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Improved toxicogenomic screening for drug-induced phospholipidosis using a multiplexed quantitative gene expression ArrayPlate assay

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Abstract

We previously showed that a toxicogenomics analysis of drug-induced phospholipidosis enabled the identification of 12 specific gene markers and the establishment of an *in vitro* real-time PCR screening assay for the assessment of the phospholipidosis-inducing potential of compounds. The purpose of this study was to transfer our PCR-based assay into a 96-well microplate-based multiple mRNAs measuring assay (ArrayPlate™ assay) in order to increase throughput. Specifically, we determined the expression of the 12 marker genes using real-time PCR and ArrayPlate in human hepatoma HepG2 cells that were treated for 24 h with each of amiodarone and 80 proprietary compounds.

The following three performance criteria were satisfied in the ArrayPlate analysis: 1. Sensitivity—the expression of mRNA for all target genes was detected at quantifiable levels. 2. Repeatability—signal intensities and fold change values of each marker gene were highly repeatable. 3. Correlation—fold change values and their average values, which were used as indices of phospholipidosis induction potential, showed apparent correlation between the ArrayPlate and real-time PCR assays.

Thus, the *in vitro* screening assay for compound-induced phospholipidosis should be transferable from a PCR-based assay to the higher-throughput ArrayPlate-based method.

Keywords: Drug-induced phospholipidosis; *In vitro* screening assay; ArrayPlate technology; Real-time PCR; Toxicogenomics

1. Introduction

Phospholipidosis is a lipid storage disorder that is characterized by the accumulation of phospholipids within cells. More than 50 cationic amphiphilic drugs (CADs), including antidepressants, antianginal, antimalarial, and cholesterol-lowering drugs are known to have the potential to induce phospholipidosis (Lullmann et al., 1978; Halliwell, 1997; Reasor, 1989). While electron microscopy has been the most reliable method for identifying phospholipidotic inclusions (Drenckhahn et al., 1976), its expense of operation and low throughput have rendered it impractical as a rapid screening tool. It has also been reported that the potential for inducing phospholipidosis can be rapidly assessed in a human monocyte-derived U937 cell line using a Nile red fluorescent stain that has a high affinity for lipids (Casartelli et al., 2003). This method can detect phospholipidosis inducing potential and the ability to recover from phospholipidosis after drug withdrawal as well. However, the Nile red staining method cannot provide the information on the induction mechanism.

DNA microarray technology allows for the simultaneous and rapid comparison of the patterns of mRNA expression of thousands of genes and has contributed to our understanding of the mechanisms underlying cellular toxicity (Aardema and MacGregor, 2002). This technology can be used to identify biomarkers of toxicity. We previously

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showed that a comprehensive gene expression analysis of drug-induced phospholipidosis enabled the identification of 12 specific gene markers that were used to develop a novel real-time PCR-based *in vitro* screening assay for assessing the phospholipidosis induction potential of compounds (Sawada et al., 2005). This assay is readily able to detect the phospholipidosis-inducing potential of multiple compounds and to provide detailed ranking scores that can be useful in structure-activity relationship studies.

The ArrayPlate qNPA™ (quantitative nuclease protection assay, High Throughput Genomics, Tucson, AZ) is a 96-well plate-based assay using an array of 16 distinct elements printed on the bottom of each well to provide a multiplexed quantitative measurement of gene expression. This assay measures multiple samples at one time and only requires that the sample be lysed. This assay does not require RNA extraction, reverse transcription, or target amplification (Martel et al., 2002).

The present study was undertaken to determine whether we could transfer our PCR-based assay into an ArrayPlate-based assay in order to improve the throughput of our *in vitro* screening system. Specifically, we measured the expression of the following 12 phospholipidosis marker genes using real-time PCR and ArrayPlate on HepG2 cells after they were treated for 24 h with each of amiodarone and an additional set of 80 proprietary compounds: ASAH1—*N*-acylsphingosine amidohydrolase (acid ceramidase) 1; MGC4171—hypothetical protein MGC4171; LSS—lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase); NR0B2—nuclear receptor subfamily 0, group B, member 2; FABP1—fatty acid binding protein 1, liver; HPN—hepsin (transmembrane protease, serine 1); SERPINA3—serine (or cysteine) proteinase inhibitor; clade A (alpha-1 antiproteinase, antitrypsin), member 3; C10orf10—chromosome 10 open reading frame 10; FLJ10055—hypothetical protein FLJ10055; FRCP1—likely ortholog of mouse fibronectin type III repeat containing protein 1; SLC2A3—solute carrier family 2 (facilitated glucose transporter) member 3; and TAGLN—transgelin. Similarly treated samples (rather than the same samples) were either lysed and frozen for ArrayPlate assay, or lysed, extracted and processed for real-time PCR assay.

2. Materials and methods

2.1. Materials

Amiodarone was purchased from ICN Biomedicals (Irvine, CA). Proprietary compounds were obtained from the chemical inventories of Takeda Pharmaceutical Company limited.

2.2. Cell culture and drug treatment

The human hepatocellular carcinoma cell line HepG2 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Cells in their log growth phase were seeded (2×10^5 cells) in 24-well plates and incubated with 10 μmol/L of a test compound in 0.25% dimethylsulfoxide (vehicle) in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Thermo Trace, Melbourne, Australia), 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen). Cells were incubated in a 37 °C incubator in an atmosphere of 5% CO_2 and 95% air, and were cultured for 24 h or 72 h, depending on whether they were being used for real-time PCR and ArrayPlate assays, or electron microscopy, respectively.

To determine the ArrayPlate assay performance characteristics of sensitivity, linearity and repeatability, treated cells (10 μmol/L of amiodarone or vehicle) were lysed and the lysates from 48 wells were pooled to create a "bulk cell lysate". In sensitivity and linearity test, serial 1:2 dilutions were made from bulk cell lysate, and 25 μL aliquots of each dilution were dispensed in the four wells in a column of a microplate. The resulting plate contained four replicates of eight samples consisting of 100,000, 50,000, 25,000, 12,500, 6250, 3125, 1562.5, and 781.25 cells. In repeatability test, four separate ArrayPlate analyses were performed with the equivalent of 1.25×10^4 cells of a bulk cell lysate and 16 replicates for each sample.

For correlation analyses between real-time PCR and ArrayPlate assays, cells were treated with each of 80 proprietary compounds. ArrayPlate assay was performed with the equivalent of 1.25×10^4 cells per well (similarly treated cells but not the identical samples as used for the real-time PCR studies). The expression data represent the average from three replicates for the real-time PCR and ArrayPlate data.

2.3. Real-time PCR assay

Following 24 h incubation with a test compound or vehicle in triplicate, cell-culture medium were aspirated and cells were stored at −80 °C until their RNA was extracted using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). The concentration and purity of total RNA was determined for each sample by measuring absorbance at 260 and 280 nm with an Ultrospec 2000 spectrophotometer (Amersham Biosciences, Piscataway, NJ). The integrity of the purified total RNA was confirmed using an RNA 6000 Nano Assay kit and Agilent 2100 Bioanalyzer (Agilent technologies, Berlin, Germany). RNA samples were stored at −80 °C until assayed.

Reverse transcription was performed using total RNA (1 μg) and oligo-dT oligonucleotide primer in 100 μL volumes using standard methods and MultiScribe Transcriptase in order to synthesize cDNA (TaqMan Reverse Transcription Reagent; PE Applied Biosystems, Foster City, CA).

Quantitative real-time PCR was performed using 5 μL of the cDNA solution, 1× TaqMan Universal PCR Master Mix, and 200 nM of primer/probe set or 1× TaqMan GAPDH control reagents (PE Applied Biosystems) in an

ABI PRISM 7000 Sequence Detection System with the following schedule: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of denaturation for 15 s at 95 °C, and annealing and elongation for 1 min at 60 °C in a final volume of 50 μL. The primer and TaqMan probe (Sigma-Genosys, Hokkaido, Japan) sequences used in this assay were detailed in our previous report (Sawada et al., 2005). Relative gene expression levels were normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and calculated using the comparative Ct method, described in User Bulletin #2 that was provided with the ABI PRISM 7700 Sequence Detection System. A phospholipidosis mRNA score was calculated for each compound as the average fold change, equal to the sum of the fold changes for the (10) genes that were induced by treatment plus the inverse of the fold changes for the (2) genes that were suppressed by treatment divided by the total number of genes (12) in the phospholipidosis signature.

2.4. ArrayPlate assay

The ArrayPlate assay was performed as previously reported (Martel et al., 2002). Briefly, following 24 h incubation with a test compound or vehicle in triplicate, the cells were lysed in the presence of probes that bound target mRNA by removing the media and then adding the lysis solution to each well. The lysates were heated to 95 °C for 10 min then frozen for shipment to and assay by High Throughput Genomics. The probe sequences used in this assay are listed in Table 1. After a 6 h incubation with probes at 60 °C, excess probes and unhybridized RNA were degraded using S1 nuclease solution (Promega, Madison, WI, supplied as a reagent integral to the kits sold by High Throughput Genomics); alkaline hydrolysis destroys the RNA component of the duplexes, leaving the probes intact. After neutralization, the solutions were hybridized with programming linker-modified ArrayPlates containing a 16-element array. Each ArrayPlate was detected by

Table 1
List of phospholipidosis markers and sequence of probes for ArrayPlate assay

Gene symbol	Gene title	Probe
ASAH1	*N*-acylsphingosine amidohydrolase (acid ceramidase) 1	GGTTGCCTCCCAGGATAAAGTAGGCTGGGGCCAATATCTTGGTCTTGGTC +GGCTGGTGCGGTTCAGACACATCTTTGCAGGCGTTCTGCGATCATCAAGG +GTGTCTTCATGTCTCAGAGGCCGCATTCTGTAGGCCAGACGTGTGCTCAC +GTCCCGTTACTCACACAGACGTGCTGGATTCAACACCCACGCTGAATCTC
MGC4171	Hypothetical protein MGC4171	CATGGGTGTCCTTGGAAACCTCTGGAACAGGTCCTCCAGACGAACCATGC
LSS	Lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	GGTGGCGAACCCTCCATCTGGATTTCTCATGTTCAGCAGCACAGCCACAG
NR0B2	Nuclear receptor subfamily 0, group B, member 2	GAGCTGTTCCTAAGGAGCCAAGTGCTGTCTATACAGGCTTGCCCCTCCAG
FABP1	Fatty acid binding protein 1, liver	CCTTCCCCTTCTGGATGAGCTCTTCCGGCAGACCGATTGCCTTCATGAAG
HPN	Hepsin (transmembrane protease, serine 1)	GGAAGATCCACTCCCGGAAGTCACTGACTTTGGTGTAGACGCCTGGCTTC +CTGCGCCATGTCACTGCCTCTTGTTAATGATTCCCTGGCTGACCTCCTGG +ACTGGAGAGGTGGACCAGGGCAATATCGTTGCTGTTCTCCTCGCTGTTGG +AGCATCCCATCATCAGGGGCACCTAGACAGGAGTCCCAGACAGCAGAACA
SERPINA3	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	GAGCTTCTTAGCTGCAGCTGAGTCCTGAAAGTCAGTGGCAAAGGCCTCGG
C10orf10	Chromosome 10 open reading frame 10	CAGATGCCTCTAGGAACTAGGGTCCTTCAGACTCCAGATGCCCTGACCTG +CAAGGTGATCAGCCAGCAAGGGTCAGAGCGAACGGGATTGGTAACATCTC
FLJ10055	Hypothetical protein FLJ10055	GTTTGGAGCCTGAGGCATTGAAGGTGATGGCAGCTAGTGTTCCCTCATGG +CCTTTTCATCCCTCTCCGGAACTCATAGAGCTTTTGCCCATCAGGGACAG +TCCAATTGCTAGGGATGTGCAGTCCTGGTTGAAAGAGAAGCAGCTGAGCG +CACTCCAGGTCGAAGGCTCTTCTGGTCGACTGTTGGTGACCTGTTCCAGC
FRCP1	Likely ortholog of mouse fibronectin type III repeat containing protein 1	CTGCTCACAGTGGAAAGAGGATGGGTACCAGGCCTGAGATTGTGGGAGTG
SLC2A3	Solute carrier family 2 (facilitated glucose transporter), member 3	CCAGATCTATCTGCACCGTGTGCCTGCCCTTCAAAGGCCCGTGTGATATC +GTCCGCAGAAGAGGCCAATAACCAAGCGACCCAGGATCAGCATTTCAACC +CTGCTGAGAGAGCTGGAGCACAATGGAAATGATGATGGGCTGTCGGTAGC +GGGTTCATCCTGATGAGGTCTCTCCCTTGTTGAGGGAGAGGTGGCTTTCC
TAGLN	Transgelin	GGCCAATGACATGCTTTCCCTCCTGCAGCTGGCTCTCTGTGAATTCCCTC
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	TGGTGCAGGAGGCATTGCTGATGATCTTGAGGCTGTTGTCATACTTCTCA
PPIA	Cyclophilin A	GCAGCGAGAGCACAAAGATTCTAGGATACTGCGAGCAAATGGGGTGGAGG
ACTIN	β-Actin	TCACACTTCATGATGGAGTTGAAGGTAGTTTCGTGGATGCCACAGGACTC
ANT	Arabidopsis thaliana ANT (AINTEGUMENTA)	CCTCTTCTTCTTCGCAGCCGCCATTGTCGTCGTCTCAAACCCAACGCTTG

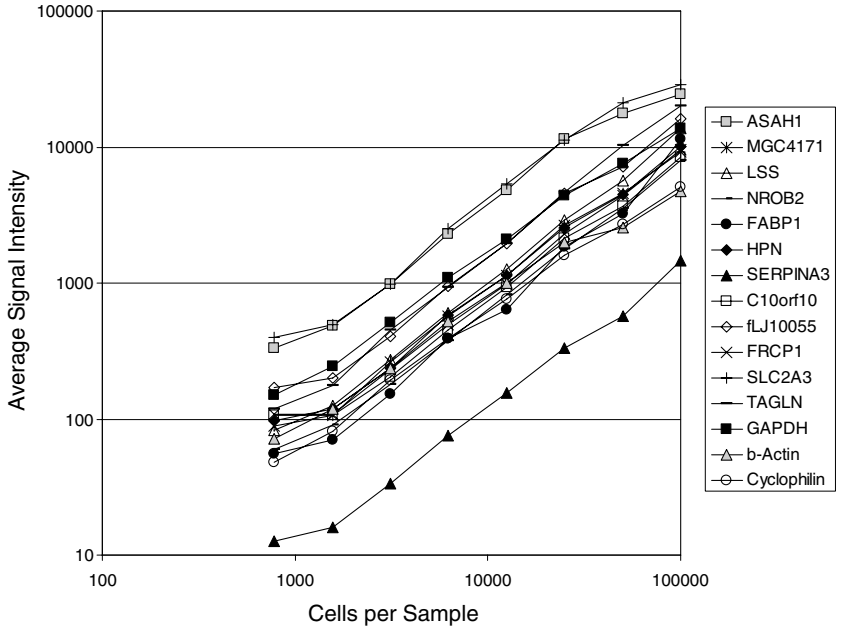

Fig. 1. Assay sensitivity. ArrayPlate analysis was carried out on eight serial 1:2 dilutions of HepG2 cells that were treated with vehicle for 24 h. The average sums of the quadruplicate were equalized for each cell concentration. This graph shows average signal intensity for all target and housekeeping genes.

consecutive exposure to detection linker solution, detection enzyme conjugate solution, and chemiluminescent peroxidase substrate (Atto-PS™, Lumigen, Southfield, MI). The ArrayPlates were imaged from the bottom with an Omix Imager (High Throughput Genomics) for 1–2 min. The digital images were analyzed using VueScript (High Throughput Genomics) to determine the expression level of each gene by calculating the average pixel intensity of each element. In order to obtain normalized within-well signal intensities, two methods were used, depending on the experiment. In the case where the cell number was intentionally varied to determine sensitivity and linearity, the method of normalization was a weighted proportional normalization to all the genes, since the only variable was cell number not treatment. This is best described by giving the equation used:

$$[\text{Normalized gene intensity}] = [(\text{gene value})/(\text{sum of intensities of all genes in the well})] * [(\text{average sum of replicate wells})]$$

Routinely, however, such as when determining the repeatability of the assay or testing samples where any variation between wells in the cell number was due to unintended variability, the gene intensities were normalized to three housekeeping genes (GAPDH, β-actin, cyclophilin A). The equation used was:

$$[\text{Normalized gene intensity}] = [(\text{gene value})/(\text{sum of 3 housekeeping gene intensities})] * [3000]$$

This adjusted for unintended variability in cell number.

In addition to target and housekeeping genes, arabidopsis thaliana aintegumenta was used as a negative control. A phospholipidosis mRNA score was calculated for each compound using the same equation as used for the PCR data.

2.5. Transmission electron microscopy

Following 72 h incubation with a test compound or vehicle, cells were fixed in 1% glutaraldehyde for 2 h after

Table 2
Average signal of phospholipidosis markers in HepG2 cells determined by ArrayPlate analysis

Gene	Vehicle treated		Amiodarone treated	
	Average signal	%CV[a]	Average signal	%CV
ASAH1	1809	12	3512	9
MGC4171	433	10	920	7
LSS	640	10	777	9
NR0B2	451	7	761	8
FABP1	465	12	671	13
HPN	548	8	1422	8
SERPINA3	110	11	175	9
C10orf10	417	10	1112	8
FLJ10055	734	11	1055	9
FRCP1	389	12	660	8
SLC2A3	2648	7	1250	9
TAGLN	1260	10	399	7

Note: ArrayPlate analysis was carried out on HepG2 cells that were treated with vehicle or 10 μmol/L of amiodarone for 24 h. The average signals were standardized against three housekeeping genes and represent the average values from 16 wells.

[a] %CV = (standard deviation/average signal) * 100%.

which they were washed with sodium phosphate buffer and post-fixed with 2% osmium tetroxide for 2 h. They were then dehydrated in increasing concentrations of ethanol and embedded in epoxy resin (Quetol 812). Ultrathin sections (80 nm) were cut using an ultramicrotome (LKB-8800 Ultrotome), double stained with uranyl acetate and lead acetate, and observed in an electron microscope (H-300; Hitachi, Tokyo, Japan). The changes indicative of phospholipidosis (formation of lamellar myelin-like bodies in lysosomes) were scored from photographs of only five cells on a scale of 0–2 (− = none, + = slight, and ++ = moderate) in a blinded fashion.

3. Results

3.1. Sensitivity and linearity of ArrayPlate analysis

The linearity of the assay was determined using eight serial 1:2 dilutions of the pooled bulk cell lysate samples, with four replicates per dilution. The averages of normal-

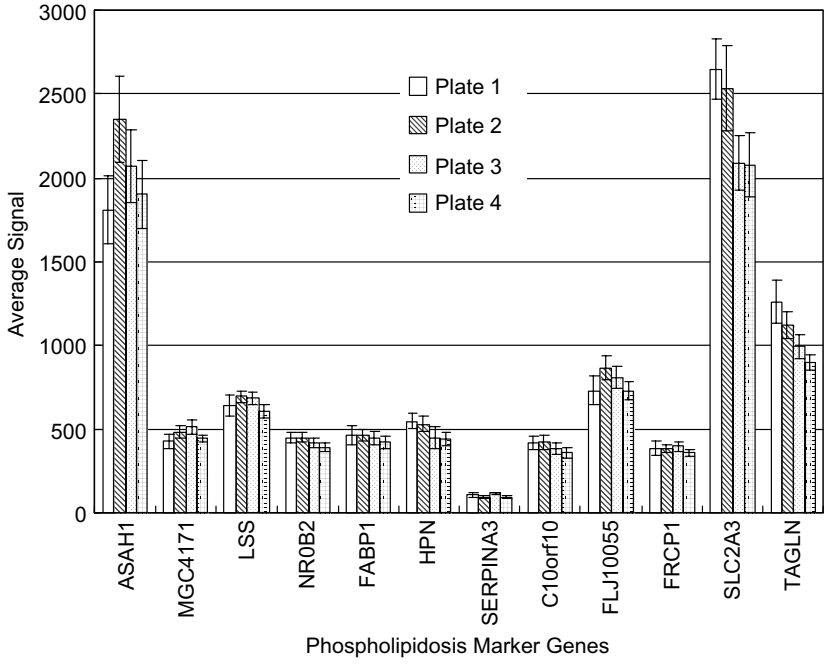

Fig. 2. Plate-to-plate repeatability of the ArrayPlate assay. ArrayPlate analysis was carried out on HepG2 cells that were treated with vehicle for 24 h. The average signals from four plates performed on different days were standardized using three housekeeping genes and represent the average values obtained from 16 wells. The error bars indicate the standard deviations.

Table 3
Plate-to-plate repeatability of fold change values of phospholipidosis markers in HepG2 cells determined by ArrayPlate and real-time PCR analysis

Gene	ArrayPlate						Real-time PCR
	Plate 1	Plate 2	Plate 3	Plate 4	Mean	%CV[a]	
ASAH1	1.94	1.89	1.90	1.86	1.90	2	2.28
MGC4171	2.13	2.06	2.02	2.10	2.08	2	2.04
LSS	1.22	1.20	1.17	1.22	1.20	2	1.20
NR0B2	1.69	1.61	1.61	1.64	1.64	2	1.66
FABP1	1.44	1.41	1.45	1.45	1.44	1	1.49
HPN	2.60	2.53	2.74	2.61	2.62	3	2.58
SERPINA3	1.59	1.55	1.53	1.59	1.57	2	1.40
C10orf10	2.66	2.56	2.55	2.61	2.60	2	2.30
FLJ10055	1.44	1.36	1.41	1.42	1.41	2	1.46
FRCP1	1.70	1.66	1.68	1.66	1.67	1	1.46
SLC2A3	0.47	0.47	0.44	0.44	0.46	4	0.46
TAGLN	0.32	0.31	0.30	0.31	0.31	2	0.35

Note: ArrayPlate and real-time PCR analyses were carried out on HepG2 cells that were treated with vehicle or 10 μmol/L of amiodarone for 24 h. ArrayPlate data from four separate plates were standardized against three housekeeping genes and represent the average values from 16 wells. Real-time PCR data were standardized against GAPDH and represent the average values from three experiments.

[a] %CV = (standard deviation/average signal) * 100%.

Table 4
Phospholipidosis mRNA scores detected by ArrayPlate and real-time PCR assays

Compound	mRNA score		Pathology score
	ArrayPlate	Real-time PCR	
1	0.97	1.14	–
2	0.99	1.01	–
3	0.99	0.97	–
4	1.01	1.12	–
5	1.01	1.06	–
6	1.01	0.98	–
7	1.03	0.98	–
8	1.04	1.04	–
9	1.04	0.96	–
10	1.04	1.06	–
11	1.04	0.97	–
12	1.05	0.97	–
13	1.06	1.09	–
14	1.09	1.16	–
15	1.09	1.16	–
16	1.09	1.17	+
17	1.11	1.13	+
18	1.11	1.41	+
19	1.12	1.11	–
20	1.13	1.15	–
21	1.14	1.28	–
22	1.15	1.15	–
23	1.16	1.01	N.D.
24	1.16	0.97	N.D.
25	1.16	1.14	N.D.
26	1.17	1.18	N.D.
27	1.22	1.25	–
28	1.24	1.10	N.D.
29	1.29	1.21	–
30	1.30	1.32	–
31	1.33	1.28	–
32	1.39	1.44	N.D.
33	1.51	1.38	N.D.
34	1.51	1.34	N.D.
35	1.52	1.42	N.D.
36	1.53	1.58	+
37	1.54	1.54	–
38	1.54	1.73	+
39	1.56	1.84	++
40	1.62	1.69	++
41	1.63	1.82	+
42	1.64	1.93	+
43	1.65	1.64	–
44	1.68	1.65	+
45	1.69	1.82	++
46	1.69	1.67	–
47	1.76	1.65	N.D.
48	1.76	2.00	+
49	1.77	1.73	–
50	1.78	2.31	++
51	1.79	1.74	–
52	1.81	1.93	+
53	1.84	1.70	N.D.
54	1.84	1.90	++
55	1.85	1.99	++
56	1.86	1.85	++
57	1.86	1.74	+
58	1.91	2.18	+
59	1.94	1.94	++
60	1.96	1.94	++
61	1.99	1.86	+
62	2.04	1.90	++
63	2.05	1.84	N.D.
64	2.09	2.03	++
65	2.12	1.96	N.D.
66	2.13	2.37	+
67	2.17	2.71	+
68	2.22	2.09	++
69	2.28	2.46	+
70	2.34	2.47	+
71	2.43	2.53	++
72	2.54	2.32	++
73	2.70	2.56	++
74	2.71	2.86	++
75	2.92	2.87	++
76	3.17	2.87	++
77	3.22	3.18	++
78	3.66	2.90	N.D.
79	4.32	4.60	++
80	5.51	5.61	++

Note: Phospholipidosis mRNA scores for HepG2 cells that were treated for 24 h of treatment represent the average fold change values of phospholipidosis markers from three replicates in the real-time PCR and ArrayPlate assays. Pathological changes in HepG2 cells (formation of lamellar myelin-like bodies in lysosomes) were scored from photographs of only five cells on a scale of 0–2 (– = no presence, + = slight, and ++ = moderate) by electron microscopy, 72 h after treatment.
N.D. = not done. (Electron microscopic analyses were performed only when the data were required to confirm the results in *in vitro* study for drug development. Some compounds lacked their pathology scores.)

ized signal intensities for 15 genes (12 phospholipidosis marker genes and 3 housekeeping genes) are shown in Fig. 1. For all genes, the assay showed a linear response of signal to sample size for samples ranging from 1563 to 25,000 cells. Therefore, all subsequent ArrayPlate analyses were performed with the equivalent of 1.25×10^4 cells per well, in which the expression of mRNA for all target and housekeeping genes were detected.

3.2. Repeatability of ArrayPlate analysis

The well-to-well repeatability of the ArrayPlate analysis was determined using the pooled bulk cell lysate samples. The average signals and coefficient of variability (%CV—the standard deviation as a proportion of the average) from a single plate are shown in Table 2. Each %CV was within 13%.

To assess plate-to-plate and day-to-day repeatability measurements were carried out using four separate plates on different days measuring vehicle treated and amiodarone treated cell lysates. The average signals from four separate plates are shown in Fig. 2 with error bars indicating one standard deviation across the 16 wells of each plate. The fold change values between vehicle control and amiodarone treated cell lysates for each of these four separate ArrayPlates are shown in Table 3. The signals and fold change values of each of the 12 phospholipidosis marker genes had high plate-to-plate repeatability. In addition,

there was an apparent correlation between the fold change values obtained for each gene by ArrayPlate and real-time PCR analyses.

3.3. Correlation of phospholipidosis mRNA scores between the ArrayPlate and real-time PCR assays

In our previous study, the average fold change values of the markers correlated well with the magnitude of phospholipidotic change obtained from electron microscopic analysis, and were established as an index of phospholipidosis induction potential—these values were referred to as phospholipidosis mRNA scores (Sawada et al., 2005). In the present study, we carried out ArrayPlate and real-time PCR assays and calculated the average fold change values of phospholipidosis markers in cells that were treated with 80 proprietary compounds (Table 4). The data in Table 4 are ranked from lowest ArrayPlate score to the highest. Our data showed that the phospholipidosis mRNA scores correlated significantly ($R^2 = 0.95$) between the ArrayPlate and real-time PCR assays (Fig. 3). And, there was also an apparent correlation between these quantitative mRNA scores determined at 24 h of treatment and the pathological scores obtained from electron microscopic analysis of the HepG2 cells determined after 72 h of treatment (Table 5).

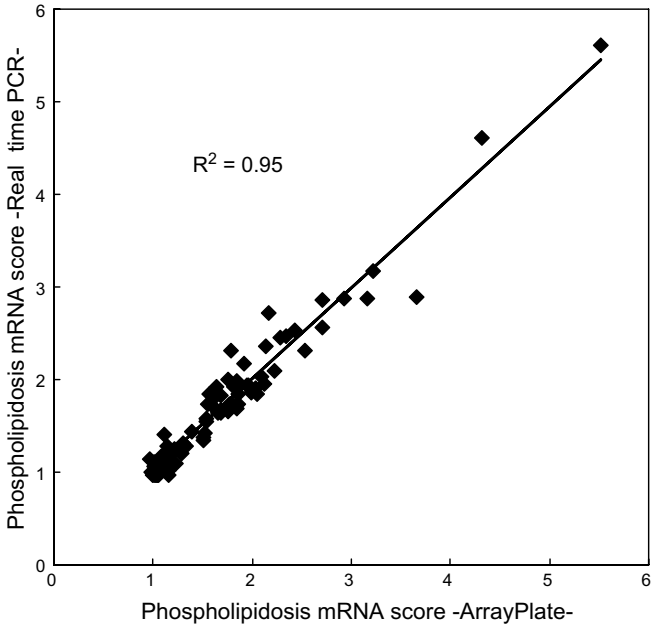

Fig. 3. Correlation of phospholipidosis mRNA scores between the ArrayPlate and real-time PCR assays. The graph plots the average fold change values of the phospholipidosis markers obtained by ArrayPlate and real-time PCR assays in HepG2 cells after they were treated with each of the 80 test compounds for 24 h.

Table 5
Correlation between phospholipidosis mRNA and pathology scores

mRNA score	≤1.5	1.5–1.8	≥1.8
Number of compounds	26	15	25
Pathology score			
−	23	5	0
+	3	6	8
++	0	4	17

Note: Sixty six compounds were determined phospholipidotic pathological scores by electron microscopy. Phospholipidosis mRNA scores were detected by ArrayPlate assay.

4. Discussion

We previously used comprehensive gene expression analysis to identify a set of 12 marker genes for phospholipidosis, and to establish a novel *in vitro* real-time PCR-based screening assay for the assessment of the phospholipidosis-inducing potential of various compounds (Sawada et al., 2005). In this study, we examined the feasibility of using the ArrayPlate assay to improve the throughput of our *in vitro* screening assay. The ArrayPlate assay that we used in this paper was designed to quantitatively detect the expression of 16 RNA targets (12 phospholipidosis marker genes, three housekeeping genes and one negative control gene) in each well of a 96-well assay microplate (Martel et al., 2002). We performed the assay on human hepatoma HepG2 cells following their treatment for 24 h with each of amiodarone and 80 proprietary compounds.

The following three performance criteria were satisfied in the ArrayPlate analysis: 1. Sensitivity—the expression of mRNA for all target and housekeeping genes were detected at quantifiable levels. 2. Repeatability—there was apparent repeatability of signals within the same plate and between plates performed on different days; fold change values of each marker gene were highly repeatable day-to-day. 3. Correlation—fold change values and their average values, which were used as indices of phospholipidosis induction potential and were termed phospholipidosis mRNA scores, determined by ArrayPlate assay correlated well with values obtained by real-time PCR. In addition, there was an apparent correlation between the mRNA scores determined by ArrayPlate and the pathological scores obtained from electron microscopic analysis. Based on these, it is concluded that the PCR-based assay was successfully transferred to the ArrayPlate assay.

The ArrayPlate-based phospholipidosis assay measures 16 genes, 12 of which comprise a phospholipidosis signature. Because it is a 96-well microplate based and does not require RNA extraction, reverse transcription or gene amplification, the ArrayPlate not only provided greater day-to-day and sample-to-sample repeatability but also significantly increased testing throughput and cost-efficiency. Compounds 1, 4, 32, 53, 65, 68, 70, 73, 77 and 79 are structurally similar but had a large diversity in mRNA scores detected by ArrayPlate (range 0.97–4.32). Compounds 50 and 64 (mRNA scores detected by ArrayPlate were 1.78 and 2.09, respectively) were tested in *in vivo* studies, and they actually induced phospholipidosis in rats (data not shown). Therefore, this assay would have the capacity to test every lead at multiple doses for phospholipidosis potential, as well as

every analog during the lead optimization process, potentially enabling only those analogs which do not appear to have phospholipidosis potential to be advanced into animal studies and on into the development pipeline.

Recently, Ploemen et al. reported that the use of physicochemical calculations of ClogP and pK_a could predict phospholipidosis-inducing potential (Ploemen et al., 2004). We calculated ClogP and pK_a of the 80 compounds as reported in the above report (data not shown). Compounds 9, 12 and 24 were ClogP <3 and calculated pK_a <8, and had low phospholipidosis inducing potentials (mRNA scores were below 1.5 and/or pathology changes were not detected). Compounds 34, 53, 65, 69, 72, 74, 75, 79 and 80 were ClogP >5.5 and calculated pK_a >8. These compounds except compound 34 had phospholipidosis inducing potentials (mRNA scores in ArrayPlate assay were above 1.8 and/or phospholipidotic pathology changes were detected). However, in other compounds (ClogP >3 and calculated pK_a <8, and ClogP <5.5 and calculated pK_a >8), their mRNA scores were ranged from low to high value. These results suggested that not only physicochemical properties (pK_a and ClogP) but also other factors were involved in the induction of phospholipidosis. In the near future, further studies of *in silico* technology in combination with other experimental tools may provide more powerful tool for drug-induced phospholipidosis.

The toxicogenomics approach that we used in our previous study will enable us to identify sets of gene markers for various toxic conditions. The data from the present study suggest that the transference of the toxicity markers identified by comprehensive gene expression analysis using DNA microarrays to the high-throughput and cost-efficient assay platform such as ArrayPlate should be quite valuable for the establishment of toxicogenomics-based practical assay systems.

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