

## Primaquine-induced differential gene expression analysis in mice liver using DNA microarrays

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### Abstract

Primaquine (PQ), a clinically important derivative of 8-aminoquinoline used against the hepatic stages (hypnozoites) of *Plasmodium vivax* and *Plasmodium ova le*, was studied to evaluate and compare between mRNA expression, and biochemical and histological parameters of hepatic stress in adult Swiss mice (*Mus musculus*). Following single oral dose of PQ (40 mg/kg, bw), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) along with hematoxylin and eosin stained liver sections did not show any signs of hepatic stress at 6, 12 and 24 h except for ALT activity at 6 h. However, analysis at RNA transcript level revealed consistent and significant deregulation ( $p < 0.01$  and two-fold) of 16 probes corresponding to important cellular processes such as protein transportation, transcription regulation, intracellular signaling, protein synthesis, hematopoiesis, cell adhesion and cell proliferation. Pathway analysis identified large number of affected genes corresponding to 40 Gene Ontology terms having a z score greater than 2. These results indicate that PQ at high doses may affect gene expression in liver and may produce undesirable outcomes if consumed for longer durations.

**Keywords:** 8-Aminoquinoline; Differential gene expression; Microarray; Primaquine; Toxicogenomics

**Abbreviations:** LOWESS, locally weighted linear regression; rRNA, ribosomal RNA; mRNA, messenger RNA; cDNA, complementary DNA; MIAME, minimum information about microarray experiment; PQ, primaquine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ANOVA, analysis of variance

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### 1. Introduction

Primaquine [8-(4-amino-1-methylbutylamino)-6-methoxyquinoline, PQ], an important anti-relapse antimalarial derivative of 8-aminoquinoline, is extensively used to combat liver stages of *Plasmodium vivax* and *Plasmodium ovale* responsible for malarial relapses. It is the most effective and perhaps the only clinically prescribed drug available against liver stages of the plasmodial spp. (Doherty et al., 1997; Karbwang and Harinasuta, 1992; Warrell, 1993). A dose of 15 mg/day PQ for 2 weeks has been recommended by the world health organization (WHO) to avoid relapses, in the infected humans, effectively (WHO, 1990). The absorption and metabolism of PQ is nearly complete

after oral administration and only a small fraction of the administered dose is excreted as the parent drug. It is metabolized to 8-(3-carboxyl-1-methyl-propyl-amino)-6-methoxyquinoline, 5-hydroxy primaquine, and 5-hydroxy-6-desmethylprimaquine however, the carboxyl derivative has been reported to be the major metabolite (Baker et al., 1982; Mihaly et al., 1984). All the three metabolites have appreciably less antimalarial activity whereas, except for carboxyl derivative, their hemolytic activity is greater than PQ (Symposium, 1987).

Other than being useful in preventing malarial relapses, PQ has various well-known undesirable effects. Mild to moderate cramps and occasional epigastric distress often results at high doses which accentuate with still higher doses. These symptoms are often accompanied by methemoglobinemia and cyanosis. Severe methemoglobinemia can occur even at therapeutic doses, in individuals with congenital deficiency of nicotinamide adenine dinucleotide (NADH) methemoglobin reductase (Cohen et al., 1968). The toxicity of PQ in individuals with glucose-6-phosphate dehydrogenase (G6PD) deficiency develops due to intravascular haemolysis even at therapeutic doses. This sensitivity is inherited on the X chromosome and haemolysis is often of intermediate severity in heterozygous females. However, there are indications that the metabolites of PQ are more active than the parent compound in causing the haemolysis (Symposium, 1987). Unfortunately, lack of an effective and less toxic substitute has resulted in the continued use of this antimalarial.

Although, gene expression studies at high throughput scale were initiated in 1995, the necessary impetus to profile global gene responses was provided by complete genome sequence of human and important laboratory animals. Recent advancements in high throughput gene profiling techniques such as DNA microarray allows simultaneous measurement of mRNA expression levels of tens of thousands of genes at once whereas previously only a handful of genes could be investigated. DNA targets, in the form of expressed sequence tags (ESTs), are arrayed onto glass slides (or membranes) and mRNA expression levels are probed by measuring the intensity of fluorescently labelled cDNAs. Thus, microarray based transcript profiling has become an important tool in determining the global gene responses in the target organs. This has resulted in the accumulation of extensive information on drug-induced, differential gene expression in response to various pharmacologically important molecules but a lack of information exists about the effects of PQ. Thus, gene responses following PQ dosing were investigated in order to identify affected

genes and to determine their direction and degree of deregulation at global scale. Additionally, microarray investigations were accompanied by traditional and established methods to identify drug-induced alterations in the biochemical marker enzymes and histological changes. The gene expression profiling was carried out in the livers of normal mice principally because it is the primary site of action of PQ and to ascertain the usefulness of microarray technology in identifying earliest gene deregulations following drug treatment. Moreover, high metabolic activity and high chemical concentration exposure renders hepatic tissue exceptionally vulnerable target for chemical-induced adverse effects (Minami et al., 2005). The results obtained in the present work indicate that PQ leads to transcriptional alterations in the hepatic genes after acute dosing and can be used as a signature of hepatic tissue response in the absence of traditional markers of hepatic stress.

## 2. Materials and methods

### 2.1. Drug administration and tissue collection

Approximately 10–12-week-old, outbred male Swiss albino mice (*Mus musculus*), weighing 25–30 g (Central Drug Research Institute, Lucknow) were randomly assigned to control and treatment groups ( $n=4$ ). All animal procedures were performed in compliance to institutional animal ethics guidelines. The animals were acclimated to optimal conditions of temperature ( $25 \pm 2^\circ\text{C}$ ) and light/dark cycle (12 h each) before drug administration. Primaquine (PQ) was purchased from Sigma (St. Louis, MO, USA), dissolved in normal saline and administered as a single oral dose (40 mg/kg bw). The dose of PQ was selected after conducting a preliminary dose finding study to ascertain robust gene expression changes. The mice of control group were administered 100  $\mu\text{l}$  normal saline. Access to food and water was 'ad libitum' before and after drug treatment. The animals belonging to control and treatment groups were sacrificed by cervical dislocation at 6, 12 and 24 h. The timing of sampling was considered appropriate as PQ gets metabolized and excreted within 24 h after oral administration (Fletcher et al., 1981). Blood was withdrawn from the animals and allowed to stand undisturbed for 30 min. Part of liver tissue was snap frozen in liquid nitrogen within 2 min of sacrifice and subsequently stored at  $-80^\circ\text{C}$  instantaneously, until further use. Perfusion of liver was avoided in order to prevent template degradation. Remaining part of liver tissue was immediately fixed in 10% formal saline for histological investigations.

### 2.2. Serum biochemistry and liver histology

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum were estimated with an automated biochemical analyzer (Beckman, Coulter, Califor-

nia, USA). Fixed liver tissue was washed overnight, dehydrated through graded alcohols and embedded in paraffin wax. Serial sections of 5  $\mu\text{m}$  thickness were stained with hematoxylin and eosin (H&E) for histological examination.

### 2.3. RNA isolation, cDNA labeling and hybridization

Approximately 50 mg frozen liver tissue was crushed in liquid nitrogen and immediately homogenized (Heidolph, Germany) in 1 ml of TRI reagent (Sigma, St. Louis, MO, USA) to isolate total RNA. RNA samples with approximately 2:1 ratio of 28S:18S rRNA and 260/280 values  $\geq 1.8$  were used for gene expression analysis. Equal amount of RNA from individuals of same group was pooled to eliminate inter-individual variations. Twenty-five microgram of pooled RNA was converted into labeled cDNA using CyScribe First Strand cDNA-labeling kit (Amersham, Buckinghamshire, UK) following manufacturer's protocol. Labeled cDNA was purified with GFX columns as per manufacturer's guidelines and subsequently concentrated by evaporation under vacuum after estimating the percent incorporation of the dyes with a spectrophotometer (Thermo, Waltham, MA, USA). Dye swap technical replicate experiments were performed with aliquots of same RNA preparation to address inconsistencies regarding dye incorporation and other technical means of variance. The Cy5 and Cy3 labeled cDNA samples were mixed in CyScribe Hyb buffer (Amersham, Buckinghamshire, UK) containing 10  $\mu\text{g}/\text{ml}$  sheared salmon sperm DNA and 10  $\mu\text{g}/\text{ml}$  yeast tRNA (Ambion, Austin, Texas, USA) as blocking agents. The labeled sample was hybridized to mouse 15k and 22.4k arrays (<http://www.microarrays.ca>) for 18 h at 42 °C.

### 2.4. Scanning and microarray data analysis

The arrays were washed and subsequently scanned to collect raw data with Array Scanner III supported with ImageQuant version 5 (Molecular Dynamics). Intensity values were extracted from the scanned images with ArrayVision version 8 (Imaging research, GE healthcare Biosciences Corp., Piscataway, NJ, USA). Raw intensity data was analyzed with Avadis Express version 4.3 (Strand life Sciences, Bangalore, India) and the background corrected intensities LOWESS normalized (Cy5 against Cy3) to obtain log (base 2) ratios. Furthermore,  $\log_2$  values of duplicate spots were averaged in order to get a single mean value to perform *k*-means clustering with MeV version 3.1 [TM4, The Institute of Genomic Research (Saeed et al., 2003)]. Each expression cluster was further clustered hierarchically with Euclidean distance matrix and average linkage to identify gene with similar expression patterns. Raw and log transformed data (series accession no. GSE 5979) has been submitted to Gene Expression Omnibus database ([www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/)) and conforms to MIAME guidelines developed by microarray gene expression data (MGED) society.

Furthermore, individual gene expression data of three time points was condensed and listed to make a representative

gene-expression dataset for identifying affected pathways using GenMAPP version 2.1 (Dahlquist et al., 2002). Furthermore, the GenMAPP gene expression dataset file (.gex) was exported to MAPPFinder to calculate percentage of genes with significant expression change, statistical score for each Gene Ontology (GO) term and respective *z* scores (Doniger et al., 2003). Functional annotation for the identified probes was inferred from the information available on NIA (<http://lgsun.grc.nia.nlm.gov>), NCBI (<http://ncbi.nlm.nih.gov/>), MGI (<http://www.informatics.jax.org/>), SwissProt (<http://www.expasy.org/sprot/>), SOURCE (<http://smd.stanford.edu/cgi-bin/source/sourceBatchSearch>) and other related databases. The final list of genes with  $p < 0.01$  and two-fold differential expression was validated with extensive data available on Gene profiles (NCBI), Comparative Toxicogenomics Database (CTD), Pubmed and related databases regarding information on direction and extent of deregulation in similar studies conducted on various mammalian species.

### 2.5. Real time-PCR

RNA samples were analyzed with real time PCR (Bio-rad) to validate differentially expressed genes identified with microarray using Quantitect SYBR Green RT-PCR kit following manufacturer's protocol (Qiagen). The names of genes and respective sequences of forward and reverse primers have been listed in Table 1. GAPDH, a housekeeping gene was used to normalize the data. The primer optimization and PCR product specificity were confirmed before proceeding for the real time-PCR. Briefly, 50  $\mu\text{l}$  reaction mixture consisted of 25  $\mu\text{l}$ ; 2X Quantitect SYBR green RT-PCR mix, 0.5  $\mu\text{M}$  forward and reverse primers, 0.5  $\mu\text{l}$  Quantitect RT mix and 500 ng template RNA. Final volume was adjusted with RNase free water. The PCR cycling protocol include reverse transcription at 50 °C for 30 min, initial denaturation at 95 °C for 15 min, 40 cycles of denaturation at 94 °C for 15 s, gene specific annealing for 30 s at 56–67.5 °C (Table 1) and extension at 72 °C for 30 s. A gradual increase in temperature from 67 to 95 °C was carried out to calculate melt curves. Following amplification the inbuilt software was used to set the baseline and threshold for each reaction. A cycle threshold ( $C_t$ ) was assigned at the beginning of logarithmic phase of PCR amplification and difference in  $C_t$  value of control and treated samples were used to determine the relative expression (fold changes) of the gene using a Microsoft excel macro.

### 2.6. Statistical analysis

Biochemical enzyme (ALT and AST) activities between the control and drug treated animals were analyzed by one-way ANOVA with Newman–Keuls post analysis test (GraphPad Prism version 3). Furthermore, the log transformed data of control and treatment groups was assessed with two-sample *t*-test to identify probes with  $p < 0.01$  and two-fold differential expression (Avadis Express version 4.3).

Table 1  
List of forward and reverse primers for quantitative real-time PCR analysis

S. No.	Gene name	Primer sequence (5' . . . 3')	Annealing temperature (°C)
1	Arhgap	FP: GATGATTGTTCCCACTGCCTACCC RP: GACCTGCGCTCTGACACACATCTCCT	67
2	H2-Eb1	FP: GCTACATGAACTGGGCTCTGAAA RP: CCGCCGCTTTAGGTTTCAGATTCAA	65
3	Zfp592	FP: TGTCTCTGTGGGTGGGAAAAATGG RP: GCAGGACAGCAGCAGAAGGAAAAC	66
4	Slc44a2	FP: GTTGATGACACAGCCTGCCCACTT RP: CAGCAGGGCACGGTGGTAGGT	56
5	Pcsk6	FP: ACAGCCGGAGGGACAGTTTGAT RP: TTCGGAAGGAGCTGACTGCGTAGA	58.5
6	Gapdh	FP: TTTGGCATTGTGGAAGGGCTCAT RP: CACCAGTGGATGCAGGGATGATGT	67.5

### 3. Results

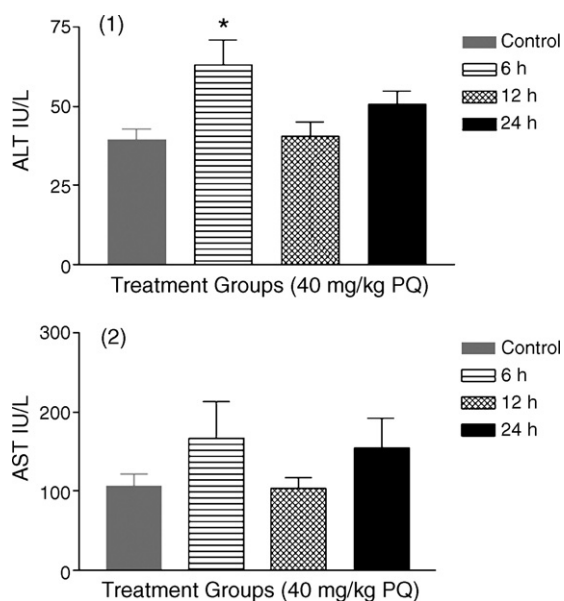
#### 3.1. ALT, AST and histopathology

Single dose of PQ administered to mice was found to be ineffective in producing classical symptoms of hepatic stress as both biochemical and histopathological marker were absent at all time points. Apart from a slight increase in ALT activity at 6 h ( $p < 0.05$ ), no significant difference in the activities of ALT and AST enzymes was observed at any time point following PQ administration (Figs. 1 and 2). Similarly, histopathological lesions characteristic of hepatic stress were absent (Fig. 3).

#### 3.2. Differential expression analysis

Following PQ exposure mRNA expression in mice liver was assessed with 15,247 unique probes. A statistically stringent criteria ( $p < 0.01$  and two-fold) was used to identify genes with significant differential expression and continuous deregulation at all time points. Presently, 16 differentially expressed probes consisting of 11 up-regulated and 5 down-regulated probes were identified. The probes that were found to be differentially expressed includes, homer homolog 2 involved in protein transport, zinc finger protein 592 involved in transcriptional regulation, rho GTPase activating protein 29 involved in the regulation of intracellular signaling, proprotein convertase subtilisin/kexin type 6 involved in the processing of precursors of alpha-factors and killer toxins, polyadenylate-binding protein-interacting protein 2 involved in protein synthesis, stromal interaction molecule 2 involved in hematopoiesis, mucin and cadherin like involved in cell adhesion, pleckstrin

homology-like domain family B member 2, SWA-70 protein, histocompatibility 2 class II antigen E beta, serine/threonine kinase 38 involved in cell proliferation, expressed sequence tag (C86400) and Solute carrier family 44, member 2. Three probes have no sequence similarity with any known gene and have not been assigned any biological function, so far (Table 2). Furthermore, analysis of microarray data at lower stringencies identified 189 probes with two-fold differential



Figs. 1 and 2. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities following PQ (40 mg/kg) administration at different time points. PQ administration did not produce significant variation in AST activity however ALT activity increased ( $p < 0.05$ ) at 6 h as compared to control (B). The control in each figure is the average of all the three control groups (6, 12 and 24 h).

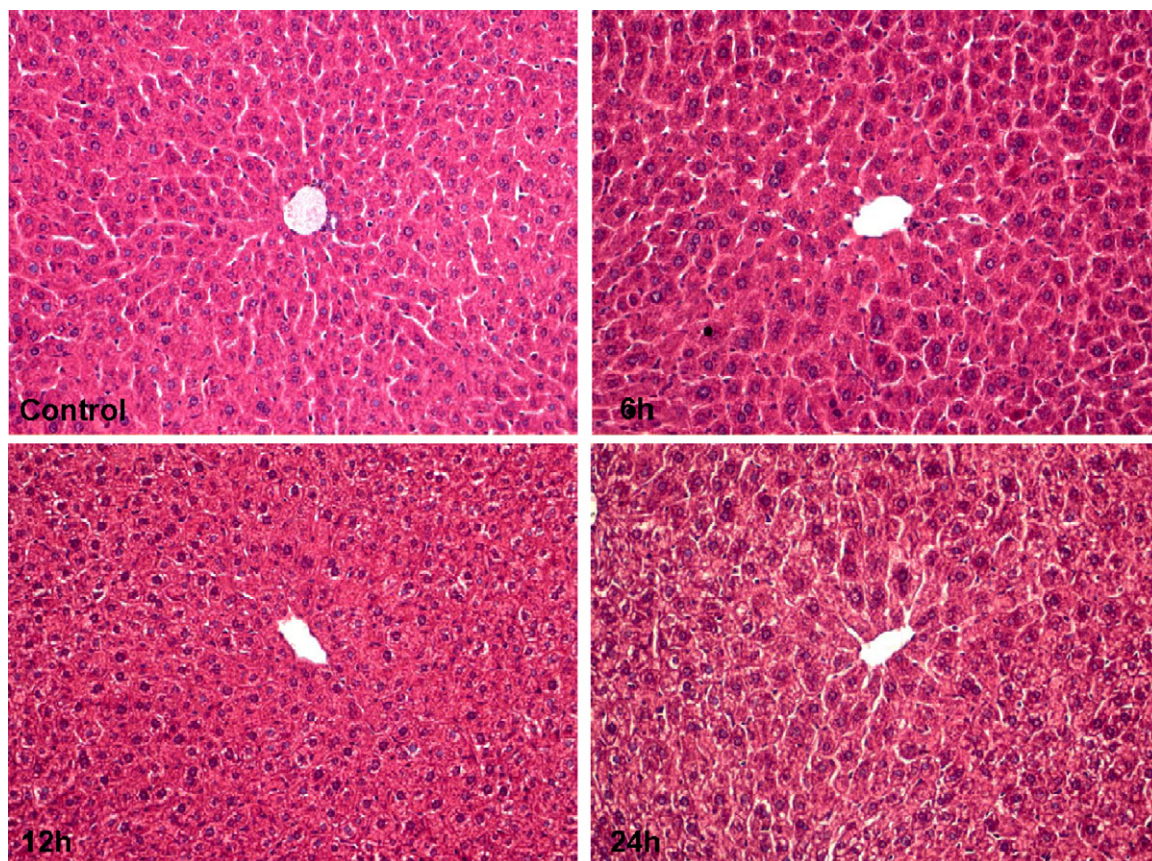


Fig. 3. Representative images of hematoxylin and eosin stained liver sections of PQ administered mice showing absence of histologically detectable damage at different time points.

Table 2

Differentially expressed genes ( $p < 0.01$  and two-fold) at 6, 12 and 24 h after primaquine (40 mg/kg) administration

Probe ID	Gene name	Symbol	Function	Expression
H3158D12	Stromal interaction molecule 2	Stim2	Hematopoiesis	▲
H3148F06	Zinc finger protein 592	Zfp592	Transcriptional regulation	▲
H3133F07	Rho GTPase activating protein 29	Arhgap29	Intracellular signaling	▲
H3054D08	Proprotein convertase subtilisin/kexin type 6	Pcsk6	Processing of precursors of alpha-factors and killer toxin	▲
H3012D06	Homer homolog 2	Homer2	Protein transport	▲*
H3154H12	Polyadenylate-binding protein-interacting protein 2	Paip2	Protein synthesis	▲*
H3009G08	Mucin and cadherin like	Mucdhl	Cell adhesion	▲
H3010C08	Pleckstrin homology-like domain, family B, member 2	Phldb2	–	▲
H3099E10	Data unavailable	–	–	▲*
H3116F02	Data unavailable	–	–	▲
H3070F12	Data unavailable	–	–	▲
H3045C06	SWA-70 protein	Swap70	Regulation of VEGF mRNA	▼
H3008F07	Histocompatibility 2, class II antigen E beta	H2-Eb1	Immune response	▼
H3060B12	Serine/threonine kinase 38	Stk38	Cell proliferation	▼
H3054F09	Expressed sequence tag	C86400	–	▼
H3146H06	Solute carrier family 44, member 2	Slc44a2	Cellular transport	▼

▲: Up-regulated probes at 6, 12 and 24 h, ▼: down-regulated probes at 6, 12 and 24 h, ▲\* up-regulated probes at 6 and 12 h only.

Table 3  
Important probes with two-fold differential expression at 6, 12 and 24 h after PQ treatment

Biological process/probe ID	Gene symbol	6 h log <sub>2</sub>	12 h log <sub>2</sub>	24 h log <sub>2</sub>
<b>Apoptosis</b>				
H3035B01	Trib3	2.178	0.09	1.002
H3156F06	Nfkb1	−3.473	−0.637	−0.154
H3033B06	Birc1c	−2.869	−0.054	−0.039
H3104G10	Camk1d	1.527	2.019	0.103
H3041E11	Elmo1	−3.268	0	0
<b>Transcription</b>				
H3148F06	Zfp592	1.116	0.962	0.61
H3113F07	Cdkn2c	2.012	0.113	0.349
H3085H09	Ankrd22	−2.074	−0.113	−0.158
H3137H06	Aes	−1.988	−0.226	−1.535
H3018H06	Gatad1	4.845	0.05	0.753
H3058D06	Zfp91	2.072	1.008	0.213
H3142H06	Gtf2h1	2.391	1.298	0.266
H3031G10	Pias1	1.176	0.831	0.835
H3140C12	Snape2	−0.64	−1.488	0
H3049G08	Narg1	0.225	2.364	0.321
H3038A09	Gata1	0	−3.11	0
H3045E10	Jarid2	−1.959	−0.258	−0.624
H3034A04	Mtf2	2.871	0	2.818
H3025G12	Purb	1.761	0.499	0.86
<b>Cell cycle</b>				
H3119H07	Ncaph	0	2.026	0
H3084D05	Ccnd1	2.279	0.213	0.035
H3022D12	Anapc7	4.447	1.243	0.361
H3069A07	Mdm2	−0.695	−2.008	−0.23
<b>Protein folding</b>				
H3044B01	Cct4	−1.734	−0.338	−0.858
H3130F12	Fkbp4	0.761	1.499	0.361
H3046H12	Upf3b	3.807	1.989	0.469
<b>Intracellular signaling</b>				
H3133F07	Arhgap29	1.167	1.033	0.398
H3074E03	Apbb2	0	−3.075	−0.216
H3042E05	Rims2	−1.565	−0.761	−0.599
H3001H03	Ywhaq	1.226	0.979	0.477
H3058F09	Lphn2	−1.988	−0.824	0
H3126F04	Gpr124	0	−2.156	−0.082
H3055G12	F2r	0.855	1.26	0
H3037A04	Snx13	−2.286	0	0
H3035D06	Adam15	−1.581	−0.567	−0.056
H3098B12	Fgfr1	1.231	0.878	0.643
<b>Metabolism</b>				
H3022H07	Topors	2.03	0.502	0.194
H3066F04	Igfbp7	−3.649	−0.041	−0.367
H3019G11	Lrp10	−1.471	−1.002	−0.666
H3118B12	Pkm2	2.606	1.934	0.558
H3030C07	Large	1.882	0.967	0.914
<b>Immune response</b>				
H3008F07	H2-Eb1	−1.257	−1.513	−1.771
H3053D08	Clec4e	−2.863	0	−1.56
H3018F06	Crry	2.385	0.64	0.464
H3045C06	Swap70	−1.246	−1.389	−0.297

Table 3 (Continued)

Biological process/probe ID	Gene symbol	6 h log <sub>2</sub>	12 h log <sub>2</sub>	24 h log <sub>2</sub>
<b>Protein biosynthesis and translation</b>				
H3027H01	Rpl15	-0.829	-1.174	-0.053
H3004D10	Eif3s10	-3.502	-1.031	-1.527
H3002F06	Eif3s10	-2.436	-0.148	-0.697
H3126F06	Rps29	1.907	0.657	0.162
H3154H12	Paip2	2.51	2.834	-0.279
<b>Cell adhesion</b>				
H3053F08	Tnr	0.872	1.332	0.279
H3130D12	Cd164	2.841	0.596	0.023
H3158D12	Stim2	1.384	1.331	0.162
H3070C07	Vcam1	0	-2.605	-0.535
H3009G08	Mucdhl	1.289	1.166	0.245
<b>Cellular transport</b>				
H3008D12	Atp5a1	-0.853	-1.429	-0.895
H3042D09	Cyp51	-2.658	-0.697	-0.979
H3046H11	Slc5a11	-1.464	-0.789	-1.131
H3002B06	Ehd1	-2.668	-0.068	-0.642
H3083G02	Lcn2	-2.467	-0.027	-0.287
H3067C10	Clta	1.867	0.626	0
H3140C11	Atp6v0b	-1.877	-0.752	-1.319
H3121G04	Cyp51	-2.913	-0.07	-0.27
H3121G05	Cyp51	-2.614	-0.041	-0.206
H3012D06	Homer2	1.353	1.253	-2.337
<b>Miscellaneous</b>				
H3068F07	Mapkbp1	0	-2.219	-0.134
H3024D02	Tubb2c	1.792	0.296	0.342
H3075H03	Cd300lg	0.805	1.298	0
H3008H09	Lmnb1	0.605	1.467	-0.691
H3003B10	Golph4	-2.049	0	0
H3067D10	BC014805	-2.072	0	-0.29
H3058B12	Gdap1	3.751	1.654	0.1
H3146H06	Slc44a2	-1.103	-1.156	-0.684
H3015G02	Rbm6	0.623	2.029	1.529
H3035C02	2310042E22Rik	1.747	0.55	1.885
H3028G11	Phf17	1.397	0.872	0
H3109A10	Tmem59	-1.888	-0.671	-0.915
H3108A06	Tnfrsf19	1.284	1.004	1.266
H3061A07	Srpk2	-1.746	-0.253	0
H3003A03	Lgals1	-1.268	-1.645	2.513
H3143C03	Ttc14	2.392	0.141	0.338
H3079A10	Mina	-0.744	-1.794	-0.031
H3035C05	Serac1	-0.933	-1.262	-0.397
H3135C05	Ilk	1.417	0.853	0.632
H3154H06	Zdhhc2	-1.123	-0.881	-0.096
H3060E07	Exod1	1.396	0.772	0.199
H3098F12	Smg6	2.515	0.007	0.281
H3118H12	Fbxo3	1.929	0.303	0.659
H3126D06	Efh2	1.689	1.347	0.441
H3150B12	Ehmt2	-3.077	-0.7	-1.317
H3155D12	Wnk1	-2.081	-0.292	-0.044
H3040D06	Slk	0.2	2.078	0
H3060B12	Stk38	-1.987	-1.779	-0.079
H3074F01	Sfpq	0	-2.266	-0.647
H3138H08	Numa1	-2.034	-0.288	-0.216
H3046D11	Elavl1	2.384	0.255	-0.097
H3010D12	Usp19	1.873	0.997	0.478
H3014D12	Rbm28	3.139	0.634	0.531

Table 3 (Continued)

Biological process/probe ID	Gene symbol	6 h log <sub>2</sub>	12 h log <sub>2</sub>	24 h log <sub>2</sub>
H3022H06	Ppm1b	-1.429	-0.916	-2.149
H3050B12	Dirc2	2.274	0.356	0.481
H3054D08	Pcsk6	1.09	0.917	0.571
Unknown biological process				
H3063B04	Abpb	-2.224	0	-0.212
H3063F12	Msi2	-0.136	-2.437	-0.727
H3150H12	Commd6	2.206	0.925	0.31
H3048C10	Auts2	-2.391	0	-1.174
H3019E11	Pfpl	-1.7	-0.411	-0.101
H3016H09	2610029G23Rik	1.636	0.59	0.67
H3040H09	C78653	0	-2.125	-0.791
H3064B04	Apobec4	-2.643	-0.467	-1.034
H3055H06	Zfp708	-2.851	-0.116	-0.341
H3068B06	9030205A07Rik	0	3.505	0.027
H3068F06	Sgpp2	-1.634	-0.718	-0.34
H3149H09	1700006H03Rik	0.197	1.943	0.297
H3054F09	C86400	-1.15	-1.087	-1.074
H3069B12	Sned1	-0.181	-1.944	-1.78
H3093D12	Gm944	0.271	1.882	0.132
H3062H06	Tmco1	2.368	1.034	0
H3082B06	Pramel5	0.649	1.575	0.131
H3146H12	1700021F05Rik	1.577	0.505	0.437
H3028E07	3110082I17Rik	2.37	0	1.108
H3052E07	Gm1070	2.233	0.552	0.112
H3135G08	AU015836	0	-2.185	-0.108
H3051A09	Mcm10	-1.658	-0.77	-0.633
H3020E03	Utp15	-2.087	-0.308	-0.455
H3024A03	Jub	-2.295	-0.037	-0.075
H3111E05	AU023434	0.022	2.296	1.648
H3143G05	9130011J15Rik	-1.507	-0.912	-1.032
H3100G05	1110021L09Rik	0.608	1.918	0
H3061E02	Wipi1	0	-2.137	0
H3093A08	A230106M15Rik	-2.886	0	-0.076
H3010C08	Phldb2	1.045	1.23	0.52
H3109A04	Malat1	-3.091	-0.4	-0.241
H3110G10	Dcun1d2	-1.509	-0.653	-0.691
H3093E11	BC026657	-2.244	-0.016	-0.344

Note directionally similar deregulation in most of the probes.

expression following PQ administration at all time points (Table 3). Moreover, real time PCR analysis of selected genes showed similar trend in differential expression to microarray results. An expression difference of up to 15-fold was detected in these genes.

### 3.3. PQ affected pathways

Clustering algorithm group data points solely on the basis of their expression profile and does not take into consideration any biological association. *k*-means clustering employed in this study identified similar pattern of expression in genes at different time points. Furthermore, microarray data pertaining to all three-time points was condensed and subsequently analyzed with GenMAPP, a pathway analysis tool, to derive better conclusions

at the level of biological pathways. Major pathways that showed signs of PQ induced perturbations include oxidative stress and FAS pathway, apoptosis, cell cycle, inflammatory response, glycogen metabolism, Krebs's cycle, electron transport chain, fatty acid beta oxidation, MAPK signaling, signaling of hepatocyte growth factor receptor, matrix metalloproteinases, steroid biosynthesis, TGF- $\beta$  signaling, translation factors, wnt signaling, regulation of actin cytoskeleton, ribosomal proteins, RNA transcription reactome, proteasome degradation and nuclear receptors in lipid metabolism.

### 3.4. Identification of most affected GO terms

Furthermore, microarray data analysis with MAPPFinder revealed that 40 Gene Ontology terms

Table 4  
Most affected Gene Ontology terms ( $z$  score  $> 2$ ) identified after PQ dosing

Gene Ontology name	Gene Ontology ID	$z$ -Score	$p$ -Value (uncorrected)
Monosaccharide metabolism	5,996	2.782	0.003
Hexose metabolism	19,318	2.782	0.003
Growth	40,007	2.782	0.005
Organelle organization and biogenesis	6,996	2.805	0.006
Glucose catabolism	6,007	2.574	0.011
Carbohydrate metabolism	6,092	2.574	0.011
Alcohol catabolism	46,164	2.574	0.011
Carbohydrate catabolism	16,052	2.574	0.011
Cellular carbohydrate catabolism	44,275	2.574	0.011
Glucose metabolism	6,006	2.574	0.011
Monosaccharide catabolism	46,365	2.574	0.011
Hexose catabolism	19,320	2.574	0.011
Gtpase activity	3,924	2.574	0.013
Alcohol metabolism	6,066	2.488	0.015
Regulation of growth	40,008	2.347	0.015
Protein complex	43,234	2.28	0.018
Cellular physiological process	50,875	2.439	0.021
Glycolysis	6,096	2.347	0.021
Actin filament-based process	30,029	2.347	0.025
Cellular process	9,987	2.265	0.028
Biological process	8,150	2.271	0.03
Zinc ion binding	8,270	2.251	0.03
Physiological process	7,582	2.109	0.039
Regulation of cell size	8,361	2.098	0.042
Cell growth	16,049	2.098	0.042
Guanyl-nucleotide exchange factor activity	5,085	2.098	0.045
Cell organization and biogenesis	16,043	2.126	0.046
Transition metal ion binding	46,914	2.02	0.05
Cytoplasm organization and biogenesis	7,028	2.098	0.051
Actin cytoskeleton organization and biogenesis	30,036	2.098	0.051
Acid-amino acid ligase activity	16,881	2.012	0.055
Ligase activity, forming carbon-nitrogen bonds	16,879	2.012	0.055
Ubiquitin-protein ligase activity	4,842	2.012	0.055
Cell-cell adhesion	16,337	2.022	0.063
Condensed chromosome	793	2.022	0.069
Protein ubiquitination	16,567	2.025	0.074
Cysteine-type peptidase activity	8,234	2.334	0.013
Cysteine-type endopeptidase activity	4,197	2.159	0.032
Hydrolase activity, acting on ester bonds	16,788	2.146	0.04
Endopeptidase activity	4,175	2.067	0.049

were affected significantly ( $z$  scores  $> 2$ ). Carbohydrate metabolism, cell structure and protein metabolism related pathways were the most affected with a significant  $z$  score. Table 4 shows the complete list of Gene Ontology terms with their corresponding scores that were found to be most affected by PQ.

#### 4. Discussion

Presently, *in vivo* differential expression analysis of hepatic genes was carried out following PQ administration in mice. A preliminary dose finding study was carried out to decide PQ dose by evaluating tra-

ditional markers of hepatic stress in serum (ALT and AST) and liver histology (data not shown). PQ did not produce any detectable damage either in serum or liver histology at any of the doses tested (i.e. 25, 35 and 40 mg/kg). Consequently, the highest dose of PQ (40 mg/kg) was administered to ascertain robust gene expression changes. Interestingly, in contrast to biochemical and histological findings, a significant ( $p < 0.01$  and two-fold) differential expression was observed in some of the probes that provided earliest evidence of the hepatic tissue response at molecular level. Some of the identified genes have well established cellular functions that may be important in maintaining the general

homeostasis of hepatic tissue under PQ stress however; others have not been assigned any specific cellular function as yet. Nevertheless, considering the complexity of intracellular signaling and multiple roles being attributed to already annotated genes, it is expected that both types of probes, annotated as well as non-annotated, have important implications. Furthermore, real-time validation of microarray result in selected genes strengthens present findings.

The exact mechanism of PQ action and its activity especially against hepatic forms of the plasmodia is presently unclear (Rang et al., 2003). However, it may be converted to electrophiles that act as oxidation–reduction mediators (Tarlov et al., 1962). Such activity could generate reactive oxygen species or interfere with electron transport chain of the host along with the parasite (Bates et al., 1990). Presently, components of oxidative stress related pathway were affected following drug administration (Fig. 4). Furthermore, in some circumstances, particularly overdosage, high-energy reactive metabolites can form covalent adducts with other cellular constituents such as essential cellular enzymes and

nucleic acids. Under these conditions analysis of differentially expressed genes, at high-throughput scale, required adoption of an appropriate statistical approach that could provide complete insight of the underlying biological events with high confidence. Therefore, we primarily observed differential gene expression between the control and the PQ treated animals at  $p < 0.01$  and two-fold at all time points in this study. Identification of genes with similar expression changes, at such a stringent statistical level, both in direction and magnitude at all three time points indicate important biological role under these circumstances. For example, one of the genes identified as being differently expressed was pro-protein convertase 6 (Pcsk6). It is a member of serine proteinase family responsible for processing precursor proteins to their active forms by selective proteolysis. One recent report indicates that Pcsk6 mRNA is also expressed in mouse uterus where it is involved in decidualization (Wong et al., 2002). Furthermore, the gene encoding poly (A)-binding protein-interacting protein 2 (Paip2) was also differentially expressed. Product of this gene has important effect on the regulation and stability

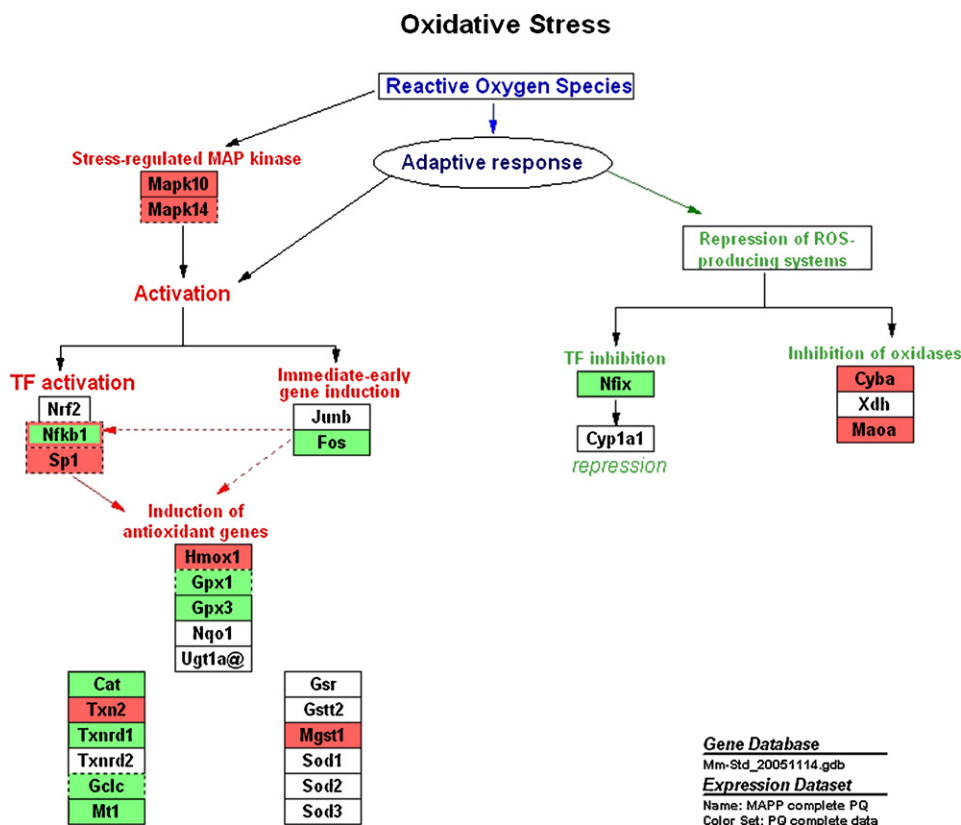


Fig. 4. Condensed data of PQ administered mice from 6, 12 and 24 h indicate different components of the oxidative stress related pathways being affected. GenMAPP generated image shows the up-regulated probes (in red boxes) and down-regulated probes (in green boxes). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

of vascular endothelial growth factor (VEGF) mRNA (Onesto et al., 2004), which is an important angiogenic factor that promotes tumor neovascularization (Kyzas et al., 2005) and is an important prognostic factor in many types of human cancers (Dvorak, 2002). Furthermore, gene encoding SWA-70 protein with an important function in regulating the cellular actin dynamics and organization was down regulated. It is an important constituent of SWAP complex and influences, targeting and binding of protein complexes to switch region of DNA (Hanakahi et al., 1997). Other genes listed in Table 3 have also important cellular function.

Since, high stringency data processing provides limited insight of the exact biological changes inherent in the microarray data; gene deregulations at lower stringency were also investigated. Expectedly, microarray data analysis at lower stringency resulted in the identification of greater number of affected probes both for differential expression and pathway level analysis, indicating that PQ does affect gene expression at high doses. Similarly, the probes corresponding to almost all the pathways considered in this study were affected whatsoever the number of affected probes and magnitude of deregulation be. Furthermore, identification of 40 GO terms with a  $z$  score greater than 2 also provides additional evidence of significant hepatic response at molecular level. Alterations in important cellular processes have been observed by workers with known hepatotoxicants in similar studies (Minami et al., 2005). Most biological process such as cell survival, cell metabolism, cell signaling and transcription regulation, etc., were significantly affected as evident in the differential expression and pathway level analysis. Since a high dose of PQ was administered in this study most gene expression changes would be more relevant to PQ induced adverse effects rather than therapeutic action. Nonetheless, consistent deregulation of these genes at all times may suggest a stress signature inherent in the present data. However, absence of clear-cut stress related signature in the present findings is difficult to explain at present but we suspect that length of PQ exposure might be one of the reasons.

One difficulty in generalizing these results as the primary response of hepatocyte is the heterogeneous cellular composition of hepatic tissue. Although, hepatocytes constitute majority of the hepatic tissue, involvement and effect of minor cell populations of liver such as Kupffer cells and endothelial cells cannot be neglected. Significant exacerbation of hepatic injury by recruitment of inflammatory cells such as neutrophils and activation of sinusoidal lining cells, particularly

Kupffer cells has already been documented (Jaeschke et al., 2002). Moreover, the involvement of the identified genes in executing useful pharmacological activity of PQ cannot be ruled out, especially in the absence of traditional biomarkers of hepatic stress. Since there is no information available about the mechanism of action of PQ, the genes identified in this study may prove helpful in undertaking further work in order to find important link with a specific cellular effect.

In summary we conclude that acute dosing of PQ in mice liver leads to significant differential expression of genes which provides the earliest clue of the hepatic response. However, we strongly believe that further studies conducted at doses closer to therapeutic level can provide better attributes to the present molecular signature. Moreover, as already discussed, many of the probes identified in this study have no biological function assigned as yet and may be important in the initiation and progression of disease or may merely represent an initial protective signature which might otherwise have led to more serious effects under chronic exposure of the drug. However, the exact outcome of these genetic responses is hard to envisage at this stage and entails further studies at both gene and protein level at a high-throughput scale both after acute and chronic PQ exposure ([www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE5979](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE5979)).

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