

## Characteristic gene expression profile of nuclear receptor superfamily induced by hepatotoxic and antimetabolic drugs in human primary hepatocytes

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### Abstract

We evaluated the effect of 19 hepatotoxicants and 20 antimetabolites on the expression of genes of the human nuclear receptor (NR) superfamily in human primary hepatocytes, utilizing NR superfamily-related data extracted from the toxicogenomics database Open TG-GATES. A considerable number of the drugs alone induced a significant fold change in the expression of a large number of NRs. The members of the NR superfamily that changed expression with more than 40% of the drugs consisted of 12 NRs common to both classes (COUP $\beta$ , FXR, HNF4 $\alpha$ , LRH1, LXR $\alpha$ , PPAR $\alpha$ , PPAR $\delta$ , PXR, ROR $\alpha$ , RXR $\alpha$ , and TR4), 3 NRs specific to hepatotoxicants (GCNF1, RAR $\beta$ , and TR $\beta$ ), and 7 NRs specific to antimetabolites (ER $\alpha$ , GR, RAR $\alpha$ , REVERB $\alpha$ , RXR $\beta$ , SHP, and VDR). Nine of these were classified into cluster I involved in reproduction, development, and growth, whereas 13 were classified into cluster II, involved in nutrient uptake, metabolism, and excretion. These were also characterized by containing members of 6 out of 8 circadian-regulated subfamilies (ROR, Rev-erb, PPAR, FXR, TR, and TR2/TR4) including circadian oscillator genes Rev-erbs  $\alpha$ ,  $\beta$ , and ROR $\alpha$  and by containing 8 out of 9 NR subfamilies controlling the expression of genes for drug-metabolizing enzymes (CAR, FXR, GR, HNF4 $\alpha$ , LXR, PXR, PPAR, RAR, and VDR). The unsupervised hierarchical clustering of the NRs mobilized by drugs showed markedly different profiles between hepatotoxicants and antimetabolites. The results suggest that the profile of the expression response is determined by coordinated changes of drug-specific NRs and homeostasis-maintaining core NRs including circadian-regulated and circadian oscillator NRs and NRs controlling the expression of genes for drug-metabolizing enzymes. The hierarchical clustering of the hepatotoxicants and antimetabolites based on their effect on NRs showed that hepatotoxicants were classified into two subfamilies, one of which consisted exclusively of those inducing coagulopathy, while antimetabolites were divided into

**4 subfamilies where functionally-related drugs were generally classified together but with some exceptions. The classification of drugs based on their effect on the NR superfamily would urge us to re-examine the profile of toxicological actions of the drugs.**

**Key Words:** nuclear receptor superfamily, gene expression, hepatotoxicants, antimetabolites, human primary hepatocytes, circadian genes, xenobiotic-sensing nuclear receptors

**Area of Interest:** Information and computing approach for drug design and ADMET study

## 1. Introduction

The nuclear receptor superfamily consists of 48 members in humans, having conservative DNA binding and ligand binding domains [1]. Nuclear receptors (NRs) are transcription factors, and their activities are mostly regulated by a variety of lipophilic ligands such as steroids, bile acids, vitamin D, and fatty acids through their interaction with the ligand binding domain. NRs play key roles in the regulation of reproduction, development, and nutrient utilization. Since NRs have been favorable targets for drugs for diseases such as endocrine disorders, immunological diseases, metabolic diseases including diabetes, hyperlipidemia, and osteoporosis, cardiovascular diseases, and various cancers [2], intensive studies on NRs from pharmacological, physiological and pathological aspects have been performed. Through these studies, some NRs were elucidated to exert their regulatory activities in cooperation with other NRs, such as the following. 1) RXR $\alpha$ ,  $\beta$ , and  $\gamma$  heterodimerize with subfamily NR1 members such as TRs, RARs, PPARs, LXRs, FXR, PXR, and CAR. The heterodimers bind to a response element in regulatory regions of their target genes and corepressors are bound to the dimer, which inhibit the transcription of the target genes. When the heterodimers are activated by either NR1-ligands or RXR-ligands, corepressor are released and coactivators are recruited, which initiate the transcription of the target genes [3]. 2) SHP is an atypical NR lacking DNA binding domain; hence, it exerts its activity through interactions with various NRs and other transcription factors such as ERs, LRH-1, PPARs, RARs, and LXRs, and negatively regulates these activities with the exception of PPARs [4]. 3) ROR $\alpha$  and Rev-erb $\alpha$  are members of the secondary feedback loop in the circadian cycle [5]. Rev-erb $\alpha$  represses the transcription of *BMAL1*, a core loop oscillator gene, whereas ROR $\alpha$  competes with Rev-erb $\alpha$  for binding to their shared DNA binding element, RORE, in the *BMAL1* promoter. 4) SF-1 transactivates the SHP promoter, and the alterations in the levels or activities of SF-1 modulate SHP expression [6]. However, there have been few systematic studies on the interactions among NRs and no systematic studies on the effect of drugs on the network of actions of NRs. Recently, anatomical and temporal profiling of the expression of the NR superfamily were studied by Mangelsdorf *et al.*, revealing that the NR superfamily forms a hierarchical transcriptional network governing distinct physiological pathways, and that the coordinated diurnal rhythmicity of expression of the NR superfamily contributes to circadian entrainment of nutrient and energy metabolism [7][8]. Meanwhile, the Japanese national toxicogenomics project performed comprehensive evaluation of the effects of drugs on the expression of human genes and compiled the data as Open TG-GATES [9]. We utilized this database to evaluate the effect of drugs on the expression of the NR superfamily. The purpose of this study was to elucidate whether and how the NR superfamily shows systematic gene expression response on exposure of a cell line to a variety of drugs.

## 2. Methods

### 2.1 Selection of compounds

Thirty-five compounds were selected for this study, which consist of 19 hepatotoxicants and 20 antimetabolites. Four compounds, acetaminophen, aspirin, clofibrate, and glibenclamide were adopted dually as a hepatotoxicant and an antimetabolite. Categorization of antimetabolites was subject to the list of categories of the compounds provided by Open TG-GATES whereas categorization of hepatotoxicants was performed by collecting the compounds described as representative hepatotoxicants in the publications by the Open TG-GATES research group [10] [11][12].

### 2.2 Data retrieval and processing

Affymetrix human U133 plus2.0 array data were obtained from the TG-GATES database (<http://toxico.nibiohn.go.jp/>). We retrieved 696 CEL files on human primary hepatocytes experiments using human primary hepatocytes exposed to each of 35 drugs for 2, 8, or 24 hours.

The drug concentrations used were the maximum tolerable concentrations and 2 or 3 serial five-fold dilutions and the maximum concentration used for each of 35 drugs is shown in Table 1.

**Table 1.** The maximum concentration used for each of 35 drugs

Drug name	Conc. ( $\mu$ M)	Drug name	Conc. ( $\mu$ M)	Drug name	Conc. ( $\mu$ M)
acarbose	10000	colchicine	4000	metformin hydrochloride	1000
acetaminophen	5000	coumarin	300	methapyrilene	600
allopurinol	140	cyclosporine A	6	omeprazole	600
amiodarone	7	dexamethasone	300	perhexiline maleate	15
aspirin	3000	diclofenac	400	phenylbutazone	400
azathioprine	72.8	disulfiram	60	propylthiouracil	4000
benzbromarone	100	ethionine	10000	rosiglitazone maleate	300
bromobenzene	200	fenofibrate	30	simvastatin	30
carbon tetrachloride	7500	glibenclamide	20	thioacetamide	10000
chlorpromazine	20	hexachlorobenzene	30	tolbutamide	2000
chlorpropamide	750	ibuprofen	150	WY14643	150
clofibrate	300	mefenamic acid	150		

Bioconductor version 2.10 was used for microarray data processing [13]. CEL files were analyzed with the Affy package, and normalization was performed by taking the RMA log<sub>2</sub> intensity.

To determine differentially expressed genes between the highest concentration and time-matched control sample groups, the t-test was applied and data on the 151 probes of NR superfamily identified by Affymetrix Annotation were retrieved. Differentially expressed NR genes were extracted with a p cut-off value of 0.05 on the condition that the probe with the lowest p-value among redundant probes was representative of the NR gene.

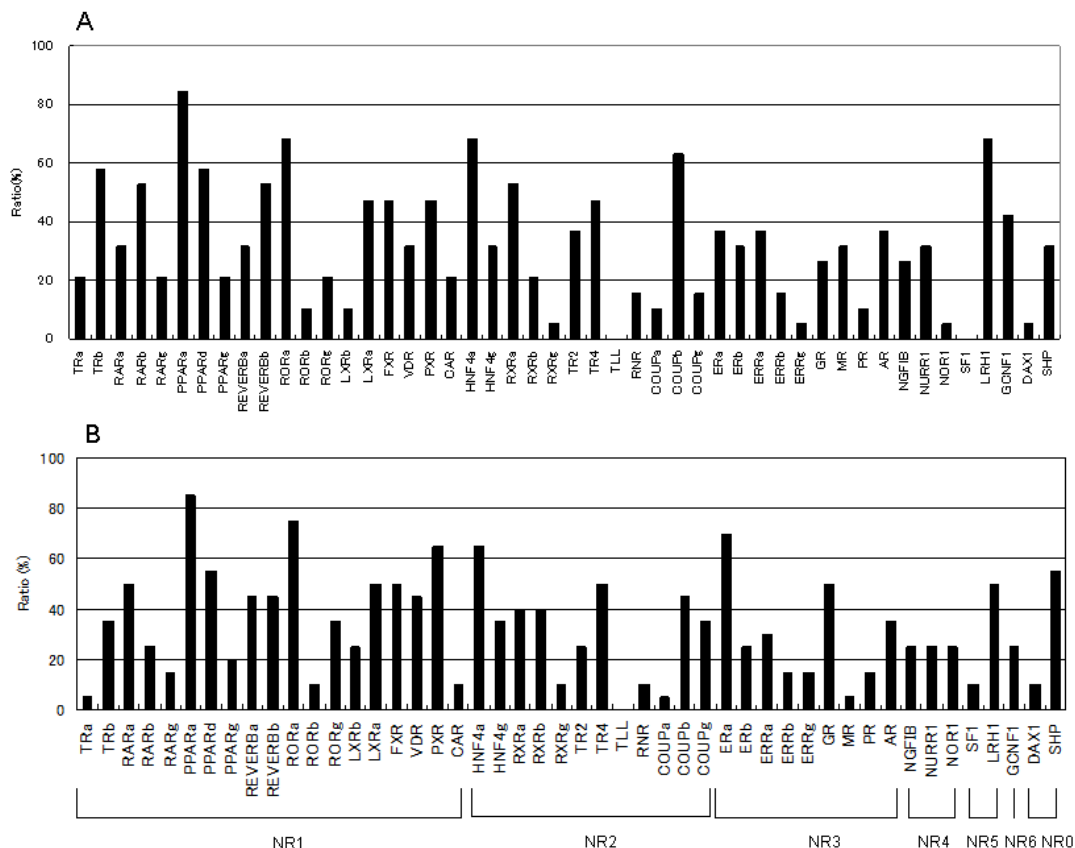
## 2.3 Cluster analysis

An NR and drug dendrogram was built using Ward's hierarchical clustering, using log<sub>2</sub> fold change values for the differentially expressed NR genes [14]. Heatmaps of the differentially expressed NR genes were generated using Excel 2007.

## 3. Results

### 3.1 Expression change profile of the NR superfamily genes induced by hepatotoxics and antimetabolites in human primary hepatocytes

Comprehensive evaluation of the effects of approximately 150 chemicals on the expression of 18,400 human genes was performed as a part of the Japanese national toxicogenomics project, and the resulting data were compiled in Open TG-GATES (<http://toxico.nibiohn.go.jp>). We utilized the NR superfamily-related data of this toxicogenomics project to elucidate the role of the network of the human NR superfamily in the toxicological and pharmacological actions of drugs. The GeneChip Human Genome U133 Plus 2.0 Array used by the toxicogenomics project contains 152 NR superfamily-related probes among its total of 49,977 probes, which represent all 48 of the human genes of the NR superfamily. We selected 19 hepatotoxics and 20 antimetabolites, and examined the fold change in gene expression of the human NR superfamily between the liver cell line untreated and treated with hepatotoxics or antimetabolites. Surprisingly, a considerable number of the drugs alone induced a significant fold change in the expression of a large number of NRs. The NR members whose gene expression was changed by more than 40% of the drugs included PPAR $\alpha$ , PPAR $\delta$ , ROR $\alpha$ , HNF4 $\alpha$ , LRH1, PXR, COUP $\beta$ , REVERB $\beta$ , RXR $\alpha$ , FXR, LXR $\alpha$ , TR4 (12 NRs, common to hepatotoxics and antimetabolites), TR $\beta$ , RAR $\beta$ , GCNF1 (3 NRs, hepatotoxicant-specific), ER $\alpha$ , SHP, RAR $\alpha$ , GR, REVERB $\alpha$ , VDR, and RXR $\beta$  (7 NRs, antimetabolite-specific) (Figure 1). The cut-off value 40% was selected as it could separate the bimodal distribution of NRs in both hepatotoxics and antimetabolites cases. These NRs were characterized by the following three points. Firstly, according to the classification of NRs proposed by Mangelsdorf *et al.* [7], 9 NRs among the 22 above-mentioned NRs were classified into cluster I consisting 28 NRs involved in reproduction, development, and growth, whereas 13 NRs were classified into cluster II consisting 20 NRs involved in nutrient uptake, metabolism, and excretion, indicating that gene expression of 65% of NRs of cluster II was changed whereas that of only 32% of NRs of cluster I was changed. Secondly, the NRs whose gene expression was changed included members of 8 out of 9 xenobiotic-sensing NR subfamilies involved in drug metabolism (CAR, FXR, GR, HNF4 $\alpha$ , LXR, PPAR, PXR, RAR, and VDR) [15]. Lastly, the NRs whose gene expression was changed also included members of 6 out of 8 circadian-regulated subfamilies (ROR, Rev-erb, PPAR, FXR, TR, and TR2/TR4) including the circadian oscillator genes Rev-erbs  $\alpha$ ,  $\beta$ , and ROR $\alpha$  [8].

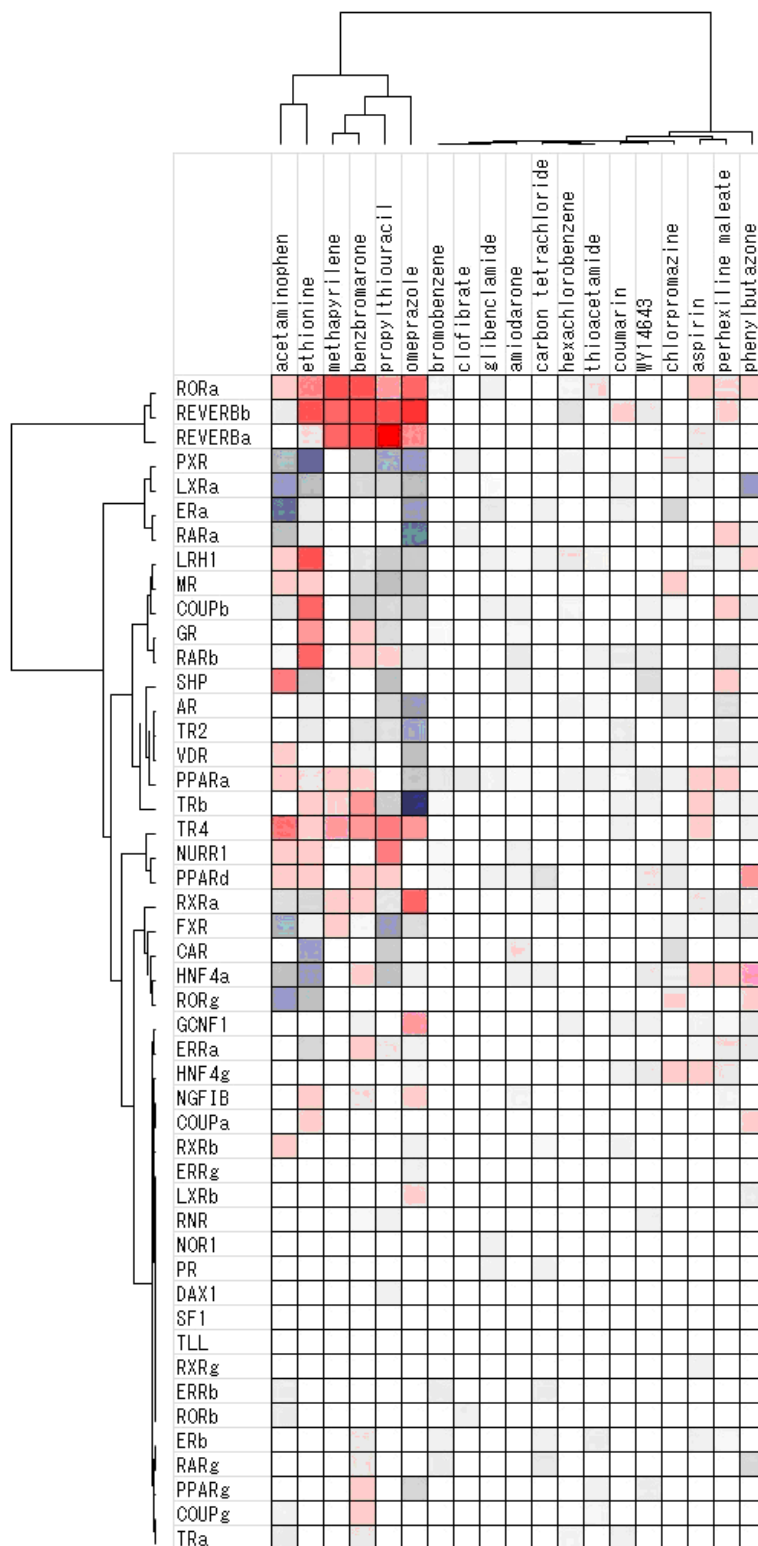


**Figure 1.** Rate of expression change of NR superfamily

The number of hepatotoxics (A) or antimetabolites (B) significantly modulating each NR as a percentage of the total number of drugs is plotted against NRs. Nomenclatures of NR subfamilies defined by IUPHAR [19] are shown below names of NRs.

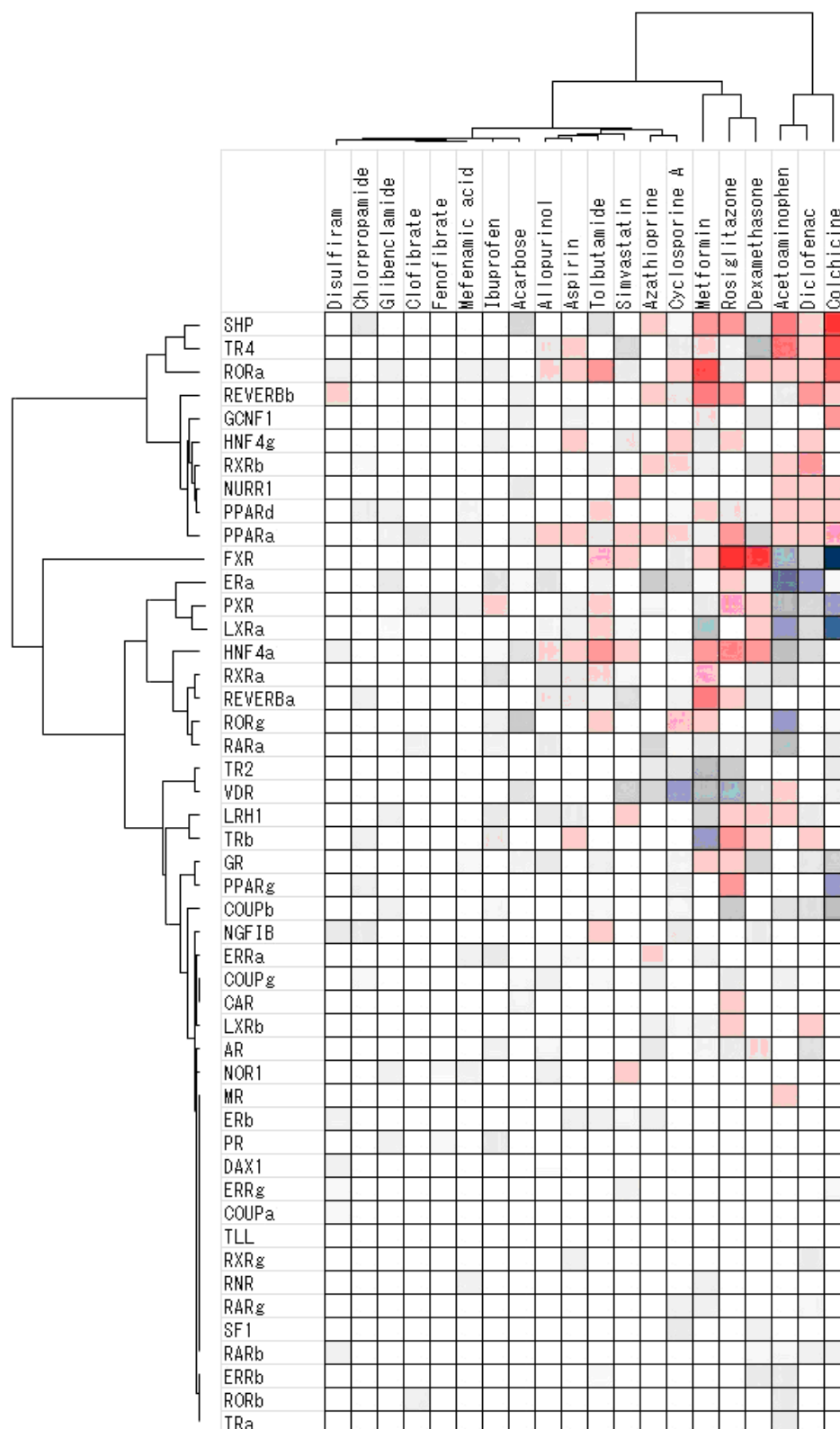
### 3.2 Cluster analysis of the NR superfamily by the gene expression response to hepatotoxics and antimetabolites

The profile of the fold change in the gene expression of the NR superfamily between the drug-treated and untreated hepatocytes was evaluated by unsupervised hierarchical clustering (Figures 2-5). The cluster profiles were markedly different between hepatotoxicant- and antimetabolite-treated hepatocytes. The NR superfamily was classified into H1-H7 subfamilies based on fold changes in gene expression by hepatotoxics, whereas the NR superfamily was classified into A1-A9 subfamilies based on those by antimetabolites (Figures 4 and 5). High-level homologies were observed between H2 and A4 subfamilies and between H7 and A9 subfamilies. H2 consisting of 4 NRs and A4 consisting of 3 NRs shared 3 NRs, while H7 consisting of 22 NRs and A9 consisting of 23 NRs shared 18 NRs whose gene expressions were markedly low. The other H- and A-subfamilies showed less homology with each others; nonetheless, local homologies dispersed among subfamilies were observed. It should also be noted that members of cluster I unevenly distributed in H1-H3, H5, and A1-A2.



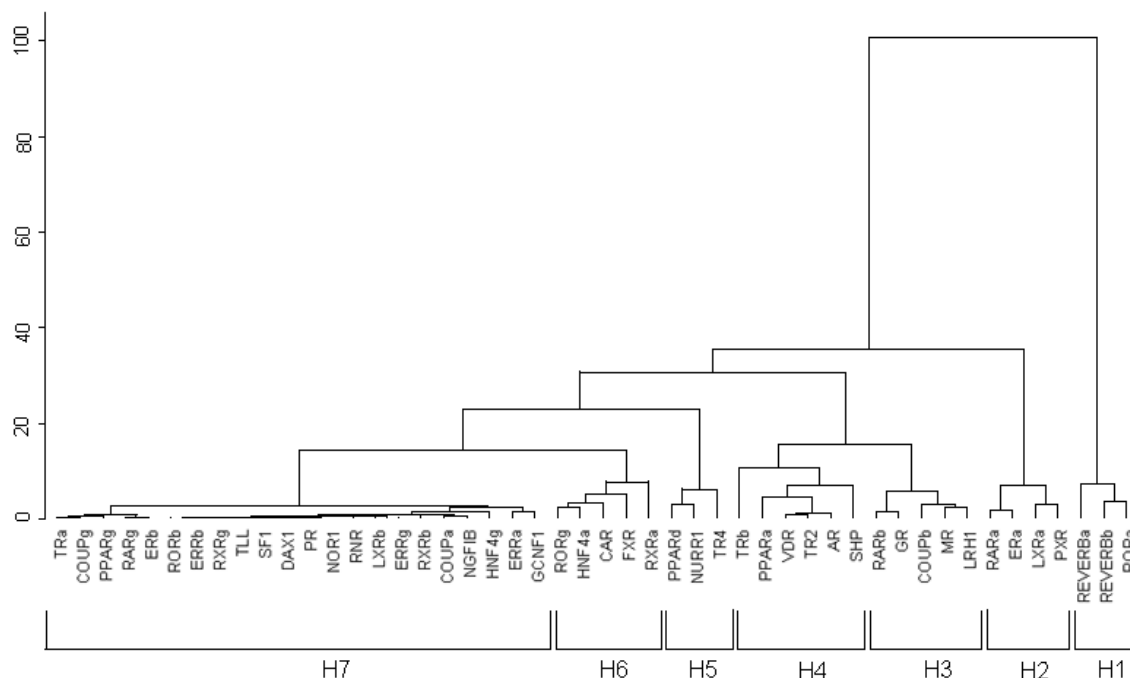
**Figure 2.** Gene expression profile of NR superfamily induced by hepatotoxins

Fold changes in expression of NRs by hepatotoxins are shown by heat maps. The darkest red is assigned to 8-fold increase whereas the darkest blue is assigned to 8-fold decrease. The detailed hierarchical clustering dendrograms were depicted separately in Figures 4 and 6.

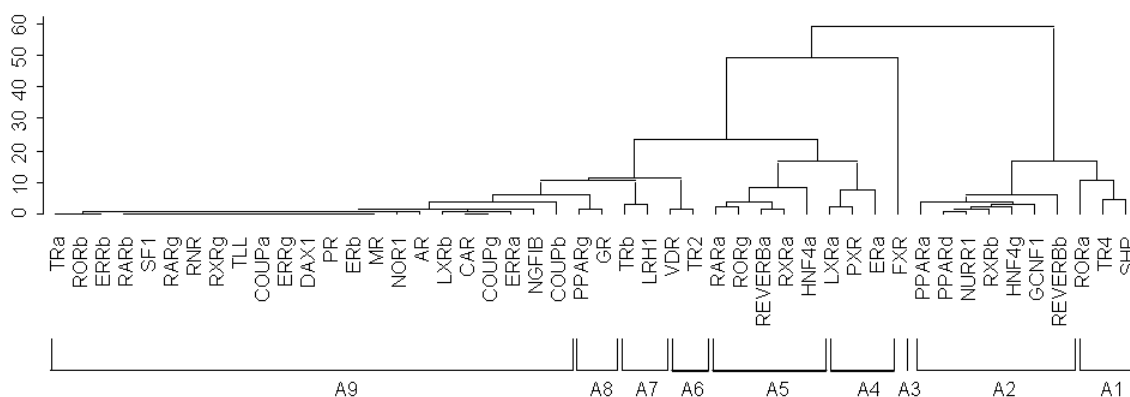


**Figure 3.** Gene expression profile of NR superfamily induced by antimetabolites

Fold changes in expression of NRs by antimetabolites are shown by heat maps. The darkest red is assigned to 8-fold increase whereas the darkest blue is assigned to 8-fold decrease. The detailed hierarchical clustering dendrograms were depicted separately in Figures 5 and 7.



**Figure 4.** Cluster analysis of gene expression profile of NR superfamily induced by hepatotoxins  
Cluster analysis was performed as described in the text, using the values of fold change of NR expression by hepatotoxins.

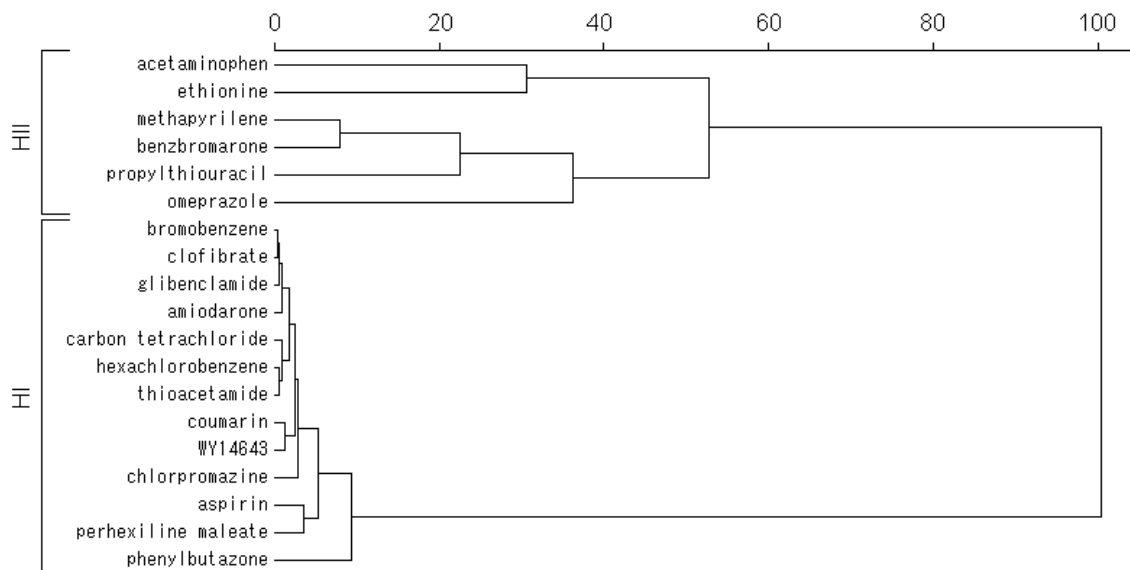


**Figure 5.** Cluster analysis of gene expression profile of NR superfamily induced by antimetabolites  
Cluster analysis was performed as described in the text, using the values of fold change of NR expression by antimetabolites.

### 3.3 Differential and convergent effects on the expression of the NR superfamily among similar functional groups of drugs

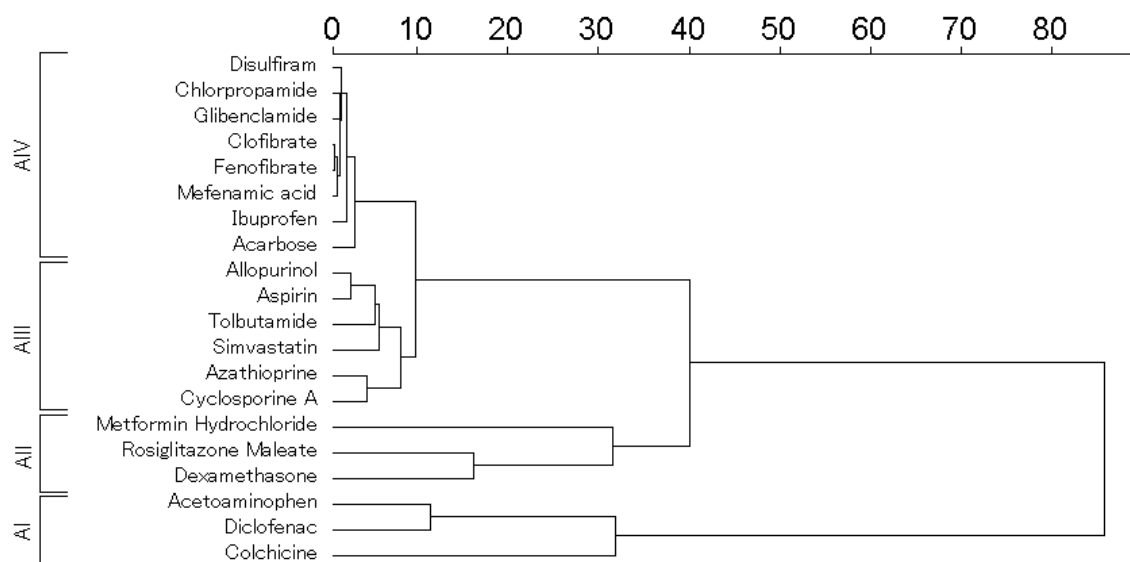
The hepatotoxins and antimetabolites were clustered based on fold changes in gene expression of the NR superfamily induced by them (Figures 6 and 7). The hepatotoxins were classified into two subfamilies, whereas the antimetabolites were classified into 4 subfamilies.





**Figure 6.** Cluster analysis of hepatotoxics according to their effect on gene expression of NR superfamily

Cluster analysis was performed as described in the text, using the values of fold change of NR expression by hepatotoxics.



**Figure 7.** Cluster analysis of antimetabolites according to their effect on gene expression of NR superfamily

Cluster analysis was performed as described in the text, using the values of fold change of NR expression by antimetabolites.

One of the hepatotoxics clusters, HIII, consisted of acetaminophen, benzbromarone, ethionine, methapyrilene, omeprazole, and propylthiouracil (Figure 6). It is noteworthy that all of these 6 hepatotoxics were distinguished by inducing coagulopathy [12]. However, the other coagulopathy-inducing hepatotoxics: amiodarone, thioacetamide, and WY14843, were classified into HII as exceptions. Regarding the antimetabolites, similarly, functionally-related drugs were included in the same subfamilies with some exceptions. Namely, the lipid-lowering drugs clofibrate

and fenofibrate were both classified into the AIV subfamily. Among four non-steroidal anti-inflammatory drugs (NSAIDs), ibuprofen and mefenamic acid were classified into AIV whereas diclofenac and aspirin were classified into AI and AIII, respectively. The sulfonylurea class antidiabetic drugs chlorpropamide and glibenclamide were classified into AIV, whereas tolbutamide, the other sulfonylurea drug, was classified into the AIII subfamily. In contrast, cyclosporine A and azathioprine, which are both immunosuppressant drugs but whose action mechanisms are entirely different from each other, were both classified into the AIII subfamily. Similarly, metformin hydrochloride and rosiglitazone maleate, which are antidiabetic drugs but whose action mechanisms are markedly different from each other, were classified into the same subfamily AII (Figure 7).

### 3. Discussion

The NR superfamily is a family of distinctive transcription factors showing domain features highly homologous with each others and the activities of most of which are modulated by a wide variety of hydrophobic ligands [1]. The NR superfamily plays key roles in the regulation of metabolism as well as regulation of reproduction, development, and growth. Recent systematic studies on anatomical and temporal profiling of the expression of the NR superfamily revealed that the NR superfamily forms a hierarchical transcriptional network governing distinct physiological pathways, and that the coordinated diurnal rhythmicity of expression of the NR superfamily contributes to circadian entrainment of nutrient and energy metabolism [7][8].

Studies on drug actions against NRs have been intensively performed to develop novel drugs since NRs, most of which have ligand-binding domains, are straightforward drug targets. Accordingly, most of those studies have been focused on the effect of drugs on the activities of NRs but not on the gene expression of NRs. Thus, only a small number of drugs have been investigated regarding their effect on the gene expression of NRs [6][16] and, to the best of our knowledge, there are no publications on the systematic effect of drugs on the gene expression of the NR superfamily. This study was aimed to elucidate whether and how the NR superfamily shows a systematic gene expression response on exposure of a cell line to a variety of drugs.

Firstly, we evaluated the fold changes in gene expression of 48 human NRs between drug-treated and untreated human primary hepatocytes, using 19 hepatotoxicants and 20 antimetabolites. The features of the profile of NR gene expression change by drugs are summarized as follows: 1) Generally, a single drug induced a significant change in the expression of a large number of NRs. 2) The NRs whose gene expression was changed significantly by more than 40% of the drugs consisted of those common to hepatotoxicants and antimetabolites and those specific to hepatotoxicants or antimetabolites. 3) The NRs whose gene expression was changed by drugs contained 8 out of 9 xenobiotic-sensing NR subfamilies involved in drug metabolism. 4) The NRs whose gene expression was changed by drugs contained members of 6 out of 8 circadian-regulated NR subfamilies including circadian oscillator genes.

These results together suggested a hypothesis that the profile of the gene expression response of the NR superfamily to drugs is determined by cooperative changes in the gene expression of drug-specific NRs and those of homeostasis-maintaining core NRs. Core NRs are characterized by including circadian-regulated and circadian oscillator NRs [8] and NRs sensing xenobiotics and controlling the expression of genes for drug-metabolizing enzymes [15]. Changes in the gene expression of circadian-regulated and circadian oscillator NRs are suggested to play a key role in maintaining the homeostasis of cells perturbed by exposure to drugs. NRs controlling the expression of drug-metabolizing enzymes are also thought to contribute to maintaining the

homeostasis by facilitating the excretion of drugs. Furthermore, these NRs are believed to trigger drug-exposure signals to other NRs through their ability to sense xenobiotics. Meanwhile, differences of the drug response of the NR superfamily between hepatotoxics and antimetabolites are suggested to be determined by hepatotoxicant- and antimetabolite-specific NRs.

We subsequently performed clustering of the hepatotoxics and antimetabolites on the basis of the profile of fold changes in gene expression of the NR superfamily induced by the drugs. Firstly, hepatotoxics were classified into 2 subfamilies, HI and HII, the latter of which exclusively consisted of hepatotoxics inducing coagulopathy. Hirode *et al.* previously extracted genes commonly mobilized by hepatotoxics inducing coagulopathy and elucidated with principal component analysis that the top 20 genes contributing PC1 and PC2 mainly consisted of aquaporins and lipid metabolism-related genes [12]. The expression of most of these genes, such as acyl-coenzyme A dehydrogenases, acetyl-coenzyme A acetyltransferase 1, acyl-coA oxidase, and aquaporin 3 genes, was reported to be regulated by PPAR $\alpha/\delta$  [17][18]. This is consistent with the finding that the expression of PPAR $\alpha/\delta$  genes was enhanced by most of the HII hepatotoxics.

Among the coagulopathy-inducing hepatotoxics, amiodarone, thioacetamide, and WY14843 were classified into the HI subfamily as exceptions. This may be related to Hirode *et al.*'s finding that thioacetamide and WY14843 diverged from the other hepatotoxics in principal component analysis [12]. Secondly, antimetabolites were classified into 4 subfamilies, AI-AIV. Functionally-related drugs were classified into the same subfamilies, although there were some exceptions. Namely, among four NSAIDs, ibuprofen and mefenamic acid were classified into the same subfamily AIV whereas diclofenac and aspirin were classified into AI and AIII, respectively. Among three sulfonylurea class antidiabetic drugs, chlorpropamide and glibenclamide were classified into AIV whereas tolbutamide was classified into the AIII subfamily. In contrast, cyclosporine A and azathioprine, which are both immunosuppressant drugs but whose action mechanisms are entirely different from each other, were both classified into the AIII subfamily. Similarly, antidiabetic drugs, metformin hydrochloride and rosiglitazone maleate, whose action mechanisms are markedly different from each other, were classified into the same subfamily AII. These differential and convergent effects on the expression of the NR superfamily among similar functional groups of drugs would urge us to re-examine whether the NR superfamily plays important roles in the differences or similarities in the toxicological actions among the groups of drugs.

Finally, this study revealed the characteristic gene expression profile of the NR superfamily induced by a variety of drugs; however, further studies are needed to elucidate the molecular mechanisms of the network of NRs involved in the toxicological actions of drugs suggested by this study.

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