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Trovafloxacin-Induced Gene Expression Changes in Liver-Derived In Vitro Systems: Comparison of Primary Human Hepatocytes to HepG2 Cells

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List of abbreviations: TVX, trovafloxacin; PHH, primary human hepatocytes; CYP, cytochrome P-450; IDR, idiosyncratic drug reaction; UGT, UDP-glucuronosyltransferase; CYR61, cysteine-rich 61; GADD45, growth arrest and DNA-damage-inducible; IL-8, interleukin-8; BCL2, B-cell leukemia/lymphoma 2; HNF, hepatocyte nuclear factor; C/EBP, CCAAT/enhancer binding protein; ACADVL, acyl-coenzyme A dehydrogenase, very long chain; TFAM, transcription factor A, mitochondrial; MYCBP, c-myc binding protein

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Abstract

Primary human hepatocytes (PHH) are a main instrument in drug metabolism research and in the prediction of drug-induced phase I/II enzyme induction in humans. The HepG2 liver-derived cell line is commonly used as a surrogate for human hepatocytes, but their use in ADME and toxicity studies can be limited because of lowered basal levels of metabolizing enzymes. Despite their widespread use, the transcriptome of HepG2 cells compared to PHH is not well characterized. In this study, microarray analysis was conducted to ascertain the differences and similarities in mRNA expression between HepG2 cells and human hepatocytes before and after exposure to a panel of fluoroquinolone compounds. Comparison of the naïve HepG2 cell and PHH transcriptomes revealed a substantial number of basal gene expression differences. When HepG2 cells were dosed with a series of fluoroquinolones, trovafloxacin, which has been associated with human idiosyncratic hepatotoxicity, induced substantially more gene expression changes than the other quinolones, similar to previous observations with PHH. While TVX-treatment resulted in many gene expression differences between HepG2 cells and PHH, there were also a number of TVX-induced commonalities, including genes involved in RNA processing and mitochondrial function. Taken together, these results provide insight for interpretation of results from drug metabolism and toxicity studies conducted with HepG2 cells in lieu of PHH, and could provide further insight into the mechanistic evaluation of TVX-induced hepatotoxicity.

Introduction

In vitro human cell systems are a critical tool for the investigation of human-specific toxicities and the ADME properties of novel molecular entities. Primary human hepatocytes (PHH) represent the most widely used and best-characterized model for the understanding of the molecular mechanisms involved in the biotransformation and toxic responses of xenobiotics in humans. For instance, PHH are a main instrument in drug metabolism research and in the prediction of phase I/II enzyme induction in humans (Vermeir et al., 2005). They are essential for the classification and elucidation of molecular transport activity, especially as they relate to new pharmaceuticals in development (Pauli-Magnus and Meier, 2006). They have been applied to predict and understand mechanisms of hepatotoxicity (Dambach et al., 2005). Furthermore, they have been implemented in the evaluation of normal liver functioning and in the study of hepatic disease (Elaut et al., 2006).

While PHH systems are clearly advantageous in the evaluation of human toxicological and ADME phenomena, their use is not without limitations. For example, PHH tend to dedifferentiate over time, eventually losing their drug metabolizing capability (LeCluyse, 2001; Boess et al., 2003). Moreover, human hepatocytes are subject to various stimuli such as exposure to a wide assortment of drugs, environmental influences, disease, diet, natural hormones, alcohol, tobacco, trauma, genetic variation, among other factors that contribute to experimental variability. Indeed, differences in gene expression have been observed across donors of human hepatocytes (LeCluyse, 2001; Liguori et al., 2005). Lastly, the sporadic availability and significant cost associated with the use of human hepatocytes must be considered.

Because of the technical and financial difficulties associated with the use of isolated human hepatocytes, liver cell lines are often utilized for experimental assessment at the in vitro level. Of these, the most prominently employed is the human hepatocarcinoma derived, HepG2 cell line, which was first established in 1979 (Aden, 1979). Since this time, the cell line has become a system of choice for the study of liver-like responses in vitro (Washington and Tchounwou, 2004). However, of particular

importance to toxicology and ADME studies, this cell line possesses lower levels of the major phase I drug metabolizing enzymes then liver, most predominately the cytochrome P450 enzymes (CYPs) (Rodriguez-Antona et al., 2002). These monooxygenases are the central machinery catalyzing the biotransformation of many xenobiotics, including pharmaceuticals. Albeit, some reports have demonstrated the utility of HepG2 cells in the study of drug metabolism enzyme regulation (Wilkening et al., 2003).

In some recent studies, investigators sought to find potential causes for differences in cytotoxicity determination between PHH and HepG2 cell culture systems (Westerink and Schoonen, 2007 (A); Westerink and Schoonen, 2007 (B)). One possible cause may be a difference in metabolism between these cell types, and thus, the expression of phase I and II enzymes was extensively evaluated. It was found that the basal level of many CYPs was substantially lower in HepG2 cells, which could account for the differences in cytotoxicity. Furthermore, mRNAs for several UDP-glucuronosyltransferases were expressed at varying levels between the two cell types, which also may contribute to the differences in cytotoxicity.

Although the HepG2 cell line is applied rather extensively in some types of ADME and toxicology research, only a handful of reports have focused on the global genomic expression patterns of HepG2 cells (Morgan, 2002; Sonna et al., 2003). Even fewer reports have focused on the transcriptomic diversity between human hepatocytes and HepG2 cells. In one study, microarray analysis was performed on two hepatic cell lines, cultured primary hepatocytes, and liver slices. Substantial gene expression differences between these systems were observed (Boess et al., 2003). In another example, Harris, et al. compared the basal gene expression profiles of HepG2 cells and human hepatocytes as well as those resulting from exposure to chemical carcinogens (Harris et al., 2004). Approximately 31% of the genes expressed basally by HepG2 were unique to the cell line, and many of these coded for transcriptions factors, signal transduction proteins, growth factors, and transporters. Not surprisingly, several transcripts differentially modulated in HepG2 cells were involved in carcinogenesis. Hence, genes that are associated with critical pathways of toxic response were transcribed at disparate levels between the transformed HepG2 cells and PHH, with potential implications for some toxicological and ADME research.

Olsavsky, et al. recently examined the transcriptomic variation of PHH in Matrigel™ sandwich cultures versus liver cell lines including HepG2 cells (Olsavsky et al., 2007). While the PHH sandwich cultures showed basal and post-xenobiotic exposure expression profiles similar to those from liver, the cell lines evaluated (HepG2 and Huh7) failed to elicit a comparable response.

Here, we sought to compare the transcriptomes of HepG2 cells and PHH before and after exposure to a panel of fluoroquinolones, a class of bacterial DNA gyrase and DNA topoisomerase IV inhibitors. The quinolones as a class are generally safe and effective with few examples of liver toxicity. One exception is trovafloxacin, where its use has been restricted because of hepatic idiosyncratic drug reaction (IDR) issues (Bertino and Fish, 2000). Our laboratory has previously demonstrated that microarray analysis can distinguish trovafloxacin from other quinolones, not associated with hepatic toxicity, in PHH (Liguori et al., 2005). Since then, other examples of unique gene expression patterns in PHH or other culture systems from IDR-causing drugs relative to pharmacologically related compounds have been reported (Kier et al., 2004; Guo et al., 2006). In these examples, several endpoints including gene expression patterns induced by the IDR drug, troglitazone, could be distinguished from other thiazolidinediones in several cell types, including PHH, rat hepatocytes, or HepG2 cells.

As such, a central question of this work was to determine how the overall gene expression profiles of HepG2 cells and PHH compared in response to TVX exposure. Currently, these types of questions have become increasingly relevant due to a variety of efforts, including the use of stem cell technology, at establishing an immortalized human hepatocyte cell line with a phenotype more similar to its primary counterparts (Hengstler et al., 2005) for potential use in ADME or toxicity studies. Data such as these may be valuable for interpretation of results arising from drug evaluation and biotransformation studies using the HepG2 cell line and other novel hepatocyte-like cell lines.

Materials and Methods

Cell Culture

HepG2 cells were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured in Minimum Essential Medium (Invitrogen Life Technologies, Carlsbad,CA) with 10% Fetal Bovine Serum under a humidified 5% CO_2 atmosphere using T-162 plastic culture flasks. The cells were split when they reached approximately 70-85% confluence after washing with sterile phosphate buffered saline and detachment of the cells with trypsin (Invitrogen Life Technologies, Carlsbad, CA). The HepG2 cells were cultured in 6-well plastic plates upon exposure to quinolone compounds. Primary human hepatocytes, obtained from *In Vitro* Technologies (IVT, Baltimore, MD) in 6-well type I collagen coated plates, were cultured with 2 mL of Hepatocyte Incubation Media (IVT) at 37°C with 5% CO_2 for 24 hours after receipt.

Cytotoxicity Assay and Cell Treatment

A MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium), assay was conducted on HepG2 cells to establish the cytotoxic range of the fluoroquinolones evaluated and to establish doses for the genomic experiments. The assay was performed according to the manufacturer's instructions (Promega, Madison, WI). HepG2 cells were cultured in 96-flat well plates and were subsequently exposed to the drugs for 24 hours. At this time, the MTS reagent was added to each well, and the cells were incubated for 2-3 hours. After incubation, the absorbance readings were determined using a SpectraMax™ Plus plate reader (Molecular Devices, Sunnyvale, CA) using a visible wavelength of 490nm.

For the genomic experiments, quinolone compounds, dissolved in 0.1 N KOH (Sigma Chemical Co., St. Louis, MO), were added to the wells with fresh media at levels of 100 µM (HepG2) or 400 µM (primary human hepatocytes) for 24 hours using at least two technical replicates. Trovafloxacin was dosed using two separate preparations of HepG2 cells and two separate donors of human hepatocytes. Vehicle control cells were dosed with an equivalent volume of 0.1N KOH as the experimental samples. For intracellular comparison (HepG2 cells vs PHH), naïve cells were harvested using TRIzol™ reagent (Invitrogen Life Technologies, Carlsbad, CA).

RNA isolation and cRNA sample preparation

Total RNA was isolated from the TRIzol™ extracts using the standard procedure from the manufacturer. O.D. at 260 nm determined RNA concentrations. RNA quality was accessed using an Agilent Technologies bioanalyzer before proceeding to microarray sample preparation. Microarray analysis was performed using the standard protocol provided by Affymetrix Inc. (Santa Clara, CA) and as previously described, starting with 5 μg of total RNA (Richert et al., 2006).

Microarray analysis

Fragmented, labeled cRNA was hybridized to an Affymetrix human genome U133A array, which contains sequences corresponding to roughly 22,200 transcripts at 45°C overnight. The arrays were washed, developed, and scanned. The microarray scanned image and intensity files were imported in Rosetta Resolver™ gene expression analysis software version 6.0 (Rosetta Inpharmatics, Kirkland, WA). Resolver's Affymetrix error model was applied and ratios were built for each treatment array versus its respective control. Agglomerative or divisive cluster analysis was performed using the average link heuristic criteria (agglomerative only) and the Euclidean distance metric for similarity measure. Some microarray data were also evaluated using the Panther Classification system (Thomas et al., 2003). The data discussed in this publication have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE9166.

Results

Cytotoxicity Assay

Using an MTS cytotoxicity assay (Figure 1A) of the various fluoroquinolones on HepG2 cells (at 24 hours), all of the drugs started to show some evidence of cytotoxicity beginning at 300 μM. An approximate rank order of the relative cytotoxicity of the drugs was levofloxacin (least cytotoxic), gatifloxacin, ciprofloxacin, clinafloxacin,

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trovafloxacin, and grepafloxacin (most cytotoxic). From these data, drug doses for the microarray studies were selected where the cells were at least >80% viable (100 μM). In this manner, any genomic artifacts due to cytotoxicity could be minimized and any potential drug-specific mechanistic effects would be more pronounced. For PHH, cytotoxicity was first observed at 800 μM for TVX, but at this dose, the other quinolones showed no appreciable loss in cell viability. Therefore, in this study, the drugs were dosed at a level (400 μM), which was previously useful for evaluating potential mechanistic effects in PHH (Liguori et al., 2005).

Expression profiles of naïve HepG2 cells vs. PHH

The expression profiles of untreated HepG2 cells and PHH were examined to establish the overall basal similarities and differences between the two cell types. Figure 1B is a plot of gene expression intensity from one donor of PHH versus that of HepG2 cells. 4306 probe sets ($p \leq 0.01$) were differentially regulated between the two cell types. 2004 of these were up-regulated in the HepG2 cells relative to PHH, and 2302 were down-regulated in the HepG2 cells relative to PHH. Many of these probe sets were modulated greater than 2-fold between the cell types. The expression level of several major cytochrome P450 monooxygenases was lower in HepG2 cells relative to PHH, consistent with prior observations (Rodriguez-Antona et al., 2002; Wilkening et al., 2003; Westerink and Schoonen, 2007 (A)). These included CYP1A1 (reduced 22.3-fold), CYP3A5 (reduced 78.9-fold), CYP2E1 (reduced 27.6-fold), and CYP2C9 (reduced 21.8-fold). Additionally, several phase II metabolizing enzymes, including UGT1A6, UGT2B15, and UGT1A6 were markedly down-regulated while others, especially members of the sulfotransferase family had heightened levels of expression in HepG2 cells.

Other transcripts that code for proteins critical for proper maintenance of the cell cycle were differentially expressed between the cell types. For example, cyclin E1 (induced 31.7-fold), cell division cycle 2 (induced 48-fold), cell division cycle associated 8 (induced 25.7-fold), and cyclin A2 (induced 16.6-fold) were all expressed at a greater level in HepG2 cells vs. PHH. The mRNAs from several histones, which are essential to

proper chromatin organization, were greatly reduced in HepG2 cells, including Histone1, H2bk (reduced 100-fold), H2B histone family, member S (reduced 32.2-fold), and Histone 2, H2aa3 (reduced 6.1-fold). A complete list of transcripts differentially regulated between HepG2 cells and PHH as shown in Figure 1B is included in a supplementary table.

These data suggest that the global transcriptome of the HepG2 cells varies considerably from that of primary hepatocytes. Transcripts present in over-abundance in HepG2 cells included many associated with cell cycle regulation and checkpoint control, regulation of transcription, DNA, RNA, and nucleotide metabolism, transport, and cell surface-linked signal transduction. Those present in diminished levels included many genes associated with cell death, lipid metabolism, and xenobiotic metabolism.

Gene expression changes in HepG2 cells induced by quinolone treatment

Clearly, much transcriptomic variation exists between HepG2 cells and isolated human hepatocytes, but it also would be beneficial to determine effects of drug treatment on gene expression for each cell type. Therefore, we sought to identify gene expression alterations after exposure to a panel of fluoroquinolones. This class of antibiotics on human hepatocytes has been previously studied in our laboratory because of the link between one member of the class (trovafloxacin) and idiosyncratic hepatotoxicity (Bertino and Fish, 2000; Liguori et al., 2005). Trovafloxacin (TVX) has previously been shown to induce a unique mRNA profile than other quinolones in PHH, stemming from a significantly larger number and magnitude of gene expression changes.

To evaluate whether TVX may have a similar effect on HepG2 cells, we exposed these cells to TVX and 5 related quinolones for 24 hours and then analyzed the resulting genomic profiles of treated cells relative to their respective vehicle controls (Figure 2A). Agglomerative cluster analysis revealed distinct expression profiles for the TVX-treated HepG2 cells compared to the other quinolones examined, similar to the prior result observed with the PHH. Likewise, the treatment of other fluoroquinolones resulted in only mild alterations in gene expression as previously observed with PHH. Of these,

only grepafloxacin treatment showed any notable gene expression changes, which is also in agreement with the observations with PHH.

We compared the gene expression changes in HepG2 cells following TVX treatment with those induced by troglitazone in rat hepatocytes, another drug associated with idiosyncratic liver toxicity, as determined by Guo et al. (Guo et al., 2006). Of the 33 cell death-related genes that were differentially expressed by troglitazone versus relevant negative control compounds, slightly more than half (17) of these genes were also uniquely regulated by TVX (as listed in Table 1).

Comparison of HepG2 cells and PHH quinolone-induced gene expression changes

Using agglomerative cluster analysis, we next compared the quinolone-induced gene expression profiles from HepG2 cells with those from PHH (Figure 2B). Two distinct major clusters were apparent for the two cell types for most of the compounds examined. However, TVX-treated HepG2 cell expression profiles clustered closer to the TVX-treated PHH and away from HepG2 cells treated with other quinolones. Irrespective of cell type, TVX treatment resulted in unique mRNA profiles compared to the other quinolone compounds, which are associated with a low risk of hepatotoxicity in humans. While the majority of probe sets were regulated differently, small areas of similarity in gene expression were also observed between TVX-treated HepG2 cells and PHH.

The differences in expression were more remarkable when a cluster analysis was performed focusing only on TVX-treated HepG2 cells and PHH from two different donors (Figure 2C). With this type of analysis, two major clusters were formed which were linked to the underlying cell type. Together encompassing HepG2 cells and PHH, TVX treatment resulted in almost 5900 gene expression changes out of the approximately 22,000 transcripts on the array. These perturbations were reproducible between donors of PHH and between preparations of HepG2 cells.

To determine whether the TVX-induced gene expression changes were truly distinct from the other quinolones evaluated, rather than due to artifacts from dosing, we next elevated the doses of ciprofloxacin, gatifloxacin, and levofloxacin to between 500-

800 μM in HepG2 cells. Figure 3A is a cluster analysis using a multidimensional scaling algorithm in Rosetta Resolver™ showing that the TVX-gene expression profiles in both cell types are distinct from those induced by high doses of ciprofloxacin, gatifloxacin, and levofloxacin. Also, apparent from this analysis is that TVX-treated HepG2 cells and PHH clustered closely together and away from the other quinolones evaluated. The analysis also was completed using an agglomerative hierarchical clustering algorithm as depicted in Figure 3B. In this manner, the HepG2 expression level of individual genes induced by the high dose quinolones could be directly compared to those from the TVX-treated HepG2 cells. The resulting profiles associated with the high doses of ciprofloxacin, gatifloxacin, and levofloxacin were clearly distinct from the TVX-induced profiles.

ANOVA methods to discern similarities and differences in expression

Using the Rosetta Resolver™ ANOVA function, differences in gene expression between distinct groups can be identified. One-way ANOVA (using a $p \leq 0.0001$ for the ANOVA to find probe sets of interest) designated 2025 probe sets that were differentially modulated between the TVX-treated HepG2 cell group and the PHH group (Figure 4A). Some of the differences were due to an anti-correlation in expression between groups, whereas a greater number were due to genes that were regulated solely in one group. Again, these ANOVA-identified probe sets were regulated similarly despite the differences in donor for the PHH or the preparation of HepG2 cells. Some major biological function gene ontologies and pathways for these ANOVA-identified probe sets are listed in Table 2A and B.

Some individual transcripts that were regulated dissimilarly by TVX between the two cell types included helicase, lymphoid-specific (repressed in HepG2 cells but unchanged in PHH), which is thought to be involved with cellular proliferation (Lee et al., 2000). Others include Cyr61 (induced by TVX 20-100-fold in HepG2 cells but unchanged in PHH), a heparin-binding protein that oversees a diverse range of critical cellular functions including cell survival, adhesion, and differentiation (Wang et al., 2005). GADD45B, a stress response factor and cell death regulator (Major and Jones,

2004), was induced, on average, over 12.6-fold in HepG2 cells vs. 2.5-fold in PHH. The proinflammatory cytokine, IL-8, a critical factor of hepatocyte repair subsequent to injury (Gomez-Quiroz et al., 2005), was induced (9.4-fold) by TVX in HepG2 cells. However, its expression was repressed in TVX-treated PHH.

Although differences in TVX-induced gene expression profiles were predominant for HepG2 cells and PHH, 929 probe sets were found that were transcribed similarly between HepG2 cells and PHH (Figure 4B), which were associated with diverse gene ontologies and pathways (Table 3A and B). Topoisomerase 1, which is responsible for adjusting the torsional state of DNA and is critical for the balance between the life and death of the cell (Kang et al., 2004), is an example of a gene that was potentiated comparably (induced ~2-fold) between these two cell types. Metallothionein 2A, 1X, and 1H, members of a family of proteins whose over-expression has been linked to increased cell death (Li et al., 2005), were induced by TVX treatment in both PHH and HepG2 cells. BCL2-associated transcription factor 1, which promotes activation of apoptotic activity (Kasof et al., 1999), was also over-expressed in both types of cells.

Comparison of a TVX-Specific Gene Expression Profile in HepG2 cells vs. PHH

In our previous investigation of the TVX-induced effects on PHH, we identified 142 probe sets whose expression modulation was unique to TVX treatment and was not associated with treatment by any other quinolones (Liguori et al., 2005). Thus, it was considered plausible that some of these probe sets ultimately could be linked with the toxic outcome. Here, we questioned whether the HepG2 cell line would also uniquely regulate this set of sequences upon TVX treatment. Figure 5 shows that the HepG2 cell line regulated this set of genes very analogously to its PHH counterparts, which may provide further insight into the mechanism of TVX-induced hepatotoxicity. Furthermore, the other quinolones tested in HepG2 cells failed to regulate the 142-member set, again consistent with the observation made using PHH. Additionally, TVX-treatment regulated key pathways, such as RNA processing and mitochondrial apoptosis, similarly between HepG2 cells and PHH. Thus, despite the numerous differences in TVX-induced

expression profiles between the cell types, it was clear that the regulation of a critical set of genes was similar.

Discussion

The HepG2 cell line is used extensively in ADME, toxicological, and other basic research, and thus an understanding of the genomic responses of these cells is paramount to enhanced interpretation and assessment of data generated with these cells. In this report, we have sought to study the native transcriptome of HepG2 cells as well as to characterize genomic changes upon exposure to several fluoroquinolone antibiotics. Approximately 4300 gene expression changes were evident between the cell types, some of which may explain some of their phenotypic differences. Not surprisingly, since HepG2 cells are immortalized, many genes associated with cell cycle and checkpoint control (for example, cyclin A1 and cyclin E2) were differentially regulated between PHH and HepG2 cells, which is in agreement with other studies (Harris et al., 2004). Other notable gene categories that were significantly different between these cell types were involved in xenobiotic metabolism, cell death, transport, and lipid metabolism. Viewing gene expression intensities between the two cell types, it was evident that there were many differences between HepG2 cells and PHH, a factor that may assist in the interpretation of data from other HepG2 studies.

Another characteristic of the HepG2 cell line is the lowered level of Phase I drug metabolizing enzymes such as the CYPs. The results presented here, in agreement with other studies, showed lowered levels of cytochrome P450 transcripts in HepG2 cells as compared to human hepatocytes (Rodriguez-Antona et al., 2002). Our results provide additional evidence that the lowered level of CYPs in HepG2 cells is most likely due to decreased transcription of CYP mRNA, rather than to protein or mRNA degradation. Thus, the lowered levels of CYPs could be linked to changes in levels or to biochemical modification of transcription factors that govern the expression of the CYPs. Although the mechanism for the reduced transcription of CYPs remains unclear, it is thought that it

may be associated with alterations in the levels of HNF-1, 3, 4, and C/EBP and other key liver enriched transcription factors (Cereghini, 1996; Jover et al., 2001; Rodriguez-Antona et al., 2002). Indeed, CEB/P beta, delta, and gamma were all under-expressed in HepG2 cells relative to PHH, and therefore, consistent with the prior hypotheses.

Other authors have described differences from PHH and HepG2 cells in gene expression responses to chemicals such as styrene, styrene-7, 8-oxide, and aflatoxin B_1 (Harris et al., 2004; Diodovich et al., 2006). Given the difference in effects, we focused on a series of fluoroquinolone compounds that had already been studied comprehensively in PHH (Liguori et al., 2005) and sought to identify the compounds' effect on HepG2 cells. One member of this class, trovafloxacin, has been associated with idiosyncratic hepatotoxicity (Bertino and Fish, 2000). In PHH, TVX was clearly shown to affect the genomic profiles more extensively than any other of the quinolones tested, which may partially relate to its hepatotoxicity potential in humans.

Here, the TVX effect on HepG2 cells was reminiscent to that seen with PHH, yielding unique gene expression profiles compared to the other quinolones examined. The modulation in expression for the other quinolones (at 100 µM levels) examined was relatively mild, implying that these compounds have a lessened impact on hepatocyte and possibly, intact liver physiology. Overall the gene expression profiles for TVX were consistent between different preparations, but some differences were also apparent. These differences were relatively small in number. An ANOVA ($p<0.0001$) was conducted to quantify the differences between the TVX HepG2 cell preparations, and of the 4344 total expression changes (with fold change at least +/- 2.0 and $p\leq0.01$ in at least one sample), only 357 probe sets (8.2%) were considered differentially regulated. While the differences were relatively minor, these results stress the importance of designing biological replicates into these types of experiments to monitor any potential variation. Furthermore, it would be interesting in future studies to determine the degree of variation in the TVX induced gene expression observed as HepG2 cells age in culture and after increased time of exposure (>24hrs.) to TVX.

Significantly, the TVX-induced gene expression profiles in HepG2 cells continued to cluster independently to those from other less hepatotoxic fluoroquinolones dosed at higher levels (500 µM ciprofloxacin and 800 µM levofloxacin and gatifloxacin).

This suggests that the TVX-induced gene expression profiles were truly distinctive to TVX-treatment and to the cell line and not due to dosing effects. This notion was verified by comparing the expression of individual probe sets from the high dose non-IDR inducing fluoroquinolones to those from TVX. While a sparse number of genes were expressed similarly between these treatment groups, the overall profiles are quite distinct. To compare the gene expression overlap of these treatments, a Venn diagram was examined, consisting of the genes regulated consistently by TVX in Figure 4B and the genes regulated consistently (in 2 of 3 treatments) by the high dose non-IDR inducing quinolones (data not shown). Of the 929 probe sets from Figure 4B, only 118 (12.7%) overlap between these treatment groups, which is more evidence of a unique TVX effect on these cells.

Another hepatic idiosyncratic drug reaction (IDR) drug, troglitazone, has similarly been shown to induce a unique set of gene expression changes in rat hepatocytes and to induce a greater number of gene expression changes than a set of negative control compounds (Guo et al., 2006). In an attempt to identify potential commonalities in the effects of IDR-inducing drugs on liver cells, it may prove worthwhile to inspect the similarities between distinct IDR-causing drugs, and therefore, potentially identify and characterize concordances in mechanism(s) (if any). Indeed, many of the transcripts identified as being regulated uniquely by troglitazone versus its negative controls (Guo et al., 2006) were also regulated uniquely by TVX in HepG2 cells. Hence, these results point to the utility of HepG2 cells as one potential model in the study of hepatic IDRs, despite the basal transcriptomic variation.

It was of interest to characterize some of the TVX-induced expression differences between PHH and HepG2 cells. We found over 2000 probe sets that were divergently modulated between the HepG2 cell line and PHH. As shown in Table 2A and B, the most significant of these were associated with cell cycle control, including cyclins, cyclin-dependent kinases, minichromosome maintenance deficient homologs, aurora kinases, and protein phosphatases. Many of these transcripts were repressed by TVX in HepG2 cells, but not significantly perturbed in PHH. Thus, genes that are regulated distinctively by TVX between cell types may be inherently related to the phenotypical variations associated with cell immortalization. Alternatively, these observations may be

related to the finding that TVX can repress cellular proliferation in some cell types, and the effects on cell cycle control expression may be reflective of this condition (Holtom et al., 2000; Zakeri et al., 2000). Since PHH do not replicate in culture, these cells may be affected less by TVX at the level of cell cycle control.

Levels of inflammation associated genes, such as IL-8, were also identified as being disparate between the cell types. Inflammation has been previously connected to the hepatic IDR-causing potential of TVX in vivo in the rat (Liguori and Waring, 2006; Waring et al., 2006). IL-8 is an important response factor in hepatic injury, and its expression is typically stimulated upon insult (Gomez-Quiroz et al., 2005). For the PHH donors evaluated here, IL-8 expression was down-regulated almost 2-fold, on average, in response to TVX. For the HepG2 cells, it was over-expressed approximately 9.4-fold. In vivo and in isolated PBMCs, TVX has diminished IL-8 levels (Purswani et al., 2000). The reason for the discrepancy in IL-8 production upon TVX exposure to these cell types is unclear. The observation is further complicated by the suggestion that TVX can diminish IL-8 transcription due to its cross-reactivity to and inhibition of mammalian topoisomerases (Purswani et al., 2000). Perhaps, an inflammation mediated reaction is regulated by distinct factors for these two cell types, and such a reaction may be complicated by the fact that inflammatory responses are usually governed by an intact immune system, which is absent in these in vitro cell systems. Another possibility stems from the actuality that PHH were still in the presence of an intact immune system several days prior to isolation, while the HepG2 cells were not, which may also influence differential inflammatory-mediated gene expression between the cell types.

Despite the expression differences between TVX-dosed PHH and HepG2 cells, a critical set of genes, previously identified as potentially associated with TVX hepatotoxicity, displayed similar expression profiles between the cell types. Hierarchical cluster analysis reveals that, with this 142 select set of genes, TVX-treated HepG2 clustered with TVX-treated PHH and away from other quinolone-treated PHH and HepG2 cells (data not shown). This observation provides additional evidence that these genes may have a role in TVX-specific effects, and also shows that HepG2 cells can respond comparably to normal hepatocytes, and for some instances can serve as a useful cell system to understand certain toxicological effects.

In this TVX-specific sequence set, several genes were associated with critical cellular processes such as RNA processing and mitochondrial function and were thus discussed as involved in the hepatotoxic response from TVX. Superimposing the gene expression of the RNA processing pathway between the two cell types yielded a pattern of noticeable concordance, suggesting that TVX-treatment may result in potentially deleterious effects on RNA processing in both types of cells. Interestingly, for the genes regulated similarly by TVX between PHH and HepG2 cells, the most significant gene ontologies are those associated with RNA processing and metabolism (Table 3A). In our previous studies with PHH, RNA metabolism was also identified as one of the most significantly impacted pathways upon TVX-treatment (Liguori et al., 2005).

TVX similarly regulated genes associated with mitochondrial function between PHH and HepG2 cells. One example was mitofusin 1, which is localized to the mitochondrial membrane where it partakes in regulation of proper mitochondrial fusion events and is critical to the lasting viability of the cell (Rojo et al., 2002). Mice with knocked down levels of mitofusins failed to progress in development past the embryo stage (Chen et al., 2003). TVX treatment has been shown to initiate oxidative stress and also can subdue the expression of mitofusin 1, both of which may result in the eventual dysfunction of the mitochondria (Liguori et al., 2005). Similarly, the level of mitofusin 1 was reduced in both PHH and in some samples of HepG2 cells ($p<0.06$), implying a similar TVX-related impact on mitochondria. A more global view of TVX effects on mitochondrial-related genes identified a large number that are analogously regulated between HepG2 cells and PHH, including ACADVL, TFAM, and MYCBP, which is again consistent with a related mitochondrial impact.

In this communication, we have extensively compared the genomic profiles of HepG2 cells and PHH at the basal level and after exposure to a panel of fluoroquinolones. The results revealed an extensive network of genes, which were regulated differentially in the native state of each cell. TVX treatment of each type of cell analogously resulted in an enhanced effect on the transcriptome relative to a panel pharmacologically and structurally related compounds. A set of genes, previously identified as potentially related to the hepatotoxic mechanism of TVX, were modulated similarly between the cell types, which may suggest some similarities in TVX-related

effects. Overall, these results could aide in the interpretation of some types of ADME, toxicity, and other data derived from HepG2 cells. As technology in ADME progresses toward the generation of hepatic-like cells from stem cell progenitors or in immortalization of hepatocytes into cell lines (Hengstler et al., 2005), a comparison of transcriptomes both before and after xenobiotic exposure may assist in the translation of effects between these new cell types and the original hepatocyte or liver.

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Legends to Figures:

Figure 1 (A) MTS cytotoxicity assay to measure approximate relative levels of cell death following exposure of HepG2 cells to various fluoroquinolones for 24 hrs. A plot of percent vehicle vs. concentration (μM) is displayed. Each quinolone is represented by a different colored line as listed in the legend. Each data point is an average of triplicate wells with error bars representing ± SEM (B). Gene expression differences between untreated primary human hepatocytes and HepG2 cells. A plot of log (intensity) of each individual probe set from the array for HepG2 cells versus PHH is shown. At a p-value of 0.01 or less, genes expressed at higher levels in HepG2 cells are represented by the red dots and those at lower levels are represented by the green dots. Blue dots indicate that there is no significant difference in expression between cell types. The red lines show the 2X-fold change cutoff range.

Figure 2. (A). Agglomerative cluster analysis of gene expression changes (Fold change of at least +/- 2.0 and p-value of 0.01 or less) upon treatment of HepG2 cells for 24 hrs. with various quinolone compounds. Individual probe sets are displayed on the vertical axis and each individual treatment is on the horizontal axis. Shades of red indicate increased expression relative to the vehicle treatment, and shades of green indicate decreased expression. Black indicates no statistically significant change in expression. Both the genes and experiments were clustered with the agglomerative method using the average link heuristic criteria and using cosine correlation for the similarity measure. 4646 individual probe sets are displayed. Two separate cell culture preparations of the HepG2 cells are presented for TVX-treatment.

(B). Comparison of gene expression profiles of HepG2 cells and PHH treated with various quinolone compounds. Clustering was performed as described above. Genes represented were regulated with a fold change of at least +/- 2.0

and p-value of 0.01 or less in at least one experiment. 5922 individual probe sets are displayed.

(C). Comparison of TVX-induced gene expression profiles for HepG2 cells and PHH. Genes represented were regulated with fold change of at least +/- 2.0 and p-value of 0.01 or less in at least one experiment. 5872 individual probe sets are displayed. PHH were from two separate donors.

Figure 3. (A). Dose response effects on gene expression profiles induced by treatment with ciprofloxacin, gatifloxacin, levofloxacin, or TVX. A multidirectional scaling algorithm using three dimensions was applied to reduce the gene expression data for each individual treatment to a single data point for increased ease in comparison (only for probe sets that were regulated at least +/- 2-fold with p-value of 0.01 or less in at least one experiment). The points are color coded by compound ID. Individual concentrations are labeled with arrows. TVX separates from the other quinolones evaluated irrespective of dose. (B). The same analysis as depicted in (A) except agglomerative cluster analysis was used to compare the gene expression profiles which also reveals a separation of TVX from the other quinolones.

Figure 4. (A). Genes regulated differently after TVX-treatment between HepG2 cells and PHH. One-way ANOVA was performed (ANOVA p-value of 0.0001 or less) to identify probe sets regulated divergently by the two cell types. 2025 probe sets met these criteria, and the genes were plotted in a heat map format.

(B). TVX-induced gene expression changes in common between HepG2 cells and PHH. 929 genes were identified that were regulated similarly between 2 donors of PHH and 2 separate preparations of HepG2 cells. These were plotted in a heat map format to show similarity in expression profiles (Fold change at least +/- 2-fold; p-value 0.01 or less).

Figure 5. 142 Probe Sets Uniquely Regulated by TVX: Comparison of PHH and HepG2. Previously, these probe sets were identified as being specific to TVX-treatment in

4 donors of PHH (Liguori 2005). Therefore, these genes were selected to compare to TVX-induced expression changes in HepG2 cells. A heatmap is displayed with the 142 TVX-specific probe sets on the vertical axis and individual treatments on the horizontal axis. Genes shown were regulated with a p-value of 0.05 or less.

Table 1. List of cell death-related genes differentially expressed by troglitazone (Guo et al.) and trovafloxacin.

Probe Set	Sequence Name	Sequence Description
202672_s_at	ATF3	Activating transcription factor 3
222343_at	BCL2L11	BCL2-like 11 (apoptosis facilitator)
210563_x_at	CFLAR	CASP8 and FADD-like apoptosis regulator
201428_at	CLDN4	Claudin 4
201693_s_at	EGR1	Early growth response 1
212373_at	FEM1B	fem-1 homolog b
202949_s_at	FHL2	Four and a half LIM domains 2
209189_at	FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog
203665_at	HMOX1	Heme oxygenase (decycling) 1
200800_s_at	HSPA1A	Heat shock 70kDa protein 1A
202581_at	HSPA1B	Heat shock 70kDa protein 1B
205302_at	IGFBP1	Insulin-like growth factor binding protein 1
205945_at	IL6R	Interleukin 6 receptor
31637_s_at	NR1D1	Nuclear receptor subfamily 1, group D, member 1
217998_at	PHLDA1	Pleckstrin homology-like domain, family A, member 1
218194_at	REXO2	REX2, RNA exonuclease 2 homolog
217591_at	SKIL	SKI-like oncogene

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Table 2A. Evaluation of genes regulated divergently by TVX (from ANOVA) between HepG2 cells and PHH for major biological process GOs(Using Panther Classification System- see M/M).

Biological Process	NCBI: H. sapiens genes - REFLIST (25431)	Uploaded ANOVA Genes (2025)	No. of Genes Expected	over/under	P-value
Biological process unclassified	11321	423	728.74	-	1.11E-53
Nucleotide and nucleic acid metabolism	3343	352	215.19	+	2.81E-19
Cell cycle	1009	144	64.95	+	8.54E-17
Protein metabolism and modification	3040	305	195.69	+	1.32E-13
Protein phosphorylation	660	98	42.48	+	1.81E-11
Protein modification	1157	141	74.48	+	1.54E-10
Other metabolism	559	79	35.98	+	7.05E-09
Cell cycle control	418	64	26.91	+	8.46E-08
Intracellular protein traffic	1008	115	64.89	+	1.74E-07
Pre-mRNA processing	291	49	18.73	+	4.76E-07
Sensory perception	506	7	32.57	-	1.76E-06
Intracellular signaling cascade	871	101	56.07	+	3.29E-06
Mitosis	382	55	24.59	+	9.83E-06
G-protein mediated signaling	834	20	53.68	-	1.70E-05
DNA metabolism	360	51	23.17	+	4.66E-05
mRNA splicing	214	36	13.78	+	7.67E-05
Cell adhesion-mediated signaling	379	4	24.4	-	7.73E-05
Stress response	200	33	12.87	+	2.52E-04
Cell communication	1213	43	78.08	-	1.02E-03
Apoptosis	531	60	34.18	+	1.02E-03
Lipid, fatty acid and steroid metabolism	770	79	49.57	+	1.66E-03
DNA repair	169	28	10.88	+	1.88E-03
Carbohydrate metabolism	592	64	38.11	+	2.00E-03
Amino acid metabolism	230	32	14.81	+	2.03E-03
Oncogenesis	472	53	30.38	+	3.33E-03
General vesicle transport	251	35	16.16	+	4.24E-03
Protein folding	186	28	11.97	+	7.22E-03
mRNA transcription	1914	167	123.2	+	7.64E-03
Cell surface receptor signal transduction	1638	69	105.44	-	9.35E-03
Phosphate metabolism	117	19	7.53	+	9.50E-03
Cell adhesion	622	20	40.04	-	9.82E-03
Cell proliferation and differentiation	1028	93	66.17	+	2.61E-02
Antioxidation and free radical removal	38	10	2.45	+	3.34E-02
Purine metabolism	65	13	4.18	+	5.90E-02
Amino acid catabolism	50	11	3.22	+	7.43E-02
Cell structure and motility	1148	99	73.9	+	7.69E-02
Neuronal activities	569	21	36.63	-	1.05E-01
Cytokinesis	115	18	7.4	+	1.31E-01
Protein targeting and localization	253	28	16.29	+	1.53E-01
Other intracellular signaling cascade	225	28	14.48	+	1.95E-01
Protein targeting	113	17	7.27	+	2.02E-01
Endocytosis	277	32	17.83	+	2.14E-01
DNA replication	155	21	9.98	+	2.16E-01
RNA localization	33	8	2.12	+	2.29E-01

Table 2B. Pathway Analysis of genes regulated divergently by TVX (from ANOVA) between HepG2 cells and PHH (Using Panther Classification System- see M/M).

Pathway	NCBI: H. sapiens genes - REFLIST (25431)	Uploaded ANOVA Genes (2025)	No. of Genes Expected	over/under	P-value
Unclassified	22565	1340	1452.51	-	2.64E-14
Ubiquitin proteasome pathway	89	23	5.73	+	5.75E-06
PDGF signaling pathway	189	32	12.17	+	2.05E-04
Apoptosis signaling pathway	131	25	8.43	+	3.75E-04
p53 pathway	136	25	8.75	+	7.07E-04
FGF signaling pathway	148	25	9.53	+	2.84E-03
EGF receptor signaling pathway	150	25	9.66	+	3.53E-03
Parkinson disease	106	20	6.82	+	4.18E-03
Huntington disease	172	27	11.07	+	4.72E-03
T cell activation	111	20	7.15	+	7.80E-03
Angiogenesis	229	32	14.74	+	8.45E-03
Ras Pathway	91	17	5.86	+	1.79E-02
p53 pathway feedback loops 2	66	14	4.25	+	1.93E-02
Cell cycle	29	9	1.87	+	1.98E-02
TGF-beta signaling pathway	154	23	9.91	+	3.49E-02
Insulin/IGF pathway-MAP kinase cascade	49	10	3.15	+	2.19E-01
B cell activation	86	14	5.54	+	2.45E-01
Oxidative stress response	69	12	4.44	+	2.98E-01
Toll receptor signaling pathway	71	12	4.57	+	3.75E-01
Circadian clock system	16	5	1.03	+	5.77E-01
5-Hydroxytryptamine degredation	17	5	1.09	+	7.42E-01
Cholesterol biosynthesis	11	4	0.71	+	8.36E-01
Axon guidance mediated by netrin	40	5	2.57	+	1.00E+00
Axon guidance mediated by Slit/Robo	35	3	2.25	+	1.00E+00
Axon guidance mediated by semaphorins	46	5	2.96	+	1.00E+00
Alzheimer disease-presenilin pathway	143	9	9.2	-	1.00E+00
Alzheimer disease-amyloid secretase pathway	77	3	4.96	-	1.00E+00
Adrenaline and noradrenaline biosynthesis	30	4	1.93	+	1.00E+00
mRNA splicing	11	1	0.71	+	1.00E+00

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Table 3A. Evaluation of genes regulated similarly by TVX (from Fig. 4B) between HepG2 cells and PHH for major biological process GOs (Using Panther Classification System- see M/M).

Biological Process	NCBI: H. sapiens genes - REFLIST (25431)	Uploaded Similar Genes (929)	No. of Genes Expected	over/under	P-value
Biological process unclassified	11321	170	336.99	-	2.55E-35
Nucleoside, nucleotide metabolism	3343	219	99.51	+	1.08E-28
Pre-mRNA processing	291	39	8.66	+	3.15E-12
mRNA splicing	214	30	6.37	+	1.49E-09
Intracellular protein traffic	1008	67	30	+	5.09E-08
mRNA transcription	1914	102	56.97	+	1.64E-06
Intracellular signaling cascade	871	55	25.93	+	3.69E-05
Protein modification	1157	67	34.44	+	4.02E-05
mRNA transcription regulation	1459	79	43.43	+	6.46E-05
Protein phosphorylation	660	44	19.65	+	2.06E-04
Cell cycle	1009	53	30.03	+	2.06E-03
Stress response	200	19	5.95	+	2.06E-03
DNA repair	169	17	5.03	+	3.88E-03
Protein metabolism and modification	3040	125	90.49	+	4.14E-03
rRNA metabolism	66	10	1.96	+	5.60E-03
Nuclear transport	135	14	4.02	+	1.09E-02
Apoptosis	531	31	15.81	+	1.24E-02
DNA metabolism	360	25	10.72	+	1.71E-02
Meiosis	84	10	2.5	+	3.89E-02
Ligand-mediated signaling	421	2	12.53	-	6.10E-02
Protein targeting	113	11	3.36	+	1.04E-01
G-protein mediated signaling	834	10	24.83	-	1.11E-01
Protein targeting and localization	253	16	7.53	+	1.42E-01
Sensory perception	506	6	15.06	-	2.15E-01
Carbohydrate metabolism	592	29	17.62	+	2.24E-01
Cell structure and motility	1148	49	34.17	+	2.61E-01
Cell proliferation and differentiation	1028	44	30.6	+	3.60E-01
Mitosis	382	22	11.37	+	4.46E-01
Cell cycle control	418	23	12.44	+	6.27E-01
Other intracellular signaling cascade	225	15	6.7	+	7.34E-01
Glycogen metabolism	52	6	1.55	+	7.42E-01
Cell motility	352	20	10.48	+	7.74E-01
Amino acid activation	38	5	1.13	+	8.77E-01

Table 3B. Pathway Analysis of genes regulated similarly by TVX (from Fig. 4B) between HepG2 cells and PHH (Using Panther Classification System- see M/M).

Pathway	NCBI: H. sapiens genes - REFLIST (25431)	Uploaded Similar Genes (929)	No. of Genes Expected	over/under	P-value
Unclassified	22565	608	671.69	-	1.73E-09
Circadian clock system	16	7	0.48	+	9.95E-05
p53 pathway	136	17	4.05	+	1.67E-04
Wnt signaling pathway	349	23	10.39	+	6.24E-02
Ras Pathway	91	10	2.71	+	6.97E-02
Angiogenesis	229	17	6.82	+	9.46E-02
PDGF signaling pathway	189	15	5.63	+	9.93E-02
EGF receptor signaling pathway	150	13	4.47	+	1.00E-01
Huntington disease	172	14	5.12	+	1.16E-01
Oxidative stress response	69	8	2.05	+	1.78E-01
Aminobutyrate degradation	2	2	0.06	+	2.38E-01
FGF signaling pathway	148	12	4.41	+	2.74E-01
T cell activation	111	10	3.3	+	3.02E-01
Axon guidance mediated by semaphorins	46	6	1.37	+	3.98E-01
p53 pathway feedback loops 2	66	7	1.96	+	5.69E-01
JAK/STAT signaling pathway	23	4	0.68	+	7.42E-01
Cytoskeletal regulation by Rho GTPase	111	9	3.3	+	9.57E-01
Axon guidance mediated by netrin	40	3	1.19	+	1.00E+00
Axon guidance mediated by Slit/Robo	35	2	1.04	+	1.00E+00
Apoptosis signaling pathway	131	9	3.9	+	1.00E+00

Figure 1A.

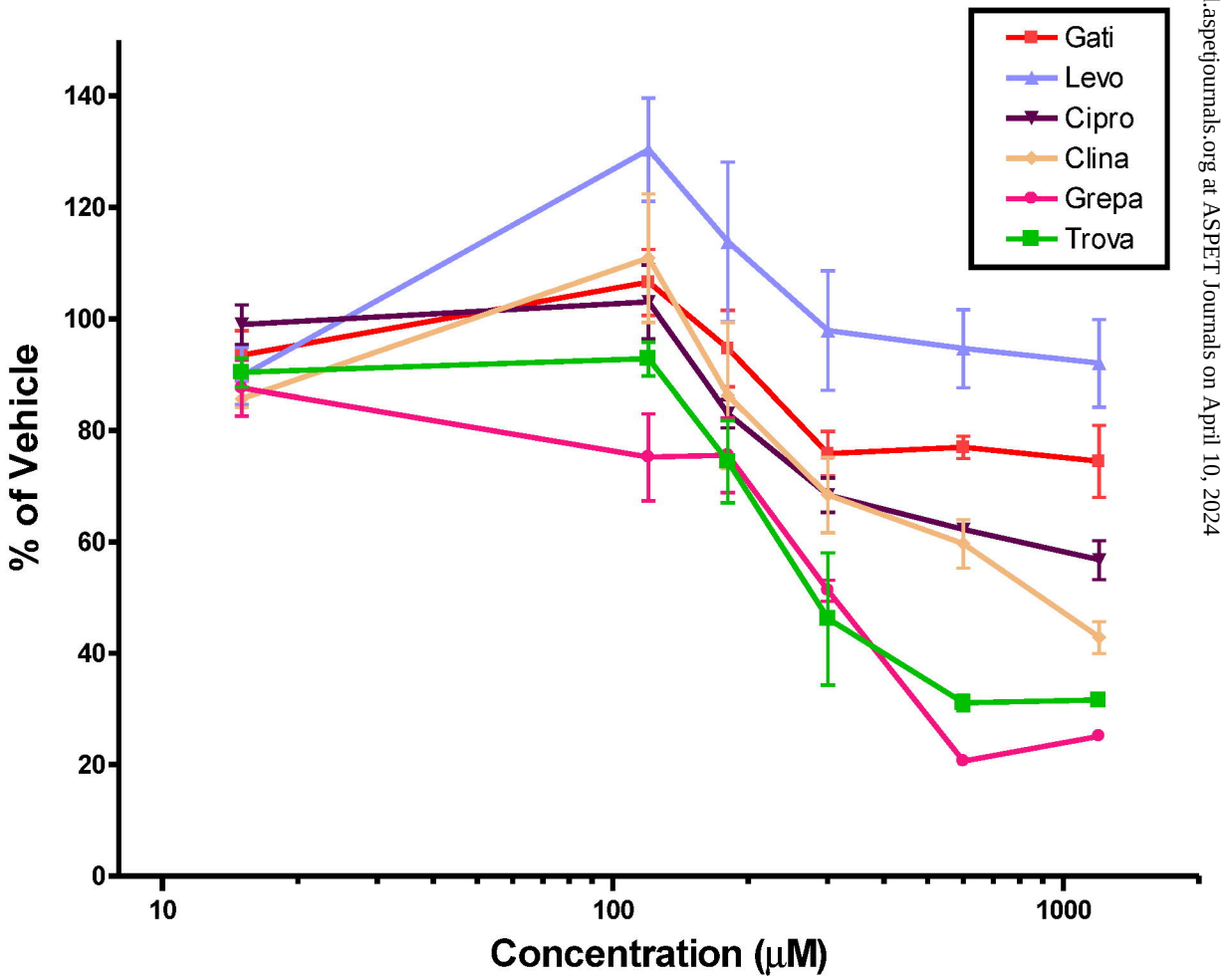


Figure 1B.

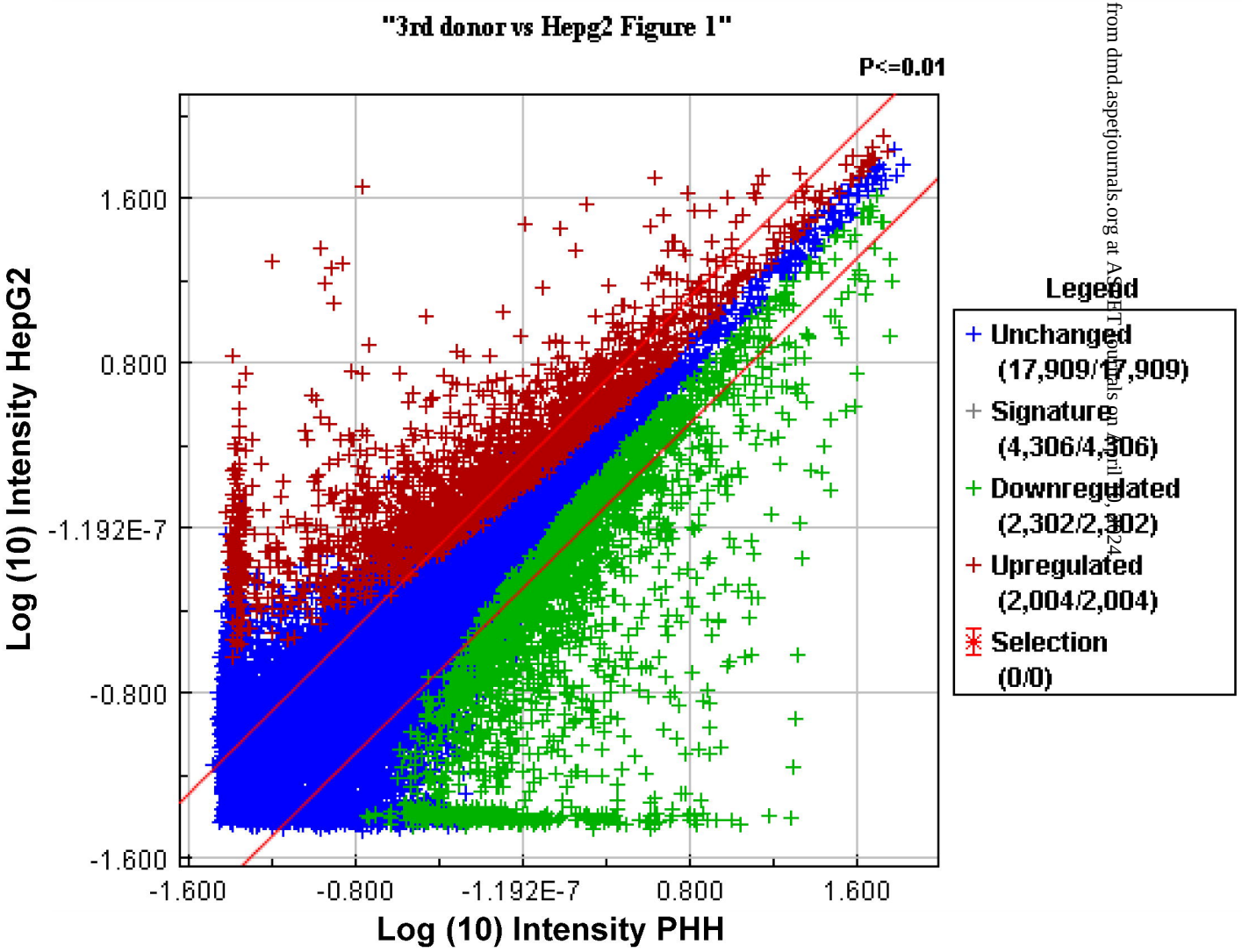


Figure 2A.

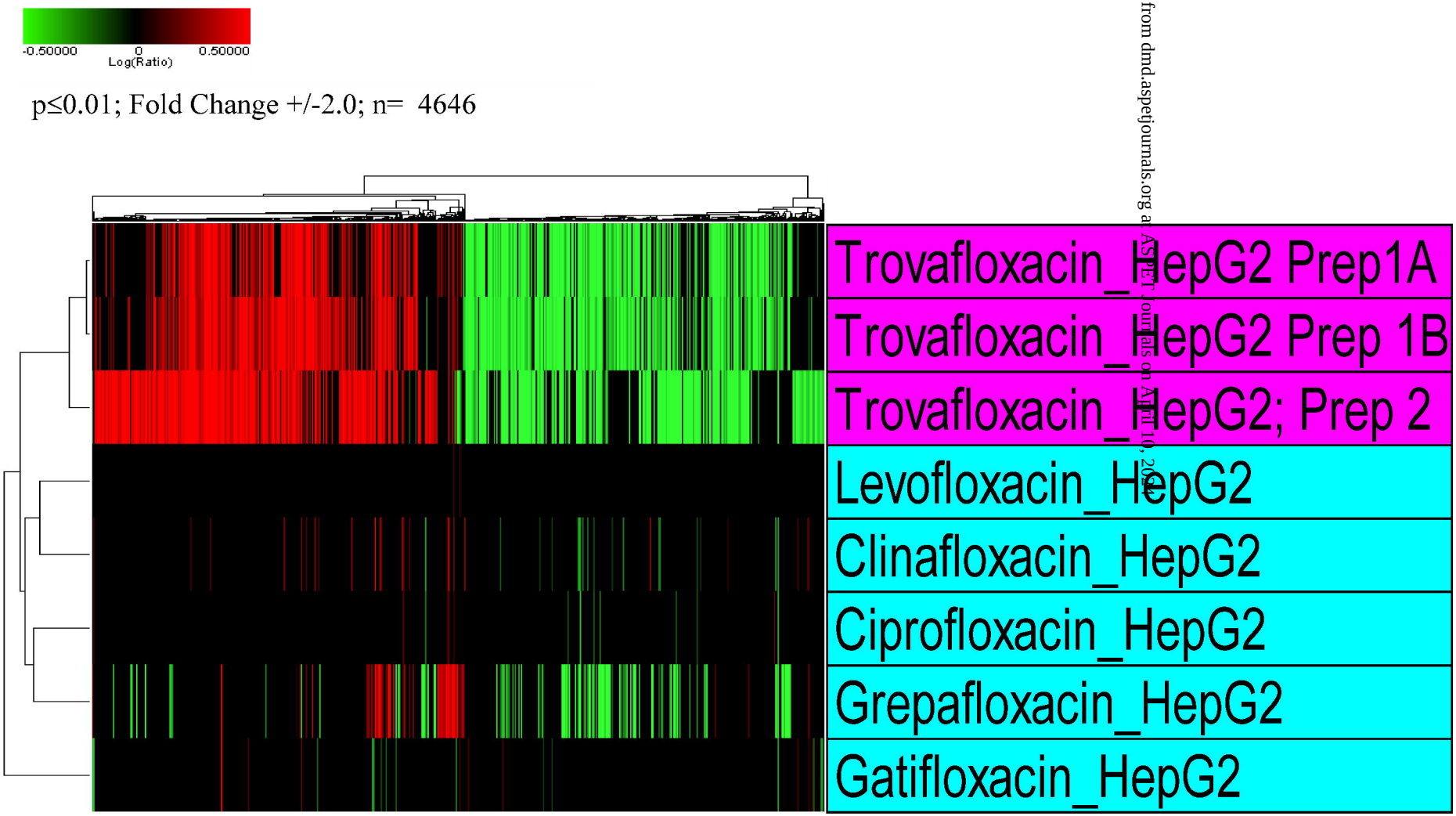


Figure 2B.

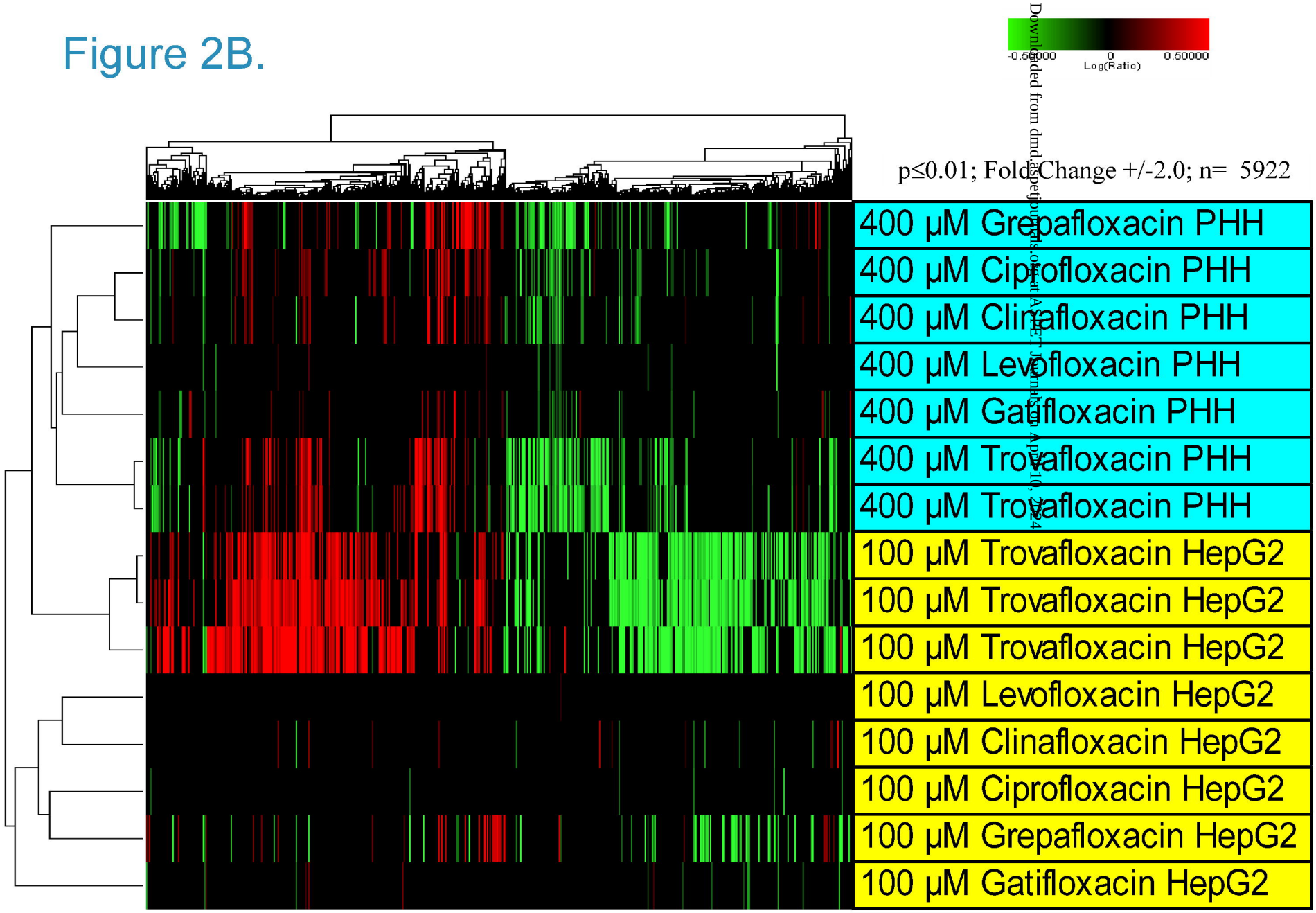


Figure 2C.

p≤0.01; Fold Change +/-2.0; n= 5872

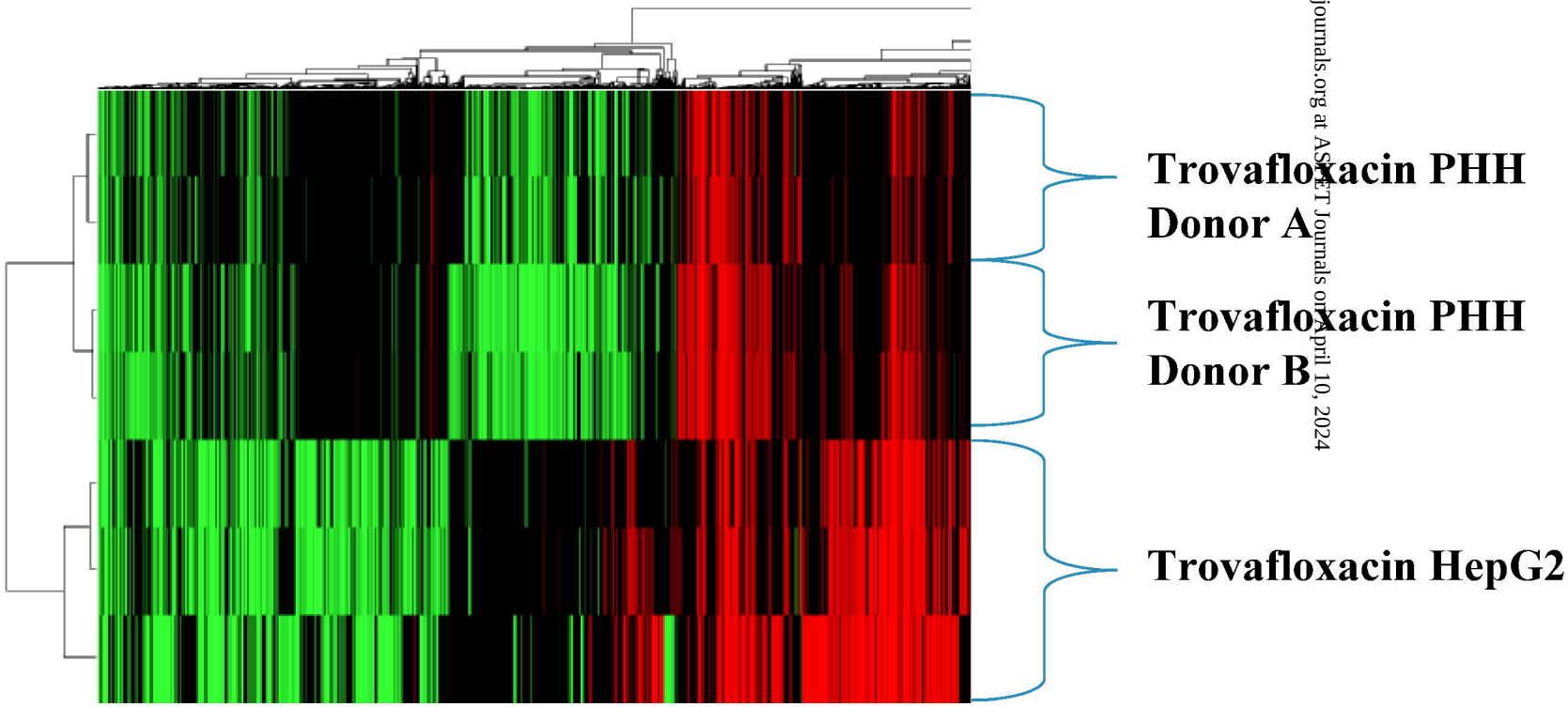


Figure 3A.

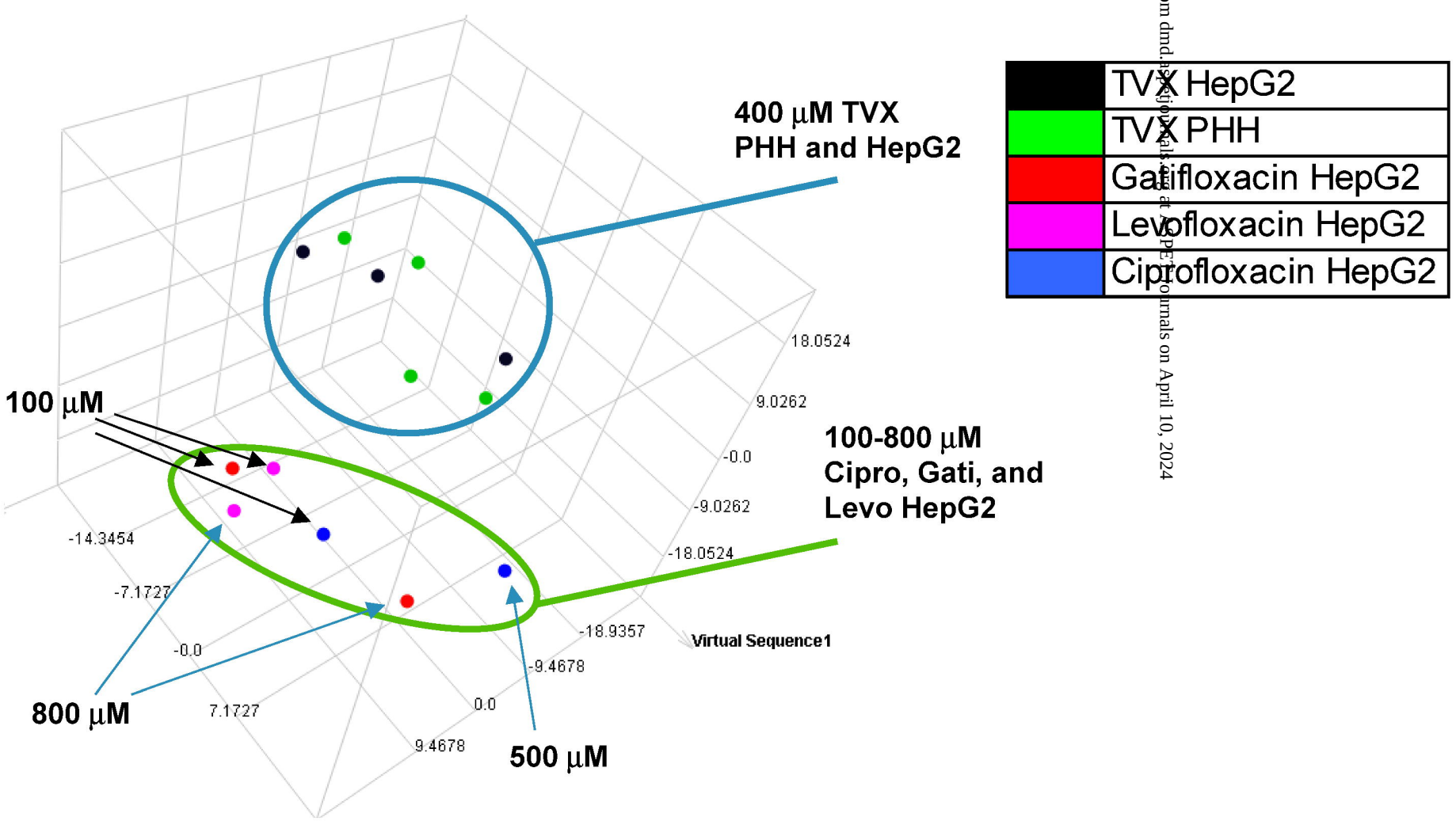
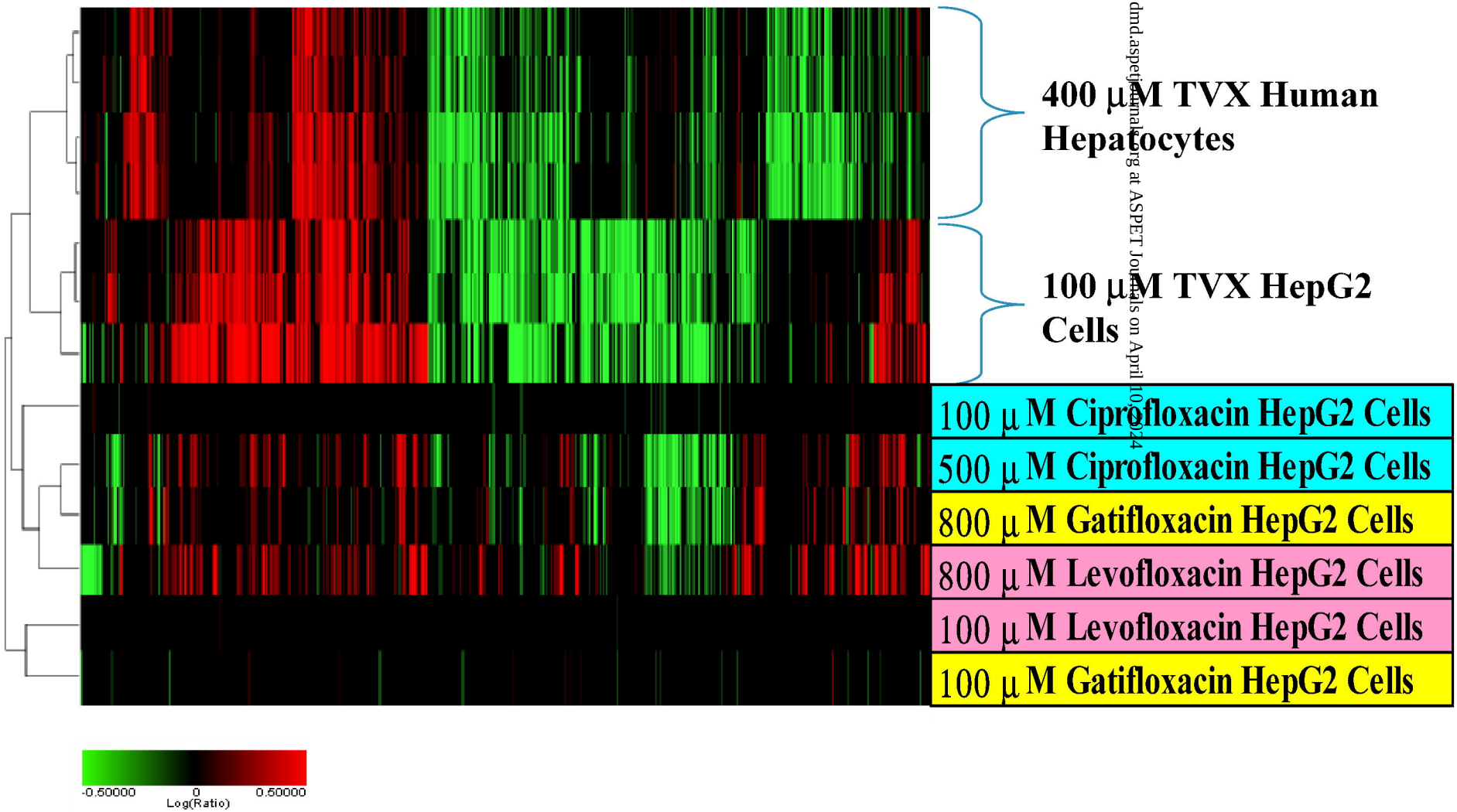


Figure 3B.



p≤0.01; Fold Change +/-2.0; n= 6775

Figure 4A.

p≤0.01; Fold Change +/-2.0; n= 2025

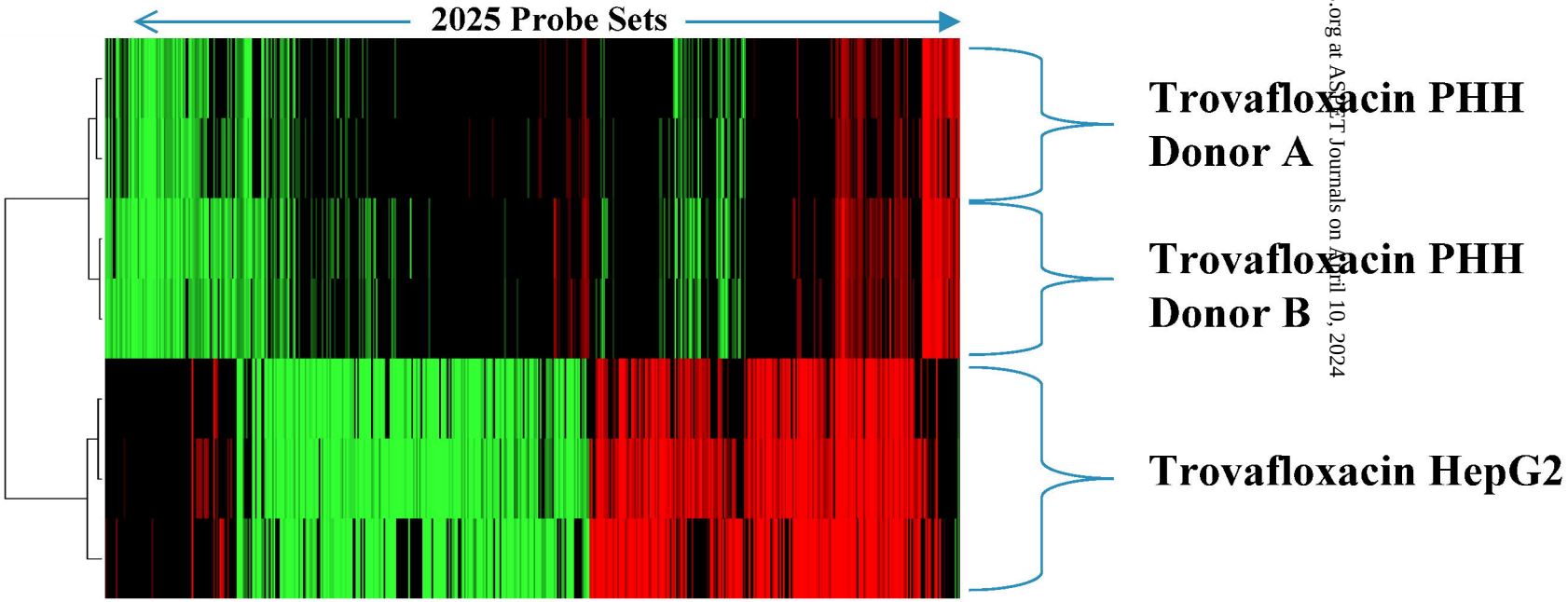


Figure 4B.

p≤0.01; Fold Change +/-2.0; n= 929

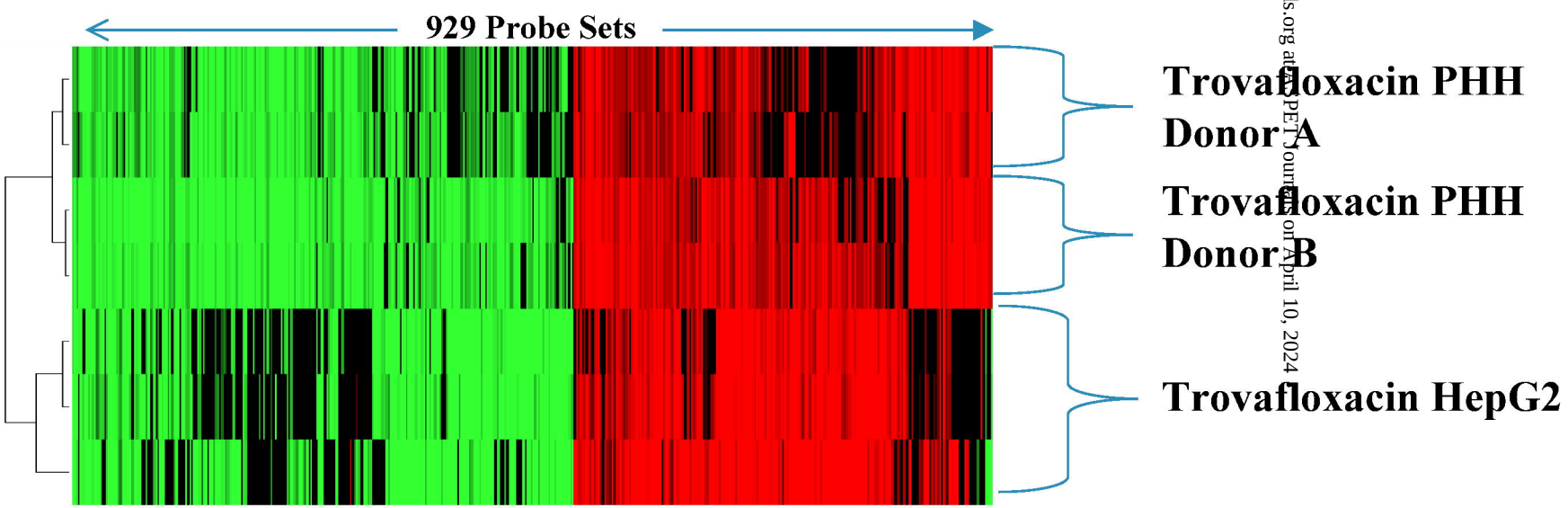


Figure 5.