Molecular Diversity (2006) 10: 349–360
DOI: 10.1007/s11030-006-9038-0

Full-length paper

Differences in hepatotoxicity and gene expression profiles by anti-diabetic PPARγ agonists on rat primary hepatocytes and human HepG2 cells

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Received 27 October 2005; Accepted 10 March 2006

Key words: apoptosis, ciglitazone, cytotoxicity, cluster analysis, JTT-501, molecular descriptors, peroxisome proliferator-activated receptor γ, pioglitazone, rat genome microarray, rat primary hepatocytes, rosiglitazone, troglitazone

Summary

Agonists of peroxisome proliferator-activated receptor γ (PPARγ) are a new class of oral drugs designed to treat insulin-resistant diabetes (i.e., type 2 diabetes). However, troglitazone, the first compound in the class approved by the US Food and Drug Administration (FDA) in 1997 was found to be hepatotoxic and was withdrawn from the market after reports of severe liver failure. The mechanism of PPARγ agonist-induced hepatotoxicity remains unknown. In this study, we examined the hepatotoxic effects of five PPARγ agonists (ciglitazone, pioglitazone, rosiglitazone, troglitazone, and JTT-501) on rat primary hepatocytes and human HepG2 cells. We also compared the gene expression profiles of rat primary hepatocytes after exposure to PPARγ agonists by using the Rat Genome Survey Microarray system from Applied Biosystems in order to understand the mechanisms of hepatotoxicities induced by PPARγ agonists. Consistent with the hepatotoxicity data, our results demonstrate that the gene expression profiles affected by troglitazone and ciglitazone can be clearly distinguished from those by pioglitazone and rosiglitazone. Genes that are differentially expressed between the more toxic troglitazone/ciglitazone group and the less toxic rosiglitazone/pioglitazone group are involved in necrotic, apoptotic, and cell proliferative pathways. The five compounds were also clustered based on a set of molecular descriptors. The clustering based on chemical structural information is in good agreement with the clustering of compounds based on cytotoxicity or gene expression data, indicating a strong relationship between chemical structure and biological endpoints. Our work suggests that microarray analysis together with toxicological observations can be used to rank drugs for hepatotoxicity and to evaluate the safety of new compounds.

Abbreviations: PPARγ, peroxisome proliferator-activated receptor γ; TZD, thiazolidinedione; PCA principal component analysis

Introduction

Adverse drug effects lead to billions of dollars in direct hospital costs every year. Drug-induced toxicities account for 10–52% of all causes of acute liver failures, and hepatotoxicity is the major cause of drug withdrawal and the rejection of new drug applications [1]. Thiazolidinediones (TZDs) are a new class of oral drugs used to treat insulin-resistant diabetes (i.e., type 2 diabetes). These drugs reduce the plasma glucose level of type 2 diabetic patients [2, 3] by improving insulin sensitivity in fat and muscle via the activation of the peroxisome proliferator-activated receptor γ (PPARγ), a ligand-activated transcription factor of the nuclear hormone receptor superfamily [4].

TZDs are PPARγ agonists with a thiazolidinedione ring as the common structural characteristic (Figure 1). Ciglitazone, the first PPARγ agonist that was put into clinical trials, was not marketed because of liver toxicity. Troglitazone, the first PPARγ agonist approved by the US Food and Drug Administration (FDA) for the treatment of type 2 diabetes in 1997, was found to be hepatotoxic [5], and was withdrawn after reports of severe liver failure and death [6, 7]. Rosiglitazone and pioglitazone,

The views presented in this article do not necessarily reflect those of the US Food and Drug Administration.

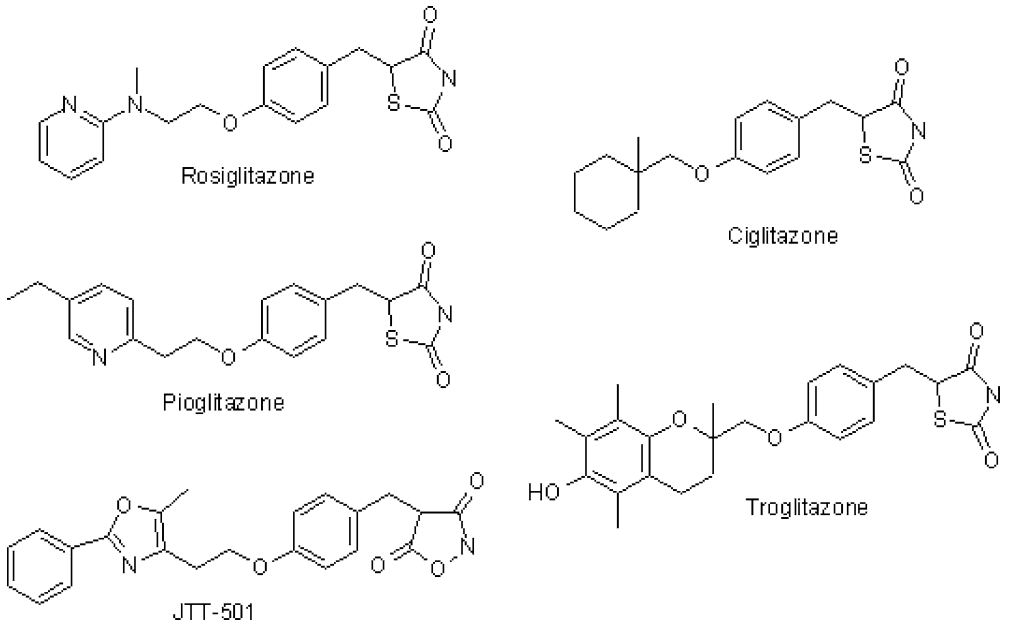

Figure 1. Chemical structures of four anti-diabetic thiazolinediones (rosiglitazone, pioglitazone, ciglitazone, and troglitazone) and a closely related compound, JTT-501.

approved by the FDA in 1999, are second generation PPARγ agonists.

Although less frequent and severe incidences of hepatotoxicity was observed with rosiglitazone and pioglitazone compared to troglitazone, hepatic failures in patients taking rosiglitazone and pioglitazone have been reported [8, 9]. The development of JTT-501, a PPARγ agonist that is structurally similar to TZDs and exhibits some degree of PPARα activity, was discontinued due to lower than expected efficacy after Phase 2 clinical trials. A variety of analogs targeting the PPAR$\alpha/\gamma/\delta$ are under development by the pharmaceutical industry. Therefore, it is of great importance to investigate the mechanisms of toxicities of various PPARγ agonists in order to develop effective and safe products.

Microarray is a powerful tool that can be used to simultaneously examine the expression levels of thousands of genes in a sample. Toxicogenomics, a new field that applies "omics" technologies in toxicological research, permits the correlation between toxicity endpoints and changes in gene expression profiles. Toxicity that occurs as a result of cellular dysfunction induced by the same class of compounds may indicate common physiological or pathological processes. Thus, a class of compounds may similarly alter gene expression patterns at the molecular level. Microarray analysis can be applied to cluster toxins based on similarities in gene expression of similar mechanisms of toxicity [10]. It can be potentially used as a tool to determine the degree of toxicity and to elucidate the mechanisms of toxicity during drug development.

In the present study, by using rat primary hepatocytes and human HepG2 cells, we compared the hepatotoxic effects and changes in gene expression profiles resulting from the treatments with five PPARγ agonists (i.e., troglitazone, ciglitazone, pioglitazone, rosiglitazone, and JTT-501) in order to examine the correlation between toxicological endpoints and gene expression patterns. Microarray analysis showed that expression profiles differed between more hepatotoxic compounds (i.e., troglitazone and ciglitazone) and less hepatotoxic compounds (i.e., pioglitazone and rosiglitazone), with JTT-501 showing gene expression profiles between those of the two groups. Genes that are differentially expressed between the troglitazone/ciglitazone group and pioglitazone/rosiglitazone group are mainly involved in pathways such as necrosis, apoptosis, and cell proliferation. Overall, there was good agreement between toxicological observations and changes in gene expression profiles. In addition, the five compounds were also clustered based on a set of molecular descriptors such as logP. The clustering based on chemical structural information was also in good agreement with the clustering of compounds based on cytotoxicity or gene expression data, indicating a strong relationship between chemical structure and biological endpoints. This study provided indications on the plausible mechanisms of hepatotoxicity of this important class of anti-diabetic drugs, and may help the assessment of safety of new drug candidates.

Materials and methods

Chemicals and cell treatments

Troglitazone and ciglitazone were purchased from BIOMOL International (Plymouth Meeting, PA, USA). Rosiglitazone was purchased from LKT Laboratories (St. Paul, MN,

USA). Pioglitazone and JTT-501 were generously donated by Chipscreen Biosciences Ltd. (Shenzhen, China) and the FDA's Center for Drug Evaluation and Research (Rockville, MD, USA), respectively. All compounds were prepared as a 40 mM stock solution in dimethyl sulfoxide (DMSO) and added to the cell cultures in final concentrations of 5–100 μM. The same amount of DMSO was added to control cell cultures. During the treatments, serum-free medium was used with supplements (see below).

Animals, hepatocyte isolation, and cell culture

Three male 6–8 week-old Sprague-Dawley rats were obtained from the breeding colony of the FDA's National Center for Toxicological Research. Rats were anesthetized with 1.5 ml/kg of the nembutal sodium solution containing 50 mg/ml of pentobarbital sodium prior to undergoing liver perfusion. All animals used in this study were handled in accordance with the principles and guidelines prepared by the National Institutes of Health, USA.

Rat primary hepatocytes were isolated by a two-stage collagenase perfusion process according to the methods described by Seglen [11] and Kreamer [12]. Rat primary hepatocytes were suspended in L-15 medium containing 2 mg/ml BSA, 18 mM HEPES, 3 mg/ml proline, 1 mg/ml galactose, 0.1% insulin-transferrin-selenite, 10 ng/ml epidermal growth factor, 50 U/ml penicillin, and 50 μg/ml streptomycin. The cells were treated with compounds six hours after plating.

Human hepatoma HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. All cell culture media and other agents including FBS were purchased from Sigma (St. Louis, MO, USA). All cultures were maintained at 37 °C in humidified incubators containing 5% CO_2.

MTT assay

The cytotoxicities of the five compounds on rat primary hepatocytes and HepG2 cells were determined using the MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2*H*-tetrazolium bromide] assay, conducted by a colorimetric micro-culture assay using an *In Vitro* Toxicology Assay Kit (Sigma, St. Louis, MO, USA). The cultured rat primary hepatocytes and HepG2 cells were plated on 96-well plates at a cell density of 1-2 $\times$ 10^4/well in 100 μl media containing serum. After being cultured for 6 h, serum-deprived cells were treated with PPARγ agonists at various concentrations for the indicated period of time. Ten μl of reconstituted MTT was added to the incubation media at the end of treatment and incubated for an additional 2 h at 37 °C. The media was removed, the resulting formazan crystals were dissolved in acidified isopropanol, and the absorbances at 570 nm and 690 nm were measured with a microplate reader (μQuant, Universal Microplate Spectrophotometer, Bio-Tek Instruments, Winooski, VT, USA). The background absorbance at 690 nm was subtracted from the 570 nm measurements. The negative control media contained the same amount of the vehicle. The viability of the cells was expressed as a percentage by comparing the absorbance of treated cells to that of negative controls. Evaluation was based on the means of values obtained from at least three independent experiments.

Apoptosis assay and quantification of apoptotic cells

Apoptosis was detected by terminal deoxynucleotidyl transferase (TdT) mediated d-UTP nick end labeling (TUNEL) of DNA fragments. Slides with rat hepatocytes or HepG2 cells were fixed in 10% neutral buffered formalin overnight at 4 °C. After washing in PBS, the cells were post-fixed in pre-cooled ethanol and acetic acid mix (2:1) for 5 min at −20 °C. The ApopTag Peroxidase *In Situ* Apoptosis Detection Kit was obtained from Serologicals Corporation (Norcross, GA, USA) and the TUNEL assay was performed according to the manufacturer's instructions. Briefly, the endogenous peroxidase was inhibited in freshly prepared 3% hydrogen peroxide with 0.1% sodium azide. The cells were enzymatically labeled with digoxigenin-nucleotide via TdT and subsequently exposed to horseradish peroxidase-conjugated anti-digoxigenin antibody. Staining was developed in diaminobenzidine (DAB), and the cells were counterstained with methyl green, dehydrated, mounted, and examined by light microscopy (BX40, Olympus, Tokyo, Japan). Approximately 6,000 to 8,000 hepatocytes per treatment group were examined with a 40X objective, and the percentage of apoptotic bodies was calculated.

RNA isolation and quality control

Total RNA from cells was isolated using an RNeasy system (Qiagen, Valencia, CA, USA). The yield of the extracted RNA was determined spectrophotometrically by measuring the optical density at 260 nm. The purity and quality of extracted RNA were evaluated using the RNA 6000 LabChip and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Only high quality RNA with RNA integrity numbers (RINs) greater than 7.5 were used for microarray experiments.

Microarray gene expression profiling

To identify signatures in gene expression profiles associated with hepatotoxic effects of chemical treatment, we used microarray technique to examine the gene expression patterns in primary rat hepatocytes exposed to various PPARγ agonists. Gene expression profiling was performed using Applied Biosystems' Rat Genome Survey Microarray platform that has 26,857 probes.

Preparation of digoxigenin labeled in vitro transcribed cRNA

All RNA targets were labeled using the Applied Biosystems RT-IVT Labeling Kit Version 2.0. Briefly, 1.5 μg of total RNA sample was reverse transcribed via 2 h incubation at 42 °C with ArrayScript RT enzyme (Ambion, Austin, TX, USA) and oligo dT-T7 primer. Double stranded cDNA was produced following 2 h incubation with E. coli DNA polymerase and RNase H at 16 °C. Double stranded cDNA was purified according to the RT-IVT kit protocol. In vitro transcription was performed by incubation of the cDNA product with T7 RNA polymerase, 0.75 mM Digoxigenin-11-UTP (Roche Applied Science, Indianapolis, IN, USA) and all other NTPs for 9 h. Labeled cRNA was purified according to the RT-IVT kit protocol and analyzed for quality and quantity using standard UV spectrometry and the Bioanalyzer.

Hybridization of labeled cRNA to microarrays and microarray imaging

Digoxigenin labeled cRNA targets were hybridized to Applied Biosystems Rat Whole Genome Survey Microarrays using the Applied Biosystems Chemiluminescent Detection Kit. Briefly, 15 μg of labeled cRNA targets were fragmented via incubation with fragmentation buffer provided in the kit for 30 min at 60 °C. Fragmented targets were hybridized to microarrays during 16 h incubation at 55 °C with buffers and reagents from the Chemiluminescent Detection Kit. Post-hybridization washes and anti-Digoxigenin-Alkaline Phosphatase binding were performed according to the protocol of the kit. Chemiluminescence detection, image acquisition and analysis were performed using Applied Biosystems Chemiluminescence Detection Kit and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer following the manufacturer's protocols. Images were autogridded and the chemiluminescent signals were quantified, corrected for background, and finally, spot- and spatially-normalized using the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer software version 1.1.

Gene expression profiles were obtained at both 6 h and 10 h after 30 μM drug treatment in three biological replicates, resulting in 36 hybridizations from 2 time points $\times$1 dose $\times$3 biological replicates $\times$6 treatments (including DMSO). To evaluate the technical reliability of microarrays, replicate microarrays were performed on several RNA samples. In total, 52 hybridizations were performed for this study.

Statistical analysis of hepatotoxicity data

Analyses were performed using SigmaStat 3.0 (SPSS, Chicago, IL, USA). The percentages of apoptotic cells were expressed as the mean $\pm$ standard deviation (SD) from three independent experiments. Statistical significance was determined by one way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test.

Microarray data analysis

Gene expression data from the Applied Biosystems' Rat Genome Survey Microarray were input to ArrayTrack, a software system developed by the FDA's National Center for Toxicological Research for the management, analysis, visualization and interpretation of microarray data [13, 14]. Raw microarray intensity data were normalized per chip to the same median intensity value of 500. The identification of differentially expressed genes based on *t*-test and fold-change cutoffs and hierarchical clustering analysis were conducted within ArrayTrack. Additional calculations were performed within S-Plus (Insightful Corp., Seattle, WA, USA) and Significance Analysis of Microarrays (SAM) [15]. ArrayTrack and Ingenuity (Mountain View, CA, USA) were used for pathway analysis.

Results

Cytotoxicities of PPARγ agonists to rat primary hepatocytes and human HepG2 cells

Five PPARγ agonists caused cytotoxicities to rat primary hepatocytes in a time- and dose-dependent manner (Figure 2A). Troglitazone, ciglitazone and JTT-501 showed strong toxicities especially after 16 h treatments, whereas pioglitazone and rosiglitazone showed less toxicity in 2 h, 6 h and 16 h treatments. Table 1 summarizes the estimated IC_{50} values (concentration causing 50% cell death) after different treatment periods. The IC_{50} of troglitazone, ciglitazone, rosiglitazone, pioglitazone and JTT-501 were 71, 92, 224, 146 and 97 μM for 6 h treatment, and 54, 59, 165, 96 and 44 μM for 16 h treatment, respectively. The cytotoxic effects of troglitazone, ciglitazone and JTT-501 were much higher than those of rosiglitazone and pioglitazone in 6 h and 16 h treatments. We noticed that even after a short period (2 h) of treatment, the cytotoxicities of troglitazone and ciglitazone were significantly higher compared to DMSO controls, whereas the cytotoxicities of rosiglitazone and pioglitazone did not appear to be appreciable.

The dose-dependent cytotoxicities of the five compounds to human HepG2 cells after 16 h treatments were also

Table 1. The cytotoxicities (IC_{50}, μM) of PPARγ agonists on rat primary hepatocytes and human HepG2 cells

	Human HepG2	Rat primary hepatocyte		
	(16 h)	2 h	6 h	16 h
Rosiglitazone	109	1518	224	165
Pioglitazone	123	1231	146	96
JTT-501	77	271	97	44
Ciglitazone	46	98	92	59
Troglitazone	55	217	71	54

Table 2. The clinical effective doses and transcription activation activities of PPARγ agonists

		Transcription activation activity (EC_{50}, μM)		
Compound	Clinical dose (mg/day)	mPPARγ	hPPARγ	PPARα
Rosiglitazone	4–8	0.06 [16]	0.018 [17]	7.3 [18]
Pioglitazone	45	0.69 [16]	0.28 [17]	
JTT-501		0.1 [19]	0.083 [4]	4.3 m- [4]
1.9 h- [4]				1.9 h-[4]
Ciglitazone		3.0 [16]		
Troglitazone	200–600	0.13 [19]	2.235 [20]	

(m: mouse; h: human).

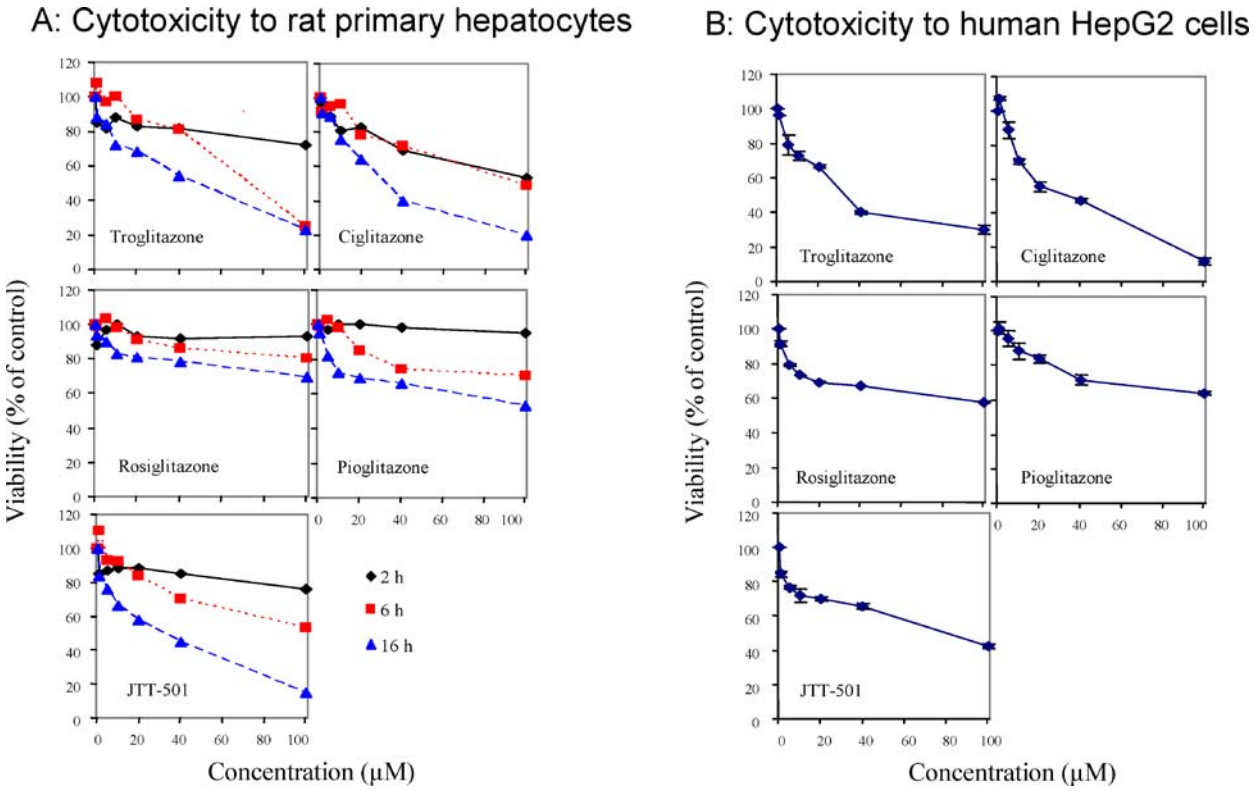

Figure 2. Cytotoxic effects of drugs on rat primary hepatocytes and human hepatoma HepG2 cells. (A). Viability of primary rat hepatocytes was examined by using the MTT assay after exposure to different concentrations (5–100 μM) for different time periods (2 h, 6 h, and 16 h). (B). Viability of HepG2 cells was examined by using the MTT assay after exposure to different concentrations (5–100 μM) for 16 h. Results are expressed as the percentage of cell viability compared to controls treated with the appropriate vehicle (DMSO). Data represent mean $\pm$ S.D. ($n = 3$–6).

examined and are shown in Figure 2B. It is clear that at a concentration of 30 μM the cytotoxicities of troglitazone and ciglitazone (viability of ~50%) are stronger than those of rosiglitazone and pioglitazone (viability of ~70%). At 30 μM, JTT-501 exhibited cytotoxicity (viability of ~70%) closer to that of rosiglitazone and pioglitazone; however, at higher concentration, its cytotoxicity increased to a level similar to that of troglitazone.

It should be noted that the variability of cytotoxicity assays with rat primary hepatocytes was much higher than that with HepG2 cells due to the difficulties in culturing and counting the primary hepatocytes and the inherent variability of the primary hepatocytes from rat liver perfusion. JTT-501, the only non-TZD PPARγ agonist, showed a stronger time-dependence of cytotoxicity in primary hepatocytes.

PPARγ agonists-induced apoptosis

Apoptosis has been considered as one of the mechanisms of hepatotoxicity induced by PPARγ agonists [21, 22]. We investigated PPARγ agonists induced apoptosis by using the TUNEL assay. Cells were incubated with PPARγ agonists at the concentration of 30 μM for 2 h and 16 h. Approximate 6000 to 8000 primary rat hepatocytes per treatment group

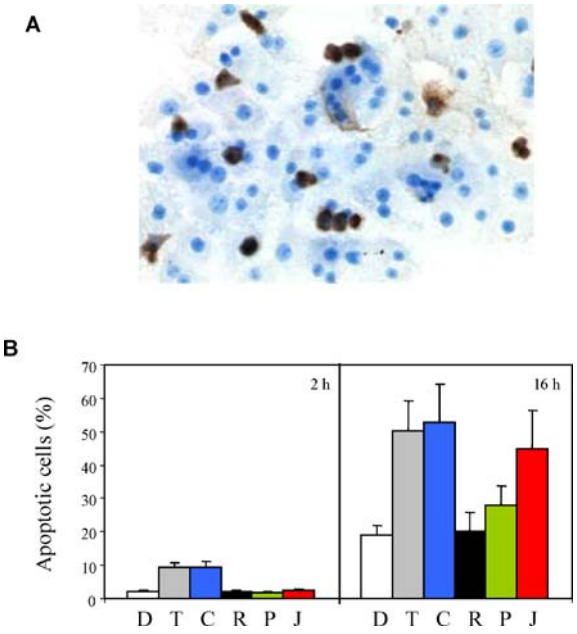

Figure 3. PPARγ agonists-induced apoptosis. (A) A representative of positive apoptotic bodies by TUNEL labeling on rat primary hepatocytes exposed to PPARγ agonists. (B) The percentages of apoptotic cells after exposure to 30 μM of the compounds for 2 h and 16 h. D, DMSO (vehicle control); T, Troglitazone; C, Ciglitazone; R, Rosiglitazone; P, Pioglitazone; J, JTT-501.

were examined and the percent incidence of apoptotic bodies was calculated. An example of TUNEL-stained apoptotic bodies is presented in Figure 3A. Figure 3B shows the percentages of apoptotic cells after treatments. Troglitazone and ciglitazone significantly increased apoptotic cells after 2 h (8–11% vs. 2% spontaneous apoptotic cells in controls) or 16 h (over 50% apoptotic cells vs. ~20% for control) treatments. The percentages of apoptotic bodies were not significantly increased by pioglitazone and rosiglitazone after 2 h treatment and only slightly increased by pioglitazone after 16 h treatment ($p < 0.01$). JTT-501 increased the percentage of apoptotic cells treated for 16 h ($p < 0.001$), but not for 2 h. All PPARγ agonists induced a time-dependent increase of apoptotic cells (16 h vs. 2 h, $p < 0.001$). The time-dependency of apoptosis induced by JTT-501 treatment is much stronger than that by other compounds, just like what has been seen for the cytotoxicity data.

Assessment of microarray data quality: identification of outlier hybridizations and animal-to-animal variability

It is expected that the majority of genes in the samples under this study will remain unchanged. Therefore, the pair-wise comparison of array data consistency may be used to identify outlier arrays. In this study, the microarray data reproducibility was assessed by the Pearson's correlation coefficient of pair-wise log2 intensity correlation. Figure 4A shows the color-coded image display of the Pearson's correlation matrix of 52 arrays based on all data points (26,857 genes). Three arrays (DMSO.6C.1.3K, DMSO.10.A.2.4M, and JTT.10.B.1.53) appeared to be quite different from the rest of arrays.

Expression data for genes marked with "present" (or of higher intensity) appear to be more reliable than those marked with "absent" (or of lower intensity) [23–25]. The large variation in lower intensity genes may dominate the pair-wise correlation analysis. Thus, we adopted a data (noise) filtering procedure proposed by Barczak et al. [24] which excludes 50% of the genes with the lowest average intensity across all 52 hybridizations, resulting in a subset of 13,428 genes (out of 26,857 genes). The reduced subset of 13,428 genes was subjected to the same procedures for data reproducibility assessment (Figure 4B). Two of the 52 arrays (CIG.6A.1.3X, DMSO.6C.1.3K) were identified as outliers and excluded from further analysis in the identification of differentially expressed genes. Figure 4B clearly shows that the clustering of the 52 arrays is mainly based on the source (animal) of primary hepatocytes. There is no obvious separation of arrays by treatment based on this clustering analysis.

Gene expression patterns associated with PPARγ agonists exposures: comparison to DMSO controls (unbiased data analysis procedure)

Replicate arrays (3–5) corresponding to treatments of each compound (rosiglitazone, pioglitazone, JTT-501, ciglitazone, and troglitazone) were compared to the control replicates (DMSO) using a *t*-test. A gene was considered significantly regulated by a compound if the two-tailed *t*-test *p*-value was less than 0.05 and the fold change was greater than 1.5. Based on these two criteria, there were 145, 171, 161, 367 and 548 genes that were altered by 6 h's treatment with pioglitazone, rosiglitazone, JTT-501, ciglitazone, and troglitazone, respectively (Figure 5). The number of genes regulated by ciglitazone/troglitazone treatment is at least twice as many as that by pioglitazone/rosiglitazone treatment. It should be noted that the data analysis at this stage did not take into consideration the class assignment of the compounds, and the emergence of the two groups of compounds (ciglitazone/troglitazone vs. pioglitazone/rosiglitazone) in terms of gene expression profiles reflected the true biological differences between the two groups of compounds.

The log2 intensity data for replicate arrays (3–5) corresponding to treatments of each compound was averaged and clustered (Figures 6A and 6C). Similarly, log2 fold changes for replicate arrays (3–5) corresponding to treatments of each compound in comparison to DMSO were also averaged and clustered (Figures 6B and 6D). In both cases (clustering based on intensity or fold change), ciglitazone and troglitazone always clustered together, as did rosiglitazone and pioglitazone. The difference between ciglitazone/troglitazone and rosiglitazone/pioglitazone is clear and is consistent with the difference in the number of genes altered (Figure 5), the differences in the degree of cytotoxicity (Figures 2) and apoptosis (Figure 3B).

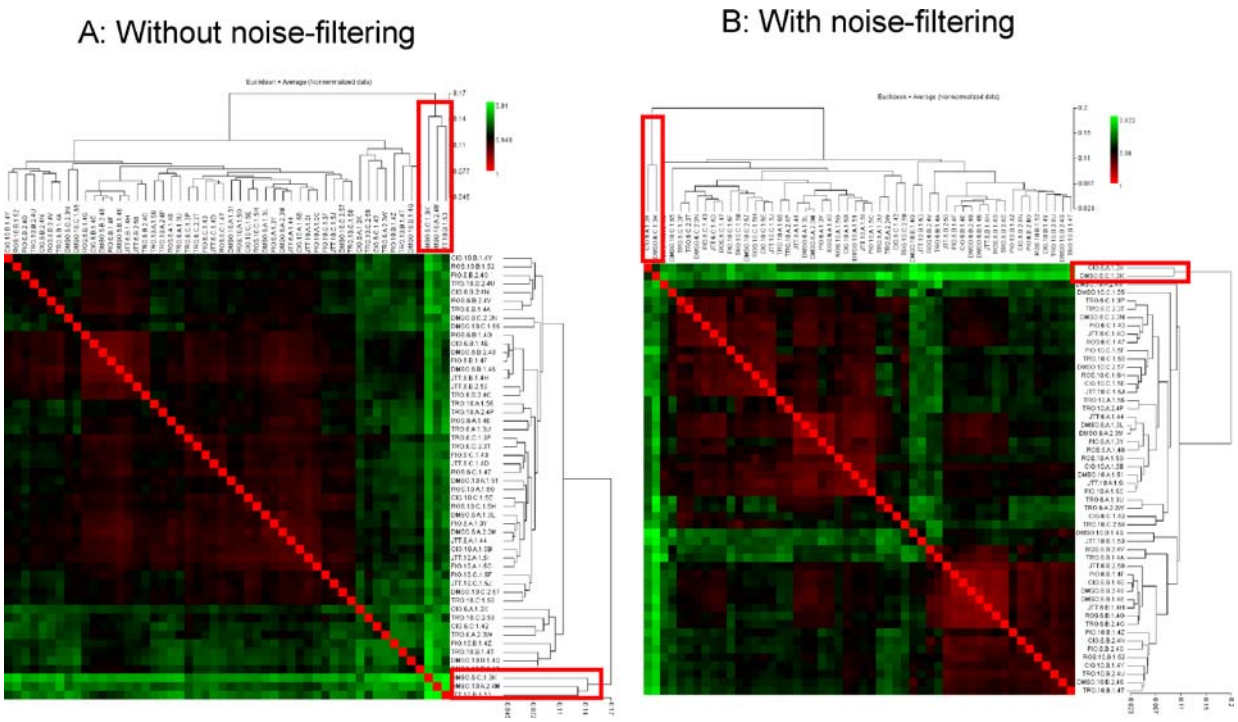

Figure 4. Identification of outlier hybridizations and animal variability. (A2) Pair-wise correlation was based on 26,857 data points; (B) Pair-wise correlation was based on 13,428 data points above the median of the overall median intensity across 52 arrays. The clustering of arrays was mainly based on the source of animals (A, B, C) from which primary hepatocytes were isolated. Obvious outlier arrays in B (CIG.6A.1.3X, DMSO.6C.1.3K) were highlighted and excluded from further analysis.

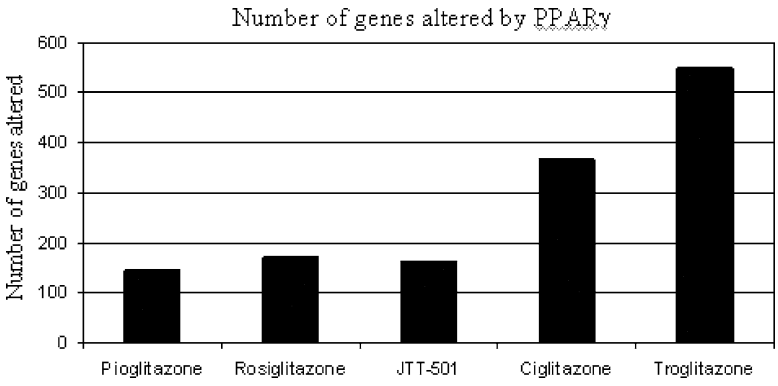

Figure 5. Number of genes altered by PPARγ agonists. There is an apparent difference in the number of genes that are significantly up- or down-regulated by the five PPARγ agonists. A gene was identified as significantly changed if the fold change (in comparison to DMSO group) was greater than 1.5 and the *p*-value was less than 0.05. Each compound treatment group consisted of at least three replicate treatments of rat primary hepatocytes isolated from three animals. 1844 genes were significantly regulated by treatments from at least one compound.

Time-dependence of gene expression in rat primary hepatocytes

Figure 6 demonstrated that gene expression in rat primary hepatocytes was affected more significantly by treatment duration (6 h vs. 10 h) than was by compounds since the group of treatments at 6 h clearly separated from those at 10 h, irrespective of the compounds. The gene expression profiles of the controls (DMSO controls) also exhibited significant differences between 6 h and 10 h, corresponding to spontaneous changes in rat primary hepatocytes that try to adapt to the new environment in cell culture medium. Such spontaneous changes in gene expression profiles without treatment are consistent with the increase of the percentage of apoptotic cells from 2 h to 16 h for the DMSO control group (Figure 3).

Differences in gene expression changes between rosiglitazone/pioglitazone and ciglitazone/troglitazone

A more explicit analysis was conducted to identify genes that were differentially expressed between the treatment groups of rosiglitazone/pioglitazone and ciglitazone/troglitazone

Table 3. Cell death related genes are differentially expressed between treatments with rosiglitazone/pioglitazone and ciglitazone/troglitazone

Gene name	Description	ABI probe ID	Locus Link ID	*p*-Value	Fold Change
Atf3	Activating transcription factor 3	21030397	25389	0.004	2.21
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	21677177	64547	0.022	1.52
Bid3	BH3 interacting (with BCL2 family) domain, apoptosis agonist	21005856	117271	0.016	2.07
Blnk	B-cell linker	21591819	17060	0.002	1.99
Cdkn1b	Cyclin-dependent kinase inhibitor 1B	22353027	83571	0.045	1.68
Cflar	CASP8 and FADD-like apoptosis regulator	20893535	117279	0.019	1.52
Cldn4	Claudin 4	22280638	12740	0	2.36
Ddit3	DNA-damage inducible transcript 3	21564969	29467	0.001	2.10
Egr1	Early growth response 1	20730028	24330	0.003	6.48
EMP2	Epithelial membrane protein 2	20764810	2013	0.001	1.53
Fem1b	Feminization 1 homolog b (C. elegans)	21889382	14155	0.018	1.51
Fhl2	Four and a half LIM domains 2	21480977	63839	0.005	1.52
Fos	FBJ osteosarcoma oncogene	22022961	14281	0.019	2.29
Gadd45g	Growth arrest and DNA-damage-inducible 45 gamma	22095143	23882	0.003	1.70
Herpud1	Homocysteine-inducible, endoplasmic reticulum stress-inducible	21193724	85430	0.008	1.62
Hmga1	High mobility group AT-hook 1	22070557	117062	0.002	1.74
Hmox1	Heme oxygenase (decycling) 1	21010125	24451	0.001	2.07
HSPA1A	Heat shock 70kDa protein 1A	22357696	3303	0.001	3.92
Igfbp1	Insulin-like growth factor binding protein 1	22115127	25685	0.009	2.87
Igfbp6	Insulin-like growth factor binding protein 6	21266211	25641	0.036	2.11
Il6r	Interleukin 6 receptor	20851835	24499	0.008	2.22
Il7	Interleukin 7	21467051	25647	0	1.67
LATS1	LATS, large tumor suppressor, homolog 1 (Drosophila)	22235056	9113	0.038	1.64
Mt1a	Metallothionein	22170383	24567	0.007	2.68
Myd116	Myeloid differentiation primary response gene 116	22152051	171071	0.01	1.71
Nkx6-1	NK6 transcription factor related, locus 1 (Drosophila)	21697943	65193	0.005	1.80
Nr1d1	Nuclear receptor subfamily 1, group D, member 1	21557648	252917	0.007	1.88
Phlda1	Pleckstrin homology-like domain, family A, member 1	21995038	29380	0.022	1.59
Pparg	Peroxisome proliferator activated receptor, gamma	21773923	25664	0.048	1.85
Sfn	Stratifin	20939212	55948	0.026	1.97
Skil	SKI-like	21164367	20482	0.024	1.87
Stk17b	Serine/threonine kinase 17b (apoptosis-inducing)	21058915	170904	0.031	1.59
Trp53inp1	Transformation related protein 53 inducible nuclear protein 1	21860958	297822	0.027	1.59

under 6 h treatments. To obtain more straightforward results, we left JTT-501 out in this comparison because of its unique profiles in cytotoxicity and PPAR activities (i.e., dual agonist). Among the subset of 1844 genes pre-selected in an unbiased manner (Figure 6), 213 genes were identified to be significantly different between the rosiglitazone/pioglitazone and ciglitazone/troglitazone groups by using a fold change cutoff of 1.5 and a *p*-value cutoff of 0.05 (Supplementary Table S1).

Genes related to apoptosis were differentially expressed between treatments with rosiglitazone/pioglitazone and ciglitazone/troglitazone

Table 3 illustrates genes that are differentially expressed between the rosiglitazone/pioglitazone and ciglitazone/troglitazone treatment groups. For example, the expression of Bid3/Hrk and Bim/Bcl2l11 was elevated in the more toxic group of ciglitazone/troglitazone treatments. Bid3/Hrk and Bim/Bcl2l11 belong to the Bcl-2 family and act as apoptosis activators. They interact with other members of the Bcl-2 protein family including Bcl-2 and Bcl-xl (anti-apoptotic) [26, 27]. Shiau et al. [28] reported that treatments with troglitazone and ciglitazone inhibited the anti-apoptotic functions of Bcl-xl and Bcl-2, leading to caspase-dependent apoptosis. Our results suggested several PPARγ agonists induced apoptosis; it may be a trigging factor which leads to hepatic cell death. Thus, the Bcl-2 family may play an important role in PPARγ agonists induced apoptosis.

Clustering of PPARγ agonists based on molecular descriptors

To examine the structural similarity of the five compounds used in this study, 486 molecular descriptors were calculated to describe the molecular characteristics of the five

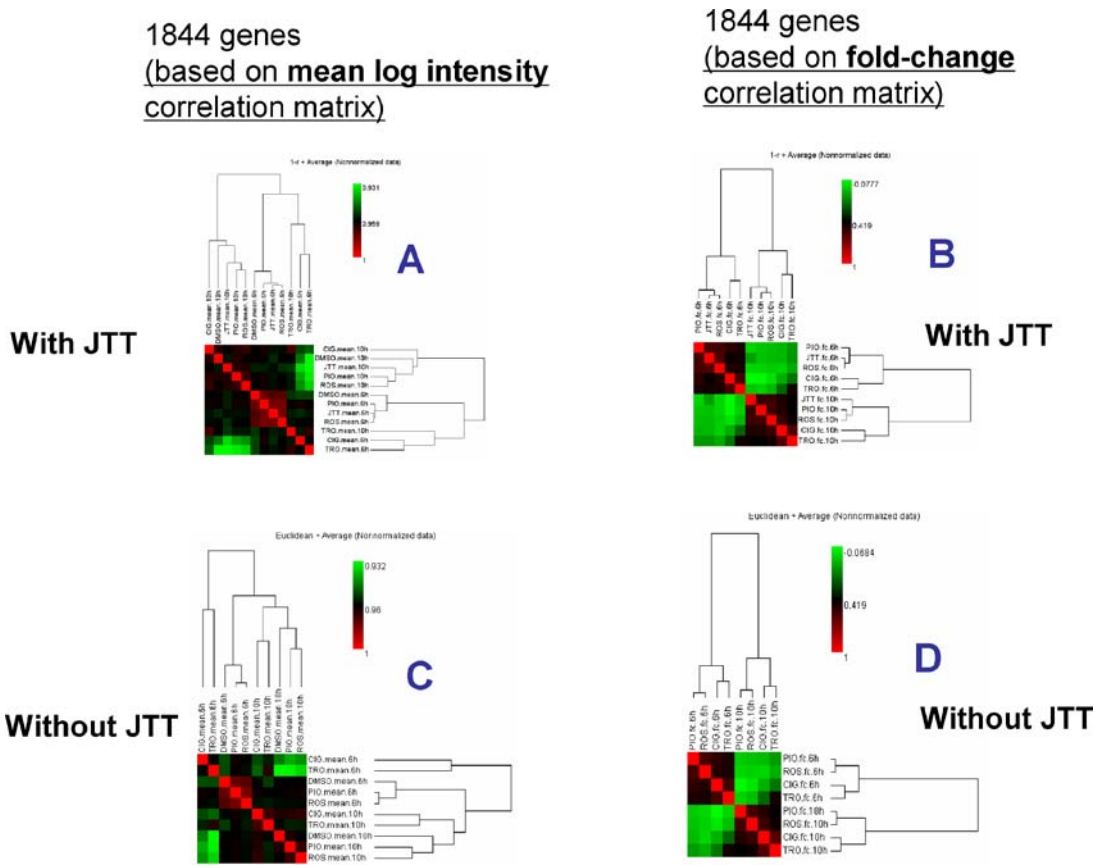

Figure 6. Two-dimensional hierarchical clustering of compounds based on averaged expression patterns of replicate treatments. (A) and (C) were based on averaged log2 intensity, whereas (B) and (D) were based on averaged log2 ratios (compared to DMSO). JTT-501 (JTT) was included in (A) and (B), but excluded in (C) and (D). Within the same treatment period (6 h or 10 h), rosiglitazone (ROS)/pioglitazone (PIO) showed appreciable different profiles compared to those of ciglitazone (CIG)/troglitazone (TRO).

PPARγ agonists by using the molecular descriptor calculation package Mold[2] developed at the FDA's National Center for Toxicological Research (Hong H et al., manuscript under review). In addition, 203 descriptors were calculated using the MOLCONN-Z package (www.eslc.vabiotech.com). The two sets of molecular descriptors were independently used for clustering the five PPARγ agonists and yield similar patterns of clustering (Figure 7). It is worth noting that rosiglitazone and pioglitazone were the most similar according to the chemical structural features (Figure 7), and they also appeared to be most similar to each other in cytotoxicity, apoptosis, and gene expression profiles.

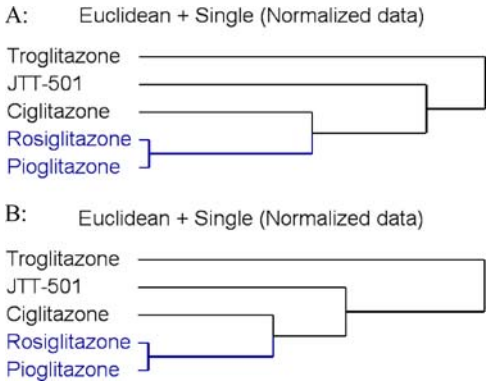

Figure 7. Clustering of five PPARγ agonists based on molecular structural properties. (A) based on 486 descriptors calculated by Mold[2]; (B) based on 203 descriptors calculated by MOLCONN-Z.

Discussion

Glucose-lowering effect of PPARγ agonists is due to the reduction of hepatic output of glucose and increase of peripheral uptake, thus reducing both pre- and after-load on the beta cells. By this action, PPARγ agonists can improve the sensitivity of endogenous insulin. It is therefore an excellent rationale for the use of PPARγ agonists in insulin resistant diabetes (type II diabetes) [29]. PPARγ agonists have high affinities to PPARγ which is a transcription factor in the regulation of gene expression and differentiation of adipocyte and metabolism of glucose and lipid. Reports of severe and fatal liver injury during clinical use finally led to the abandonment of troglitazone, the first in the PPARγ agonist class of oral anti-diabetic agents.

In this study, we compared the hepatic effects of five PPARγ agonists (including two therapeutic drugs) in order to understand whether hepatotoxicity is a class effect for PPARγ agonists or exclusively related to troglitazone. It was reported the maximal peripheral plasma drug concentrations was 0.5–2.5 μM and the drug concentration in the liver reached about 40 μM when the rats were gavaged by 5 mg/kg of troglitazone [21], and the maximal plasma concentration was 3.6–6.3 μM when humans were administered by therapeutic doses, 400–600 mg/day of troglitazone [30]. We treated primary rat hepatocytes and HepG2 cells with a range (5–100 μM) of PPARγ agonists in serum-free media that promises the best sensitivity for the assays. In addition, the concentrations of PPARγ agonists used in this study were within the appropriate tested range. Our data showed that troglitazone and ciglitazone strongly induced drug concentration- and time-dependent manner while rosiglitazone and pioglitazone only slightly induced cell death (Figure 2). This result is in accordance with clinical experience (reviewed in [31]).

It was reported that troglitazone (at concentration above 50 μM) reduced the viability of HepG2 cells while rosiglitazone and pioglitazone did not cause cytotoxicity with MTT assay [22, 32]. The disagreement between these results and our observations might be due to the treatment media contained 5–10% FBS in their experiments. It is likely that the addition of bovine serum albumin decreases toxic effect of troglitazone because of the binding of the drug to albumin [21, 33]. In the plasma, most PPARγ agonists bind to albumin, thus their distributions into red blood cells are low [34]. However, relatively high concentrations and/or prolonged exposure of hepatocytes to PPARγ agonists induce cell damage even in the presence of BSA [21]. Although the incidence of rosiglitazone- and pioglitazone-related hepatotoxicity is considered extremely rare, there are now 12 reported cases of hepatotoxicity associated with these agents (6 cases for each drug, reviewed in [35]). Considering that rosiglitazone is toxic to hepatocytes in 19% of human donors [36], and pioglitazone dose-dependently inhibits cell growth in human gastric cancer cells [37], our results suggest rosiglitazone and pioglitazone may damage hepatocytes after long exposures.

Apoptosis plays an important role not only in organ development and differentiation but also in the pathogenesis process such as in response to toxicant stimulations. Using the TUNEL assay, we demonstrated that troglitazone and ciglitazone induced apoptosis even with 2 h treatment, whereas apoptotic events induced by rosiglitazone and pioglitazone were not observed (Figure 3B). After exposure for 16 h, rosiglitazone and pioglitazone slightly induced apoptosis compared with the control, whereas troglitazone and ciglitazone strongly induced apoptosis (Figure. 3B). Our data are in agreement with previous studies in which troglitazone caused apoptosis in a wide variety of both cancer and normal cells [21, 34]. In addition, troglitazone caused apoptosis in cultured human liver cancer cells through caspase activation [38].

Using gene expression analysis, we found the expression of Bid3/Hrk and Bim/Bcl2l11 was up-regulated by troglitazone/ciglitazone compared to pioglitazone/rosiglitazone treatment (Table 3). Bid3/Hrk and Bim/Bcl2l11 belong to the Bcl-2 family and are apoptosis activators. They interact with other members of the Bcl-2 protein family including Bcl-2, Bcl-xl (anti-apoptotic) [26, 27]. It has been reported that treatment with troglitazone and ciglitazone inhibited anti-apoptotic functions of Bcl-xl and Bcl-2, leading to caspase-dependent apoptosis [28]. Our results suggested PPARγ agonists induced apoptosis (Figure 3B); it may be a triggering factor which leads to hepatic cell death. The Bcl-2 family may play an important role in apoptosis induction.

We also showed that more genes are regulated by treatment with the hepatotoxic compounds, troglitazone and ciglitazone, than the less hepatotoxic compounds, pioglitazone and rosiglitazone (Figure 5). This is in agreement with the finding of Kier et al. [39], who demonstrated that more gene expression changes occur with troglitazone than pioglitazone or rosiglitazone in isolated human hepatocytes. It is also in agreement with the recent finding of Liguor et al. [40], who demonstrated that the highly hepatotoxic compound, trovafloxacin produced more gene expression changes than other compounds in the same class.

Microarray technology has shown a major impact on the field of toxicology and drug safety evaluation because of its ability to cluster toxins based on expression profiles. Recent studies showed that by using microarray analysis, hepatotoxins with similar toxic mechanisms can be classified according to gene expression profiles [10, 41]. In this study, we applied microarray technique to challenge its capacity on classification of structurally similar compounds. We treated rat primary hepatocytes with five PPARγ agonists. We clustered the gene expression profiles by significant genes. We employed a two-dimensional hierarchical clustering method based on mean log intensity across the same compound treatment (Figure 6A and C) and fold change (Figure 6B and D). Within the same treatment period, rosiglitazone/pioglitazone showed appreciably different profiles compared to ciglitazone/troglitazone. Interestingly, DMSO control showed similar expression profiles to the group of rosiglitazone/pioglitazone rather than the group of ciglitazone/troglitazone (Figure 6C). It should be noted that the approaches we used for data analysis did not take into consideration the class assignment of the compounds, and the emergence of the two groups of compounds (ciglitazone/troglitazone vs. pioglitazone/rosiglitazone) in terms of gene expression profiles reflected the true biological differences of these compounds. It is noticeable that time was the important factor in determining gene expression patterns (Figure 4) and classification of rat primary hepatocytes (Figure 6). We conducted more explicit analysis to identify genes that were differentially expressed between the group of DMSO controls, resulting in 268 genes significantly changed between 6 and 10 h. Many genes are changed over the time course of the culture of rat primary hepatocytes

irrespective of the treatment conditions. These genes are mainly involved in cell death pathways (data not shown). This is in agreement with our toxicological finding that rat primary hepatocytes undergo spontaneous apoptosis (Figure 3B) and also in agreement with Wang C who reported that primary hepatocytes undergo spontaneous changes in cell culture media [Wang C, personal communication].

In conclusion, we have demonstrated that microarray analysis, together with toxicological observations, can be used to rank drugs for hepatotoxicity and to evaluate the safety of new compounds. There is a general agreement between the clustering of compounds based on chemical structural information and that based on cytotoxicity data or gene expression profiles.

Acknowledgments

Helpful discussions, comments, and criticisms offered by Drs. Tucker Patterson and James Fuscoe from NCTR are greatly appreciated.

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