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High throughput expression profiling facilitates prediction of toxicity and interpretation of mechanism of toxicity based on distinct gene expression changes. The simplest approach to identify genes of potential interest through several related experiments is to search for those that are consistently either up or down-regulated. Presently, in vivo differential expression analysis of hepatic genes was carried out in mice liver following administration of 8-aminoquinoline derivatives namely PQ and BQ, in a time dependent manner at different statistical stringencies using different data analysis algorithms. Furthermore, an attempt was made to correlate 8-aminoquinoline-induced gene expression changes with those of model hepatotoxicants namely APAP and CCl₄. Although, the primary goal of this study was to investigate gene expression alterations, additional parameters indicative of hepatic stress were also evaluated in drug treated animals. This study was expected to provide insight about the relative safety of the two 8-aminoquinoline derivatives, unearth gene expression signatures related to hepatotoxicity, if any, and establish microarray technology as a predictive tool to understand tissue responses at an early stage following acute dosing of 8-aminoquinoline derivatives.

Generally, the activities of ALT and AST increases significantly following acute drug or xenobiotic exposure, however, presently the activity of these enzymes was not affected following administration of PQ or BQ. Alternatively, the administration of model hepatotoxicants affected the activities of ALT and AST to varying extents. Presently, serum ALT and AST activities were significantly affected following CCl₄ treatment but remained comparable after APAP treatment except for AST activity at 6 h (Fig. 10). These observations might either be due to the dose used or the duration of exposure of the drug. In this context, it is important to mention that a preliminary dose finding study was carried out to decide the dose of PQ by evaluating traditional markers of hepatic stress in serum (ALT and AST) and liver histology. However, PQ did not produce any detectable damage in both the serum and liver histology at any of the doses tested (i.e. 25 mg/kg, 35 mg/kg and 40 mg/kg). Consequently, the highest dose of PQ (40 mg/kg) was administered to ensure robust gene expression changes at a high statistical stringency i.e. p<0.01 and 2 fold. Similarly, BQ (40 mg/kg) did not affect the activities of ALT and AST enzymes. The dose of BQ was approximately six times higher than the recommended therapeutic dose and is equimolar to the PQ dose. However, in comparison to the LD₅₀ values of both 8-aminoquinolines (LD₅₀ PQ= 80 mg/kg and LD₅₀ BQ= 681 mg/kg), the administered
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doses can not be considered toxic (Puri et al., 1989). The dose of APAP used in this study is not acutely toxic and doses closer to the present dose are used to achieve analgesic effects in rodents, however, the dose of CCl₄ was toxic. Furthermore, the time of tissue sampling i.e. 6, 12 and 24 h were decided, first, because both the 8-aminoquinoline derivatives get metabolized and excreted within 24 h after administration (Fletcher et al., 1981; Mehrotra et al., 2007) secondly, to investigate earliest gene deregulations following drug treatment. Thus, absence of biochemical markers of toxicity following 8-aminoquinolines and APAP (except for AST at 6 h) treatment is not surprising as alterations in serum biochemistry are often reported at doses closer to toxic levels (Morishita et al., 2006). Furthermore, present biochemical results may show the affect of availability of food at all times during the course of this study and/or species differences. The rationale for observed difference in ALT and AST activities is based on the observation that most toxicity studies include withdrawal of food before the administration of drug in an attempt to aggravate the toxic outcomes (Lucas et al., 2000; Placke et al., 1987). The reason for permitting food and water throughout the experiment was to simulate normal physiological state and to obviate chances of affecting gene expression with altered physiology during fasting.

Furthermore, histopathological lesions were absent in PQ, BQ or APAP treated animals, suggesting inefficiency of these drugs at administered doses to produce recognizable hepatic damage. The absence of histological lesions further confirmed the biochemical results and lack of any hepatic damage. On the contrary, CCl₄ treated animals showed hepatic pathological changes such as necrosis, eosinophilia and inflammation. The progressive trend of histological lesions observed in CCl₄ treated animals indicates a continuous and noteworthy hepatic damage that could be detected even at 24 h following single intraperitoneal injection.

Chromosomal aberrations were absent in animals treated with PQ, BQ, APAP or CCl₄. This showed that none of the doses used in this study was clastogenic in nature. These finding, particularly regarding 8-aminoquinoline derivatives, are in confirmation to previous preclinical data available in the laboratory.

The biochemical, histopathological and chromosomal aberration assay results indicate that PQ, BQ and APAP do not produce hepatic tissue damage or affect chromosomal integrity at doses used in this study following single acute treatment. However, different tissue responses may be produced if they are administered for longer durations and/or at much higher concentrations. This can particularly be true for
APAP, which produces classical symptoms of hepatic damage both at biochemical and histological level, at higher doses (Zidek et al., 2007; James et al., 2003).

The analysis of hepatic mRNA expression using DNA microarray identified differentially expressed genes in animals treated with PQ, BQ, APAP or CCl4. This finding is particularly important, first because traditional markers of hepatic stress were absent following most drug treatments, especially after 8-aminoquinolines and second, stringent statistical criteria ($p<0.01$ and 2 fold differential expression) was used in this study, which allows only extremely robust gene expression changes to be detected. The aim to study differential expression primarily at high stringency and in a time dependent manner was to increase the statistical confidence in the detection of important genes and cellular processes with a probable role in the initiation and propagation of toxicity as well as those possibly involved in regeneration during the early phase of tissue response. Furthermore, inherent biological variations among the members of the same group were reduced by pooling RNA samples. Technical means of error were corrected by performing dye swap experiments and normalizing the microarray data. This ensured detection of probes corresponding to significantly deregulated genes with a high statistical confidence following drug treatment. Furthermore, validation of microarray findings using qRT-PCR further substantiates these results, which is the most sensitive and accurate method of validating microarray based differential expression of genes (Chuaqui et al., 2002). Therefore, identification of genes with similar expression changes, at such a high stringency, both in direction and magnitude at all three time points indicate important biological functions under these circumstances.

One of the differentially expressed genes identified after PQ dosing was proprotein convertase 6 (Pcsk6). It is a member of serine proteinase family responsible for processing precursor proteins to their active forms by selective proteolysis. According to a recent study 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 significantly affected. Product of this gene has important effect on the regulation and stability 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).

Furthermore, the expression of fatty acid synthase (*Fasn*) was down regulated in APAP treated animals as compared to the controls. Interestingly, Clustering of microarray data revealed that *Fasn* was regulated identically following APAP or CCl₄ administration, however, the extent of deregulation was more pronounced in the former case (Fig. 18C). FASN is involved in the formation of long-chain fatty acids from acetyl-coA, malonyl-coA, NADPH and plays an important role in regulating apoptosis. Recent findings indicate that inhibition of FASN in cancer cells causes accumulation of malonyl-coA, which leads to inhibition of CPT1 and up-regulation of ceramide and induction of the proapoptotic genes (eg. *Bnip3, Trail, and Dapk2*) resulting in apoptosis (Bandyopadhyay et al., 2006). Furthermore, specific silencing of the *Fasn* in cancer cells substantially decreased palmitic acid synthesis. Subsequent depletion of the cellular pool of palmitic acid is associated with induction of apoptosis concomitant with the formation of reactive oxygen species (ROS) and mitochondrial impairment (Chajes et al., 2006). Moreover, inhibition of fatty acid synthesis has been attributed to caspase mediated degradation of FASN protein, independent of the *Fasn* mRNA levels (Zhang and Kiechle, 2006). Thus, down-regulation of *Fasn* in present study might reflect apoptotic events following APAP and CCl₄ administration. Similar expression changes in *Fasn* expression were observed after high doses of both APAP and CCl₄ in male Sprague-Dawley rats (Zidek et al., 2007).

The probe corresponding to disabled homolog 2 (DAB2), an ATP dependent RNA helicase, required for the processing and cleavage of 35S pre-rRNA to produce mature 18S rRNA during 40S ribosomal subunit biogenesis, was found to be down regulated in mice liver after acute exposure to APAP. *Dab2* codes a mitogen-responsive phosphoprotein that binds to the SH3 domains of GRB2 via its C-terminal proline-rich sequences and modulates growth factor/RAS pathways by competing with SOS.

Alpha thalassemia/mental retardation syndrome X-linked homolog (*Atrx*) was found to be consistently down-regulated following APAP treatment. It encodes a large multiprotein, chromatin remodeling complex of the SWI/SNF family of proteins. It has also been associated with the regulation of key stages of meiosis in mouse oocytes. RNA interference (RNAi) mediated ablation of ATRX suggests that centromeric
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ATRX is crucial for correct chromosome alignment and organization of bipolar meiotic metaphase II spindle (De La Fuente et al., 2004). Administration of CCl4 also resulted in down regulation of Atrx at all time point but could not cross high stringency criteria applied in this study (Fig. 18 D).

Serine (or cysteine) peptidase inhibitor, clade A, member 6 (SERPINA6) or corticosteroid-binding globulin (CBG) is a major transport protein synthesized in liver for glucocorticoids and progestins in the blood of almost all vertebrate species. Expression of SerpinsA6 was down-regulated after APAP administration consistently at all time points. Recently, Morishita et al. (2006) also found down-regulation of this gene both in young and adult rats following high doses of APAP. Plasma SERPINA6 regulates the levels of glucocorticoids subsequently influencing fetal development. Immunoreactivity studies conducted on fetal tissues reveal detectable levels of CBG at different time points suggesting that temporal and spatial changes in the localization of CBG and its mRNA may influence the developing tissues (Scrocchi et al., 1993). Moreover, low stringency data analysis revealed down-regulation of SerpinsA6 after CCl4 administration.

The gene encoding 5'-3' exoribonuclease 1(XRN1), a multifunctional protein that exhibits both DNA strand exchange and 5'-3' exonuclease activities in vitro, was down-regulated following APAP treatment. The purified mouse protein (mXRN1p) exhibits a preference for G4 RNA tetraplex-containing substrates demonstrating its role in RNA turnover (Bashkirov et al., 1997). Furthermore, in vitro studies suggest that it preferentially degrades single-stranded DNA (ss-DNA) and can renature complementary ss-DNA, catalyze the formation of heteroduplex DNA from circular ss-DNA and homologous linear ds-DNA. Mutations in 5'-3' exoribonuclease affects nuclear fusion, reduces chromosome stability and spindle pole body duplication and/or separation. It also acts as a microtubule-associated protein, which interacts with cytoplasmic microtubules through beta-tubulin.

Presently, CCl4 administered animals showed maximum number of differentially expressed genes both with p<0.01 and 2 fold differential expression or 2 fold differential expression alone (Table 4 and 8). Identification of large number of differentially expressed genes related to oxidative stress, protein modification, cytoskeleton proteins, transcription regulation, carbohydrate metabolism, lipid metabolism, protein metabolism and other important cellular processes, and alterations in biochemical and histopathological parameters provides an opportunity to anchor
gene expression changes with traditional markers of hepatic stress. This is particularly important as a large number of stress and metabolism related genes were found in the present signature. These findings are in good correlation with previous studies, in spite of high stringency criteria used in this study that permits identification of very few probes, which identified similar expression changes following CCl₄ treatment.

However, one of the biggest disadvantages of using high stringency criteria for microarray data analysis is the loss of large amount of relevant biological information, though it helps to reduce the unwanted noise in the data. 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 with 2 fold differential expression (Table 5-8). It is evident from these results that all drugs used in this study, including 8-aminoquinoline derivatives, affect gene expression under present experimental conditions. 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 GO terms with a z score greater than 2 also provides additional evidence of significant hepatic response at molecular level. The consistent deregulation of these genes at all times is suspected to provide important biological information. Unfortunately, some of the identified genes have not been assigned any specific cellular function as yet. But, considering the complexity of intracellular signaling and multiple roles being assigned to already annotated genes, it is expected that both type of probes, annotated as well as non-annotated, have important implications. However, exact interpretation of present findings is difficult especially due to lack of sufficient gene expression studies, particularly in case of 8-aminoquinoline derivatives.

Thus, identification of highly significant (p<0.01 and 2 fold) differential gene expression within 24 h of PQ and BQ administration, in the absence of biochemical and histological markers, provides earliest evidence of the hepatic tissue response at molecular level. These findings are particularly important because previous reports indicate that administration of BQ does not affect the mixed function oxidase (MFO) system till 7 days and noticeable inhibition of various components of the MFO system occur only after extended exposure (21 days) (Pandey et al., 1990). Furthermore, exact mechanism of action of PQ and BQ and their tissue schizonticidal activity against
hepatic forms of the *P. vivax* and *P. ovale* is presently unclear (Rang et al., 2003). It is however, suspected that they may be converted to electrophiles that (Tarlov et al., 1962) could generate reactive oxygen species or interfere with electron transport chain of the host along with the parasite (Bates et al., 1990). 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. However, absence of straightforward alterations in stress responsive and toxicologically relevant genes following exposure to 8-aminoquinolines may indicate non-toxic nature of the compounds at present doses. Nevertheless, identification of genes with similar expression changes, at such a high statistical stringency, both in direction and magnitude at all three time points seems to be biologically important.

Furthermore, hepatotoxicants can often be separated distinctly from each other on the basis of strong signature set of genes (McMillian et al., 2005). Identifying patterns of gene expression and grouping genes into expression classes provide much greater insight into their biological function and relevance. Clustering of microarray data is a useful method to understand inherent association between the probes based on their gene expression profiles irrespective of any biological pre-information. However, no particular association could be deciphered when microarray data pertaining to all four compounds was analyzed simultaneously. On the contrary, clustering analysis of APAP and CCl₄ data grouped most of the stress related genes in the same cluster that were found to be differentially expressed above the assigned threshold level. Simultaneous grouping of APAP affected genes with CCl₄ affected genes in the same cluster implies that they behaved identically but could not pass the significance criteria filter (Fig. 18 A-D).

In general, microarray study revealed distinct expression patterns of each compound. This observation is easily comprehensible considering that 8-aminoquinoline derivatives and the two model hepatotoxicants belonged to different chemical classes. While differential genes expression was detected in case of both the 8-aminoquinoline derivatives, fewer number of genes in case of BQ confirms previous report that claim it to be less interfering with microsomal enzymes (Pandey et al., 1990). Furthermore, considering the number of probes used in each case (22,827 in BQ and 15,247 in PQ) it appears that BQ affects far less number of genes than PQ. On the other hand more GO terms identified after BQ administration may be due to greater number of probes used for pathway analysis without statistical filtering. However, a lack of similarity between
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the gene expression profiles of two 8-aminoquinolines is difficult to explain. It is assumed that different gene expression signatures obtained after 8-aminoquinoline exposure might reflect difference in the metabolism of the two derivatives studied.

A number of cellular responses have been recognized following drug treatment, particularly in case of well known hepatotoxicants. This includes increased production of reactive oxygen and nitrogen species that damage DNA, lipid and proteins, over expression of proteosomal proteins, and induction of cell proliferation (Klaunig et al., 1998; Williams, 2001). Oxidative damage can further modulate many signaling pathways including modulation of transcription factors and kinase cascades. Another prominent response related to oxidative stress, particularly in case of the model hepatotoxicants, is the increased expression of genes encoding proteosomal subunits. This might reflect the elimination of misfolded or damaged proteins during drug-induced hepatic stress (Gomes-Marcondes and Tisdale, 2002; Ding et al., 2003). Furthermore, chemically-induced cell proliferation may provide favorable environment for tumor development (Cunningham, 1996; Cohen, 1995). Thus, deregulation of genes likely playing a role in stress, apoptosis, cell structure remodeling, cell signaling and cellular metabolism presumably represents a toxic response. Furthermore, chemicals that damage mitochondrial structure, enzymes or DNA synthesis can disrupt β-oxidation of lipids and oxidative energy production within the hepatocytes (Froment and Pessayre, 1995; Bissel et al., 2001). Prolonged interruption of β-oxidation leads to microvesicular steatosis within hepatocytes. Mild insult leads to macrovesicular steatosis. In severe cases microvesicular steatosis, hepatic failure and death can result. Some drugs may inhibit β-oxidation (aspirin, valproic acid, tetracyclines) and others may disrupt oxidative phosphorylation alone or in addition (bile acids, amiodarone) depleting the hepatocytes of energy.

Typically, expression of mRNA in hepatic parenchyma cells generally changes on exposure to a single toxic dose of compound. By and large these transcriptional changes are adaptive, ensuing survival of stressed cells. However, heterogeneous cellular composition of liver makes it difficult to conclude that these transcriptional changes are solely due to the hepatocytes. Although, hepatocytes constitute majority of the hepatic tissue, involvement and effect of minor cell populations of liver such as Kupffer cells and endothelial cells can not 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 (Bissel et al., 2001).

Conclusively, it can be stated that application of high statistical stringency during microarray data analysis provides distinct expression profiles for PQ, BQ, APAP and CCl₄, however, analysis at lower stringency helps to identify common genes at least between the hepatotoxicants. Most likely, this disagreement between the stringent and less stringent analysis is due to lack of robust gene expression changes which are not detected in the stringent analysis in spite of having similarities among themselves. Nevertheless, probing gene expression data with multiple analysis tools at different statistical stringencies facilitates the understanding of underlying molecular events during drug-induced hepatic stress. Furthermore, present gene expression analysis has proven helpful to uncover important genes following administration of 8-aminoquinoline derivatives. However, lack of clear cut correlation between the 8-aminoquinolines and the hepatotoxicants related gene expression changes might reflect non hepatotoxic nature of the former at present dose regimen. Yet, identified genes are expected to hold important information regarding the 8-aminoquinoline metabolism or mechanism of action. But, assigning precise molecular functions to these genes is difficult at present and entails further studies at different doses both at gene and protein level. 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. Furthermore, identification of robust gene expression alterations in the absence of biochemical and histological markers, encourage microarray technology as a useful tool to understand molecular events at an earliest stage.