypyrimidine tract binding protein 1	1.5	1.6	−0.9	0.1
X74565	Ptbp1	Polypyrimidine tract binding protein 1	1.4	1.5	−0.6	0.3
AA900476	Cited2	Cbp/p300-interacting transactivator, with ED-rich tail2	0.8	1.5	−0.1	0.9
AI014091	Cited2	Cbp/p300-interacting transactivator, with ED-rich tail2	0.5	1.2	0.0	0.4
U48596	Map3k1	MAP kinase kinase kinase 1	1.2	1.4	−0.6	−0.6
U48596	Map3k1	MAP kinase kinase kinase 1	1.1	1.2	−0.8	−0.8
AI175959	Jun	v-jun sarcoma virus 17 oncogene homolog (avian)	0.6	1.2	−0.6	−0.2
X54686	Junb	pJunB	1.0	1.1	−0.8	−0.2
D17309	Akr1d1	Aldo-keto reductase 1D1	1.3	1.1	−0.1	−0.7
AI008863	—	Similar to RIKEN cDNA 1200007D18 (LOC287177), mRNA	1.0	1.0	−0.2	−0.1
L18889	Canx	Calnexin	0.7	1.0	0.1	−0.1
U65007	Met	HGFR/met proto-oncogene	1.0	0.8	0.3	−0.1
U34963	Bcl2l1	Bcl2-like 1 (Bcl-XL)	2.2	2.5	−1.3	−0.2
S78284	Bcl2l1	Bcl2-like 1 (Bcl-XL)	2.3	2.4	−1.0	−0.2
U72350	Bcl2l1	Bcl2-like 1 (Bcl-XL)	1.0	1.2	−0.2	−0.4
AA963674	Eef2	Eukaryotic translation elongation factor 2	1.7	1.8	−1.0	0.0
X94185	Dusp6	Dual specificity phosphatase 6	1.0	1.4	−1.0	−0.9
U73174	Gsr	Glutathione reductase	1.3	1.3	0.8	1.2
U20643	Aldoa	Aldolase A	1.1	1.3	−1.1	0.0
AA892248	—	—	0.2	1.3	−1.3	−0.4
J02722	Hmox1	Heme oxygenase (decycling) 1	0.3	0.8	1.2	2.5
K00136	Gsta2	Glutathione S-transferase, $\alpha 2$	−0.2	0.1	0.1	2.5
S82820	Gsta2	Glutathione-S-transferase $\alpha 2$	0.2	0.4	0.5	1.3
J02679	Nqo1	NAD(P)H:Quinone oxidoreductase-1 (NQO1)	0.2	0.5	0.8	2.3
K01932	Gsta5	Glutathione S-transferase A5	−0.3	0.0	0.6	2.0
X78848	Gsta5	Glutathione S-transferase A5	−0.2	0.1	0.4	1.7
H32189	Gstm1	Glutathione S-transferase μ1	−0.3	−0.1	0.8	1.6
X04229	Gstm1	Glutathione S-transferase μ1	−0.2	0.0	0.1	1.0
L17127	Psmb4	PSMB4 (proteasome (prosome, macropain) subunit $\beta 4$)	−0.3	−0.6	1.2	1.3
AA945054	Cyb5	Cytochrome b5	−0.1	−0.1	1.3	1.2
AF007107	Cyb5	Cytochrome b5	−0.2	−0.4	1.2	1.0
AI171506	Me1	Malic enzyme 1	−0.2	0.0	0.9	1.2
M60322	Aldr1	Aldehyde reductase 1	−0.1	−0.1	0.2	1.2
AI070295	Gadd45a	GADD45α	0.1	0.0	0.8	1.1
AI070295	Gadd45a	GADD45α	−0.1	−0.1	0.7	1.0
AI011746	—	mRNA similar to transmembrane protein induced by TNFa	0.4	0.5	1.3	1.1
AA956114	—	mRNA similar to ubiquitin-conjugating enzyme HR6A	−0.3	−0.2	1.1	1.1
AA799525	—	mRNA similar to NADH dehydrogenase (ubiquinone) 1α9	−0.3	−0.5	1.1	0.9
AI009098	Hyou1	Hypoxia up-regulated 1	0.3	0.0	1.1	0.8
M86870	Erp70	Protein disulfide isomerase related protein (CaBP, intestinal-related)	0.3	0.1	1.0	0.6
AA892248	—	—	0.1	0.9	−1.1	−0.2
AI227887	Cdc42	cdc42 homolog(yeast)	0.1	0.3	−1.1	−0.6
AI009141	—	Chromobox homolog 1 (Drosophila HP1 beta) (predicted)	−0.1	0.6	−1.4	−0.8
D49785	Map3k12	MAP kinase kinase kinase 12	0.7	0.9	−1.4	−0.8
M11794	Mt1a	Metallothionein	−0.2	−0.4	−0.4	−1.0
X63594	Nfkbia	NFkBα	0.1	0.1	−1.0	−1.0
AA926129	—	—	−0.4	−0.3	−0.7	−1.0
AA819776	Hspca	Heat shock protein 1α	0.2	0.5	−0.9	−1.2
(B) GM						
U15211	Rara	Retinoic acid receptor α	0.3	1.0	0.1	0.5
L17127	Psmb4	PSMB4 (proteasome (prosome, macropain) subunit, $\beta 4$)	−0.3	−1.0	0.3	−0.3
L17127	Psmb4	PSMB4 (proteasome (prosome, macropain) subunit, $\beta 4$)	−0.7	−1.6	0.1	−0.8
AA892248	—	—	−1.1	0.1	−0.8	−0.3
AA799499	—	mRNA similar to NADH dehydrogenase (ubiquinone) 1$\beta 3$	−0.7	−1.1	−0.3	−0.5
AA818604	Hspa1b	Heat shock 70 kD protein 1B	0.8	1.5	1.0	3.0
AA848563	Hspa1b	Heat shock 70 kD protein 1B	1.2	1.7	0.7	2.8
L16764	Hspa1b	Heat shock 70 kD protein 1B	−0.1	0.5	0.1	1.5
Z75029	Hspa1a	Heat shock 70 kD protein 1A	0.7	0.9	0.7	2.2
E00717	Cyp1a1	Cytochrome P450, 1a1	0.3	2.0	0.7	2.0
AA685903	—	EST, similar to Glucose regulated protein, 94 kDa (GRP94)	0.3	1.0	0.9	1.8
X53363	Calr	Calreticulin	0.8	1.0	1.0	1.7
U73174	Gsr	Glutathione reductase	1.1	1.2	0.6	1.4
U09540	Cyp1b1	Cytochrome P450, 1b1	0.9	2.4	0.3	1.0
X17163	Jun	v-jun sarcoma virus 17 oncogene homolog (avian)	0.0	1.6	0.1	1.3

Table 5. (*Continued*)

Probe ID	Symbol	Description	6 h		24 h	
			(1/10)[a]	(1/3)[b]	(1/10)	(1/3)
J02722	Hmox1	Heme oxygenase (decycling) 1	0.2	0.8	0.7	2.1
U30186	Ddit3	GADD153 (DNA-damage inducible transcript 3)	0.1	0.9	0.5	1.8
M86870	Erp70	Protein disulfide isomerase related protein (CaBP, intestinal-related)	0.7	0.8	1.1	1.7
U34963	Bcl2l1	Bcl2-like 1 (Bcl-XL)	0.6	0.8	1.0	1.6
AI229421	—	EST, similar to mouse MAPKAPK-2	0.6	0.7	0.7	1.3
D17309	Akr1d1	Aldo-keto reductase 1D1	0.6	0.8	0.7	1.1
L18889	Canx	Calnexin	0.3	0.3	0.6	1.0
M14050	Hspa5	Heat shock 70kD protein 5	−0.3	0.4	0.6	1.0
S82820	Gsta2	Glutathione-S-transferase $\alpha 2$	−0.2	0.4	0.2	1.0
AI009098	Hyou1	Hypoxia up-regulated 1	0.3	0.6	1.0	0.9
AI070295	Gadd45a	GADD45α	−0.7	−0.3	−0.9	−1.0
AI013297	—	EST, similar to NADH dehydrogenase (ubiquinone) Fe-S protein 4	−0.5	−0.6	−0.5	−1.0
AI171243	—	Similar to replication protein A3 (LOC296883), mRNA	−0.8	−0.5	−0.4	−1.0
AA956114	—	mRNA similar to ubiquitin-conjugating enzyme HR6A	−0.4	−0.4	−0.5	−1.2
AI009141	—	Chromobox homolog 1 (Drosophila HP1 beta) (predicted)	−1.1	−0.5	−0.8	−1.3
D49785	Map3k12	MAP kinase kinase kinase 12	−1.5	−1.1	−0.6	−1.3
X62952	Vim	Vimentin	−0.2	0.0	−0.3	−1.6
(C) DF						
L17127	Psmb4	PSMB4 (proteasome (prosome, macropain) subunit, $\beta 4$)	−1.3	0.4	0.4	0.6
AI010292	—	—	1.1	0.1	0.5	0.3
X63594	Nfkbia	NFkBα	−0.4	−1.0	0.1	−0.5
D49785	Map3k12	MAP kinase kinase kinase 12	−0.7	−1.3	−0.6	−0.9
K00136	—	LOC494499 protein	0.8	1.2	0.1	1.3
U05014	Eif4ebp1	Translation initiation factor 4E binding protein 1	0.0	1.0	0.3	1.2
U05014	Eif4ebp1	Translation initiation factor 4E binding protein 1	0.0	0.9	0.2	1.1
J02722	Hmox1	Heme oxygenase (decycling) 1	−0.4	0.9	0.6	2.4
U30186	Ddit3	GADD153 (DNA-damage inducible transcript 3)	−0.1	0.2	−0.1	1.8
J02679	Nqo1	NAD(P)H:Quinone oxidoreductase-1 (NQO1)	−0.6	−0.6	0.6	1.3
AI070295	Gadd45a	GADD45α	−0.1	−0.2	0.2	1.0
AI070295	Gadd45a	GADD45α	−0.1	0.0	0.3	1.0
(D) CsA						
X53363	Calr	Calreticulin	1.4	1.0	0.9	0.3
D49785	Map3k12	MAP kinase kinase kinase 12	−1.0	−0.7	0.0	0.1
U73174	Gsr	Glutathione reductase	1.3	1.1	1.3	2.1
U05014	Eif4ebp1	Translation initiation factor 4E binding protein 1	0.5	1.2	0.0	1.0
U05014	Eif4ebp1	Translation initiation factor 4E binding protein 1	0.3	1.0	0.0	0.5
AA893328	Canx	Calnexin	0.5	0.7	0.8	1.2
D83796	Ugt1a1~8	UDP glycosyltransferase 1A	0.4	0.1	0.7	1.2
AA892248	—	—	−0.5	0.5	0.6	1.1
AA892248	—	—	−0.5	0.3	0.6	1.0
U17697	Cyp51	CYP51 (Sterol 14 alpha-demethylase)	0.3	0.6	0.7	1.1
AI014091	Cited2	Cbp/p300-interacting transactivator, with ED-rich tail2	0.1	−0.1	1.0	0.9
U12187	Rrad	Ras-related associated with diabetes	−0.4	−0.9	−0.6	−1.4
(E) Zn						
X53363	Calr	Calreticulin	0.4	0.4	0.2	1.1
M11794	Mt1a	Metallothionein	0.3	1.6	0.0	0.7
J05035	Srd5a1	Steroid 5α-reductase 1	−0.2	−0.2	1.1	0.3
AA945054	Cyb5	Cytochrome b5	−0.3	−1.0	−0.2	0.0
AA892248	—	—	−1.1	−0.8	0.0	0.0

[a] One-tenth of TC_{50}.
[b] One-third of TC_{50}. — no symbol or no description.

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In vitro gene expression analysis of hepatotoxic drugs in rat primary hepatocytes

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ABSTRACT: **The study examined the feasibility of screening for hepatotoxicity by an *in vitro* gene expression analysis using rat primary hepatocytes and Affymetrix Rat Toxicology U34 arrays. Hepatocytes were exposed for 6 or 24 h to eight drugs, with different mechanisms of hepatotoxicity, at one third of the cytotoxic concentration TC_{50}, i.e. acetaminophen, cyclophosphamide, clofibrate, chlorpromazine, lithocholic acid, cisplatin, diclofenac and disulfiram. The types of transcriptional changes observed in this study were generally consistent with previously reported *in vivo* data, although there were some differences. In hierarchical cluster analysis, drugs formed clusters depending on their mode of toxicity against cells. The number of transcripts affected by the cholestatic hepatotoxicants (lithocholic acid and chlorpromazine) or the drugs that rarely cause of hepatotoxicity (cisplatin, diclofenac and disulfiram) were limited compared with the other drugs (acetaminophen, clobifibrate and cyclophosphamide), where they did not induce transcriptional changes apparently related to toxicity. It is concluded that *in vitro* gene expression analysis of hepatocytes using microarray is a useful tool for evaluating the toxicological profile of drugs and in screening for the direct toxicity of drugs against hepatocytes.**

KEY WORDS: *in vitro*; primary culture; hepatotoxicity; microarray; gene expression

Introduction

Hepatotoxicity is a critical toxicity caused by many drugs, indicated by a report which showed that it is one of the major reasons for drug withdrawal from the market (Lee, 2003). This study evaluated the feasibility of an *in vitro* gene expression analysis approach, using microarray as a testing tool to screen for drug-induced hepatotoxicity. *In vitro* approaches are important especially in the early stage of drug development, where only a small amount of test item is available. This study used the primary hepatocyte as a test cell, representing normal cell function compared with cell lines. The MicroArray Quality Control (MAQC) consortium showed reproducibility across different microarray platforms, and revealed good correlation between TaqMan and microarrays including the Affymetrix GeneChip (Shi *et al.*, 2006; Patterson *et al.*, 2006). Their data indicated reliability of expression analysis using the GeneChip. This study used the Affymetrix Rat Toxicology U34 array, which contains probes for more than 850 genes and expressed sequence tags (ESTs). The study first compared the transcriptional difference between two different culture conditions of primary rat hepatocytes without drug exposure, both of which are utilized in our laboratory, i.e. conventional culture on collagen I plates and culture on collagen I-gel coated plates. After culture condition optimization, expression analysis of *in vitro* hepatotoxicity was performed with eight test drugs, acetaminophen (APAP), clofibrate (CF), cyclophosphamide (CPA), chlorpromazine (CPZ), lithocholic acid (LCA), cisplatin (CDDP), diclofenac (DF) and disulfiram (DSF), using the collagen I-gel culture condition. APAP-induced hepatotoxicity is attributed to covalent binding of a reactive metabolite to proteins and DNA after glutathione depletion, which is caused by the reactive metabolite produced in the hepatocytes (Mitchell *et al.*, 1973). CF-induced hepatotoxicity is attributed to peroxisome proliferator-activated receptor (PPAR) activation, which leads to mitochondrial damage, oxidative stress (Qu *et al.*, 2001) and induction of cell growth related genes (Peters *et al.*, 1998). CPA-induced hepatotoxicity is attributed to covalent binding of reactive metabolites to macromolecules such as proteins and DNA (Esterbauer *et al.*, 1991) after glutathione depletion. Although the mechanism of hepatotoxicity is similar to that of APAP, the principal target of CPA-induced hepatotoxicity *in vivo* is the sinusoidal endothelial cell

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(SEC) due to its greater susceptibility compared with the hepatocyte (DeLeve, 1996). This study evaluated the direct effects of CPA on the hepatocyte. The hepatotoxicity of CPZ and LCA is attributed to cholestasis. CPZ-induced cholestasis is not well understood, although various mechanisms whereby CPZ impairs the bile flow have been proposed, i.e. formation of insoluble precipitates with bile acids (Carey *et al.*, 1976), increased prostaglandin secretion (Akerboom *et al.*, 1991), impairment of bile acid excretion (Tavoloni *et al.*, 1979) and inhibition of actin polymerization (Elias and Boyer, 1979). Cholestasis from LCA, a secondary bile acid, has been related to the retention of the bile salt in the liver, increased cholesterol content in the bile canalicular membrane (Kakis *et al.*, 1980; Kakis and Yousef, 1978), bile salt crystalline formation in the bile canaliculi (Miyai *et al.*, 1977) and down-regulation of bile salt export pump (Yu *et al.*, 2002). Therefore, both CPZ and LCA were selected on the supposition that they would not induce transcriptional effects related to toxicity in the hepatocyte primary culture. CDDP induces cytotoxicity by forming CDDP-DNA adducts, whereas the liver is not the major target of CDDP *in vivo* and it rarely induces hepatotoxicity at standard doses (Cavalli *et al.*, 1978). DF, a nonsteroidal antiinflammatory drug (NSAID), is an idiosyncratic hepatotoxicant. DF-induced idiosyncratic hepatotoxicity is not well understood, but adduct formation of protein with reactive metabolites (Miyamoto *et al.*, 1997; Pumford *et al.*, 1993) and hypersensitivity (Romano *et al.*, 1994; Salama *et al.*, 1991; Kretz-Rommel and Boelsterli, 1993) have been proposed. Both DF and DSF have been reported to be idiosyncratic hepatotoxicants (Boelsterli, 2003; Forns *et al.*, 1994). Therefore, CDDP, DF and DSF were selected on the supposition that they would not induce drastic transcriptional changes in the same setting as typical hepatotoxicants.

The test concentration of each drug was set at one third of the cytotoxic concentration (TC_{50}) in order to avoid cytotoxicity but to induce transcriptional changes. In order to capture transcriptional changes of drugs, regardless of their modes of action or degrees of cytotoxicity, two time points were set. Our results were compared with previously reported *in vivo* studies.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (Nippon SLC Inc., Shizuoka, Japan) 6–7 weeks old were used. The liver was isolated under anesthesia with 60 mg kg^{-1} sodium pentobarbital intraperitoneally. This experiment was approved by the company committee for the ethical use and care of laboratory animals.

Primary Cell Preparation

Hepatocytes were isolated using a modified collagenase perfusion method described elsewhere (Klaunig *et al.*, 1981) and by Percoll centrifugation. Briefly, livers were perfused *in situ* via the portal vein, first with Ca^{2+}/Mg^{2+} free Hank's balanced salt solution (HBSS-; GibcoBRL, NY, USA) containing 0.5 mM EGTA (Wako Pure Chemical, Osaka, Japan) and 10 mM HEPES (GibcoBRL) for 2–3 min, and then with HBSS with calcium and magnesium (HBSS+; GibcoBRL) containing 10 mM HEPES, 0.5 mg ml^{-1} collagenase (*Clostridium histolyticum* origin; Wako Pure Chemical) and 0.05 mg ml^{-1} soybean type II-S trypsin inhibitor (Sigma) for 6–7 min, at a flow rate of 20 ml min^{-1}. Perfused livers were isolated, decapsulated on ice, and dispersed in ice-cold HBSS–. Dispersed cells were filtered through 100 μm nylon meshes, rinsed, suspended in ice-cold 35% (v/v) Percoll (Amersham Biosciences, Piscataway, NJ, USA) and centrifuged at 150 *g* for 10 min at 4 °C . Hepatocytes were rinsed and suspended in Williams' E (GibcoBRL) culture media supplemented with 10% (v/v) FCS (GibcoBRL), 2 mM L-glutamic acid (GibcoBRL), 100 U ml^{-1} penicillin (GibcoBRL), 100 μg ml^{-1} streptomycin (GibcoBRL), 10^{-7} M insulin (GibcoBRL), 10^{-6} M dexamethasone (Sigma) and 10 mM HEPES. Cell cultures yielding more than 80% viability, based on trypan blue exclusion, were used for further experiments. All cultures were maintained at 37 °C in a water-saturated 5% CO_2–95% air incubator, and culture media were changed every day.

Culture Plate Preparation

For optimization of the culture condition, two different culture plates were used. For one type of culture plate, a solution of 1000 μl of 2.3 mg ml^{-1} collagen I (3.25 mg ml^{-1} rat tail collagen I, BD Biosciences, Bedford, MA, USA) was poured onto each well of 6-well plastic plates (9.5 cm^2/well, Iwaki, Tokyo, Japan) and a gel layer was formed according to the manufacturer's instructions. The liquid inside the gel was replaced by culture media three times before use. For the second culture plate type, conventional 6-well collagen I plates (Iwaki, Tokyo, Japan) were used.

For the expression analysis of *in vitro* hepatotoxicity, the 6-well collagen I-gel plates were used.

Culture Condition Optimization

The transcriptional profiles of rat primary hepatocytes were compared in two different culture conditions. The cells from the same animal were divided and seeded onto two different culture plates: 6-well collagen I plates (5.25×10^4 cells/cm^2) and 6-well collagen I-gel plates

(10.5×10^4 cells/cm^2). The cells on monolayer cultures (collagen I plates) became flattened and spread more compared with the cells on the collagen I-gel; therefore, a lower number of cells was needed to form a confluent culture. The culture media were changed 3 h after seeding to remove non-adherent cells. Total RNA was extracted from both freshly isolated hepatocytes and the primary cells after a 24 h culture. Cell experiments were performed in triplicate to generate triplicate microarray.

In vitro Hepatotoxicity Expression Analysis

Test Drugs

Test concentrations were set at one-third the cytotoxic concentration TC_{50}, which was deemed not to be severely cytotoxic but should cause some transcriptional effects. The TC_{50} values used were cited in a previous study by our group (Wang *et al.*, 2002) in which rat primary hepatocytes were maintained as a monolayer culture on conventional collagen I plates and the TC_{50} was determined from the WST-1 colorimetric assay after 24 h exposure. The test concentrations were 4671.3 μM for acetaminophen (Sigma), 1219.0 μM for cyclophosphamide (Sigma), 1079.0 μM for clofibrate (Sigma), 15.2 μM for chlorpromazine HCl (Sigma), 31.7 μM for lithocholic acid (Wako Pure Chemical), 17.7 μM for cisplatin (Sigma), 87.7 μM for diclofenac sodium (Sigma) and 11.7 μM for disulfiram (Sigma). Drugs were dissolved in dimethyl sulfoxide (DMSO, Wako Pure Chemical) and a 1/100 volume was added to the culture media. Negative controls were treated with 1% DMSO.

Drug Exposure and RNA Extraction

In each independent experiment, a negative control culture was prepared for each exposure duration of 6 or 24 h. The cells were seeded onto the collagen I-gel plates. Three hours after seeding, the cells were exposed to test culture media for either 6 h or 24 h as follows. First, the culture media of the 24 h exposure groups were exchanged with test culture media. At the same time, the culture media of the 6 h exposure groups were exchanged with new, drug-free, culture media. Second, 18 h after the first culture media exchange, the culture media from the 6 h exposure groups were exchanged with the test culture media. The cells were harvested for RNA extraction 24 h after the first culture media exchange. Experiments were replicated three times and equivalent amounts of total RNA were pooled to generate one microarray for each condition.

Hybridization Cocktail Preparation

Total RNA was extracted with Sepasol I (Nacalai Tesque, Kyoto, Japan) and purified with an RNeasy Mini kit (Qiagen, Valencia, CA, USA). Double-stranded cDNA was synthesized from 5 μg of total RNA with a Superscript II Reverse Transcriptase system (Invitrogen Life Technologies, Carlsbad, CA, USA) with T7-$(dT)_{24}$ primer (5′-GGCCAGTGAATTGTAATACGACTCACTATAG-GGAGGCGG(T)24-3′) (Sigma Genosys Japan KK, Hokkaido, Japan). Biotin-labeled cRNA was synthesized from the cDNA template with a T7 MegaScript kit (Ambion, Austin, TX, USA) in the presence of biotin-labeled NTP, and purified with an RNeasy Mini kit. The cRNA was fragmented at 95 °C for 35 min in a buffer containing 40 mM Tris-acetate, pH 8.1, 100 mM KOAc and 30 mM MgOAc. The final hybridization cocktail included 0.05 μg μl^{-1} cRNA, 0.1 mg ml^{-1} herring sperm DNA (Promega, Madison, WI, USA), 0.5 mg ml^{-1} acetylated BSA (Sigma), 0.05 nM biotinylated-control oligonucleotides and 0.1 M MES buffer (pH 6.7).

Hybridization, Staining and Scanning

Rat Toxicology U34 arrays (Affymetrix, Santa Clara, CA, USA) which contain 25-mer probes for more than 850 genes and ESTs were used. Microarrays were pretreated with a solution containing 0.5 mg ml^{-1} acetylated BSA, 0.5 mg ml^{-1} herring sperm DNA and 0.1 M MES buffer at 40 °C for 15 min and then incubated with the hybridization cocktail at 45 °C for 15 h. Microarrays were stained with a solution containing 10 μg ml^{-1} streptavidin R-phycoerythrin conjugate (Molecular Probes, Eugene, OR, USA). Microarrays were scanned with a laser scanner system (Affymetrix).

Data Analysis

Signal intensity, reliability of each probe and 'Detection Call' expressed as Present, Absent or Marginal, were calculated with the Affymetrix Microarray Suite ver5.0 software (MAS 5.0). The average signal intensity of each array was scaled to one value of 150 in order to compare multiple arrays. The mean ± SD and CV% of 3′/5′ ratio for a housekeeping gene GAPDH was 1.1 ± 0.1 (9.7%), which indicated good quality of the RNA sample. Pair-wise comparison analyses were performed by MAS 5.0, where the Signal Log Ratio (log 2 ratio) and the 'Change Call' (Increase, Decrease, or No-Change) were calculated. Hierarchical cluster analysis of the Signal Log Ratio was performed with TIGR MeV ver3.1 software (Saeed *et al.*, 2003) using squared Pearson with average linkage. Probes which satisfied all the following criteria in at least one comparison were identified as differently expressed: (i) signal intensity of 100 or more and 'Detection Call' of Present; (ii) 'Change Call' other than No-Change; (iii) Signal Log Ratio of 1 or more, or −1 or less (i.e. ≥ two fold change). In the culture condition optimization experiment, each condition consisted of triplicate arrays; pair-wise

Table 1. Transcripts that expressed differently between monolayer culture and collagen I-gel culture

Probe ID	Symbol	Description	Relative signal intensity		
			Freshly isolated hepatocytes	Conventional monolayer culture	Culture on collagen I-gel
AA818122	Sult2a1	STase family, cytosolic, 2A, DHEA-preferring, member 1	1	0.07 ± 0.03[a]	0.15 ± 0.10
M31363	Sult2a1	STase family, cytosolic, 2A, DHEA-preferring, member 1	1	0.07 ± 0.04	0.14 ± 0.08
AA942685	Cdo1	Cytosolic cysteine dioxygenase 1	1	0.14 ± 0.06	0.29 ± 0.10
AA926149	Cat	Catalase	1	0.18 ± 0.06	0.25 ± 0.08
M86758	Ste	STE (STase, estrogen preferring)	1	0.28 ± 0.06	0.44 ± 0.13
AA892248	EST	—[b]	1	0.79 ± 0.12	1.62 ± 0.26
AA892248	EST	—	1	0.85 ± 0.08	1.56 ± 0.32
D86086	Abcc2	ATP-binding cassette, subfamily C (CFTR/MRP), member 2	1	1.29 ± 0.63	0.82 ± 0.07
AA685903	Tra1 predicted	Tumor rejection antigen gp96 (predicted)	1	2.13 ± 1.52	1.11 ± 0.21
D14014	Ccnd1	Cyclin D1	1	2.28 ± 1.56	0.97 ± 0.51
D14014	Ccnd1	Cyclin D1	1	3.23 ± 3.52	0.80 ± 0.41
X13933	Calm1	Calmodulin 1	1	3.31 ± 0.56	2.29 ± 0.48
U05989	Pawr	PRKC, apoptosis, WT1, regulator	1	4.27 ± 1.12	1.78 ± 0.45
L38615	Gss	Glutathione synthetase	1	5.88 ± 2.85	3.11 ± 1.67
M11794	Mt1a	Metallothionein	1	4.84 ± 4.77	12.02 ± 15.05
U30186	Ddit3	GADD153 (DNA-damage inducible transcript 3)	1	13.7 ± 6.9	23.7 ± 6.1
AA859372	EST	—	1	16.3 ± 23.9	7.9 ± 11.4
Y00396	Myc	Myelocytomatosis viral oncogene homolog (avian)	1	22.8 ± 10.1	14.9 ± 7.8
M55534	Cryab	Crystallin, alpha B	1	37.4 ± 39.8	7.0 ± 8.0
M81855	Abcb1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	1	45.0 ± 9.8	14.9 ± 2.6
J02722	Hmox1	Heme oxygenase (decycling) 1	1	47.6 ± 30.9	102.7 ± 48.5
X62952	Vim	Vimentin	1	151.1 ± 171.8	56.8 ± 68.9

[a] Mean ± SD (n = 3).
[b] No symbol or no annotation.

comparisons were performed between two different culture conditions of corresponding animals. Signal intensities of the primary cells relative to the freshly isolated hepatocytes were calculated in MS Excel. In the *in vitro* hepatotoxicity experiments, each condition consisted of one array; pair-wise comparisons were performed between the drug exposure group and the corresponding negative controls.

Results and Discussion

Culture Condition Optimization

The expression of 22 transcripts were identified as being different depending on the two culture conditions (Table 1). There was a tendency for the expression alterations in the collagen I-gel cultures to be less drastic than those in the conventional monolayer culture when they were compared with freshly isolated hepatocytes. In addition compared with conventional monolayer cultures, induced Ccnd1 was not observed and induced Myc was suppressed in the collagen I-gel cultures, both of which are cell cycle associated genes. From these results, the collagen I-gel culture was adopted for expression analysis of *in vitro* hepatotoxicity.

In Vitro Hepatotoxicity Expression Analysis

Overall Changes

Drugs were grouped depending on their mode of toxicity against cells in the hierarchical cluster analysis. APAP and CPA formed a cluster, along with CDDP at 24 h (Fig. 1). This grouping appeared reasonable for APAP and CPA given the known hepatotoxicity associated with them, but on first consideration, it seemed unusual for CDDP, which rarely induces hepatotoxicity at standard doses (Cavalli *et al.*, 1978), and unlike APAP (Table 3A) and CPA (Table 3C), did not induce any apparent toxicity

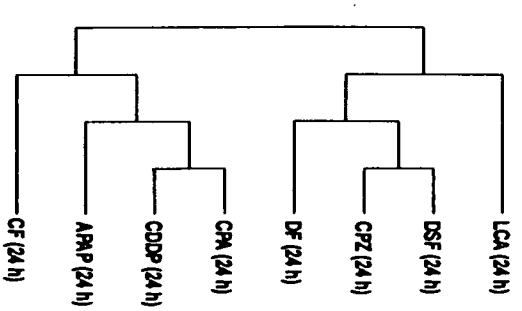

Figure 1. Hierarchical cluster analysis of gene expression profiles of all the test substances at 24 h