

Microarray Analysis in Human Hepatocytes Suggests a Mechanism for Hepatotoxicity Induced by Trovafloxacin

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Idiosyncratic drug toxicity, defined as toxicity that is dose independent, host dependent, and usually cannot be predicted during preclinical or early phases of clinical trials, is a particularly confounding complication of drug development. An understanding of the mechanisms that lead to idiosyncratic liver toxicity would be extremely beneficial for the development of new compounds. We used microarray analysis on isolated human hepatocytes to understand the mechanisms underlying the idiosyncratic toxicity induced by trovafloxacin. Our results clearly distinguish trovafloxacin from other marketed quinolone agents and identify unique gene changes induced by trovafloxacin that are involved in mitochondrial damage, RNA processing, transcription, and inflammation that may suggest a mechanism for the hepatotoxicity induced by this agent. In conclusion, this work establishes the basis for future microarray analysis of new compounds to determine the presence of these expression changes and their usefulness in predicting idiosyncratic hepatotoxicity. Supplementary material for this article can be found on the HEPATOLOGY website (<http://interscience.wiley.com/jpages/0270-9139/suppmat/index.html>). (HEPATOLOGY 2005;41:177–186.)

Adverse drug reactions are a significant source of morbidity and mortality. In 1994 for instance, in the United States alone, more than 2 million cases of hospitalization and more than 100,000 deaths occurred as a result of adverse drug reactions. In addition, adverse drug reactions may lead to \$2 to \$4 billion in direct hospital costs per year.¹ In most cases, the reactions are dose dependent and predictable based on the pharmacology of the agent.² However in certain cases, unexpected toxicity can occur in a small subset of people receiving the drug. In these cases, the adverse reaction is not related to the pharmacology of the drug and is not dose dependent, because most people tolerate the drug at standard levels. Because the toxicity occurs in low per-

centages of animals and people, it usually cannot be predicted from preclinical studies; these reactions are referred to as *idiosyncratic*.³ In most of these cases, the liver is the primary target of toxicity.

In addition to the potential hazard to patients, these types of reactions are a major obstacle to drug development and can result in the withdrawal from the market of an otherwise efficacious and life-saving drug. A significant number of drugs that showed little to no toxicity in preclinical and clinical trials subsequently were removed from the market because of a small percentage of people who experienced life-threatening or fatal drug reactions; such drugs include troglitazone (withdrawn in May 2000 because of liver toxicity), bromfenac (withdrawn in June 1998 because of liver toxicity), fenfluramine (withdrawn in September 1997 because of heart valve disease), and cerivastatin (withdrawn in August 2001 because of rhabdomyolysis).⁴ A number of theories exist regarding the underlying mechanism for idiosyncratic drug toxicity. The two most prevalent are the presence of polymorphisms in enzymes involved in the metabolism of the drug or an allergic response to a drug or to one of its metabolites.^{2,5} Other possibilities include drug-induced mitochondrial injury or increased toxicity in certain individuals resulting from inflammation induced by bacterial infection.^{6–9} It is likely that there is no single expla-

Abbreviation: GSH, glutathione.

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nation to account for all of the reported cases of idiosyncratic drug toxicity. Rather, idiosyncratic reactions probably occur because of a number of factors, both biological and environmental.¹⁰ Nonetheless, drugs that have the potential to cause idiosyncratic drug toxicity may regulate common physiological or biochemical processes. These events could lead to clinical toxicity when combined with other factors such as environment, underlying bacterial infection, or drug metabolism gene polymorphisms. Understanding the response to such drugs at the molecular level has potential both to elucidate the mechanisms of toxicity and to predict idiosyncratic toxicity during drug development.

Microarray analysis has been previously shown to be a useful tool in identifying molecular mechanisms underlying drug-induced toxicity.^{11–13} We have applied gene expression analysis toward understanding the mechanism of idiosyncratic toxicity caused by the antibacterial quinolone agent trovafloxacin. Quinolones act by inhibiting bacterial DNA gyrase and DNA topoisomerase IV.¹⁴ As a class they are generally well tolerated.¹⁵ One exception to this is trovafloxacin, which was approved in 1997. Before its regulatory approval, there were no cases of hepatic failure or death in more than 7,000 patients. More than 2 million people since have received trovafloxacin. From this initial use, 150 cases of liver toxicity were reported, including 14 cases of acute liver failure. Four patients required liver transplants and an additional five patients died.¹⁶ The mechanism underlying this adverse effect has not yet been determined.

To develop a system to assay for warning signs for idiosyncratic toxicity, we treated isolated human hepatocytes from four different donors with six quinolone agents: trovafloxacin, levofloxacin, grepafloxacin, gatifloxacin, ciprofloxacin, and clinafloxacin. Of these, in addition to trovafloxacin, three are currently on the market (levofloxacin, gatifloxacin, and ciprofloxacin). Trovafloxacin has severe restrictions placed on it because of the hepatotoxicity, and it can be administered only in life-threatening situations. Grepafloxacin was withdrawn from the market in 1999 as a result of concerns about its cardiovascular safety.¹⁶ Clinical development of clinafloxacin was terminated in 1999 because of phototoxicity.¹⁷ Neither grepafloxacin nor clinafloxacin showed significant signs of hepatotoxicity.

Microarray results show substantial gene expression differences occur as a result of treatment of hepatocytes with trovafloxacin as compared with the other quinolones. Many of these gene changes involve crucial biological pathways that may be involved in the mechanism underlying trovafloxacin-induced hepatotoxicity. Overall, our results functionally distinguish trovafloxacin from

other marketed quinolones and suggest a possible method for screening new compounds in this class.

Materials and Methods

In Vivo Studies. All animals used in the research were treated humanely according to Abbott IACUC guidelines. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were administered levofloxacin orally and were administered trovafloxacin twice daily for 7 days. Rats (four per treatment group) were administered levofloxacin at a dose of 600 mg/kg/d and were administered trovafloxacin at a dose of 200 mg/kg/d, twice daily formulated in 0.2% hydroxypropylmethyl cellulose. This dose resulted in a C_{\max} of 34.8 $\mu\text{g/mL}$ for trovafloxacin and 47.4 $\mu\text{g/mL}$ for levofloxacin. Previous studies conducted internally have shown that these dose levels result in equivalent exposures of levofloxacin and trovafloxacin (data not shown). After 7 days of treatment, rats were necropsied. A serum chemistry panel was performed using an Abbott Aeroset clinical chemistry analyzer (Abbott Laboratories, Abbott Park, IL). One slice of liver was processed for histopathological evaluation. Approximately 100 mg from each liver was placed into TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) and immediately was homogenized using a Polytron tissue grinder (Kinematica, Cincinnati, OH) for microarray analysis.

Hepatocyte Culture and Treatment. 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% CO₂ for 24 hours after receipt. 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 30, 100, 400, or 800 μM . Vehicle control cells were dosed with an equivalent volume of 0.1N KOH as the experimental samples. For one experiment, 176 μM etoposide or 1.0 mM diethylmaleate were added to the wells using DMSO or ethanol as the vehicle solvent, respectively. Etoposide and diethylmaleate doses were selected from literature reports that used these reagents during hepatocyte experiments.^{18,19} Naive cells received no dosing. The cells were treated for 24 hours before harvesting with TRIzol reagent. Primary rat hepatocytes were isolated using a two-step perfusion method as previously described.²⁰

Intracellular Glutathione Levels. Intracellular glutathione (GSH) levels were determined essentially as described.²¹ Glutathione content was determined colorimetrically with a Packard SpectraCount (Packard Bioscience, Meriden, CT) at 415 nm.

MTT and DCFDA Assay. The MTT and DCFDA assays were performed as previously described.^{22,23}

Cell Proliferation Assay. Cell numbers in each well were determined in a separate plate with the CyQUANT Cell Proliferation Assay from Molecular Probes (Eugene, OR).

RNA Isolation and cRNA Sample Preparation. Total RNA was isolated from the TRIzol extracts using the procedure from Invitrogen. Optical density at 260 nm determined RNA concentration. RNA quality was assessed using an Agilent Technologies bioanalyzer (Palo Alto, CA) 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 μ g of total RNA.²⁴

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. For the rat studies, microarray analysis was performed using the Affymetrix RT-U34 array (in-life) or RAE-230A (rat hepatocytes).

Microarray Data Analysis. The microarray scanned image and intensity files were imported in Rosetta Resolver gene expression analysis software version 3.2 (Rosetta Inpharmatics, Kirkland, WA). Resolver's Affymetrix error model was applied, and ratios were built for each treatment array versus its respective vehicle control. Using Rosetta Resolver, a *P* value was calculated for every fold change using the Rosetta Resolver error model. For all of the gene changes shown, the *P* value was less than or equal to .01. If the *P* value was more than .01, the fold change was shown as 1.0 (or black on a heatmap). Principal components analysis was completed using Spotfire Decision Site version 7.1 (Spotfire, Somerville, MA). Gene ontologies were deciphered using NETAFFX (Affymetrix).²⁵

Results

Rat In Vivo and In Vitro Studies. To determine whether standard preclinical toxicology studies could distinguish trovafloxacin from other quinolone agents, cytotoxicity and animal toxicology studies were performed. Of the five quinolone agents, levofloxacin, trovafloxacin, ciprofloxacin, clinafloxacin, and gatifloxacin, tested in this assay, none showed marked differences in cytotoxicity, and no cytotoxicity was observed when cells were treated with as high as an 800- μ M concentration of any of the quinolones.

Previous research has not identified overt toxicity in rat toxicology studies that distinguished trovafloxacin from other quinolones.¹⁶ To confirm this, a 1-week toxicology

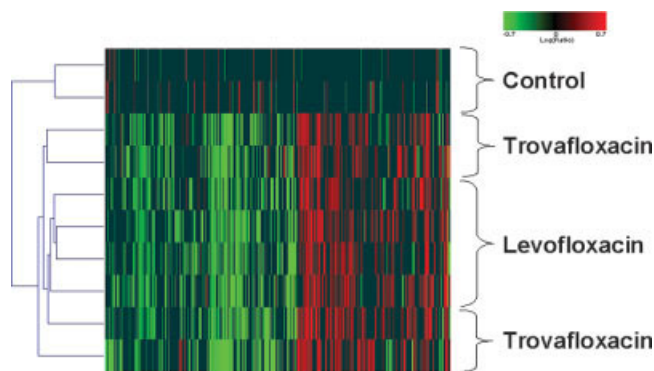


Fig. 1. Microarray analysis from rats treated with trovafloxacin and levofloxacin. Male rats dosed with trovafloxacin or levofloxacin have liver gene expression patterns that cluster together, indicating that trovafloxacin does not yield a unique expression pattern in rat liver. Each horizontal lane indicates an individual rat. Agglomerative clustering reveals a dendrogram showing compounds that exhibit similar gene expression patterns. Genes represented have a *P* value of .01 or less and are regulated at least \pm two-fold. Increases in messenger RNA (mRNA) levels are represented as shades of red and decreases in mRNA levels are represented by shades of green.

study was performed in rats treated with levofloxacin and trovafloxacin at a dose approximately 10-fold higher than human serum concentration.²⁶ No significant changes in clinical chemistry or histopathological results were seen in the rats treated with either trovafloxacin or levofloxacin when compared with control rats (data not shown). In addition, microarray studies were performed on liver RNA from the rats. Similar to the histopathological and clinical chemistry results, no significant gene changes were seen that differentiated rats treated with trovafloxacin compared with rats treated with levofloxacin (Fig. 1). Thus, in agreement with previous studies, standard preclinical toxicology models did not distinguish trovafloxacin from other quinolone agents.

Microarray Results in Isolated Human Hepatocytes. Because the idiosyncratic hepatotoxicity associated with trovafloxacin was observed only in humans, we used a combination of isolated human hepatocytes and microarray analysis to determine if trovafloxacin could be distinguished from other quinolones and to identify gene changes that may be related to the mechanism of idiosyncratic toxicity. Hepatocytes from four separate donors were used. A brief history of each of the donors is included in Table 1. A difficulty in using isolated human hepatocytes for gene expression analysis is the degree of variability in gene expression from donor to donor.²⁷ Thus, it was possible that more variability in gene expression would be seen across donors than between the different quinolone agents. However, analysis of microarray data using both agglomerative clustering and principal component analysis showed that expression profiles clustered according to

Table 1. Donor Background Information for Hepatocyte Isolations 1 through 4

	Donor 1	Donor 2	Donor 3	Donor 4
Age (yr)	48	42	31	59
Race	White	White	White	White
Sex	Female	Female	Male	Male
Height	5'2"	5'1"	6'1"	5'11"
Weight (lb)	110	111	265	250
HIV status	Negative	Negative	Negative	Negative
Hepatitis B/C status	Negative	Negative	Negative	Negative
CMV status	Positive	Negative	Negative	Positive
Cause of death	ICH	CVA	Anoxia	ICH
Alcohol use	1-2 beers/day	None	Social use	None
Tobacco use	Half pack/day for 29 years	2 packs/day for 20 years	None	None
Drug use	None	None	Benzodiazepams	None
Medications	Atenolol	None	Paroxetine, quetiapine fumarate	HTN medications
Medical history	2 Caesarean sections	Depression, HTN	UTI, acid reflux disease, asthma	HTN, type II diabetes

Abbreviations: HIV, human immunodeficiency virus; CMV, cytomegalovirus; ICH, intracranial hematomas; CVA, cerebrovascular accidents; HTN, hypertension; UTI, urinary tract infection.

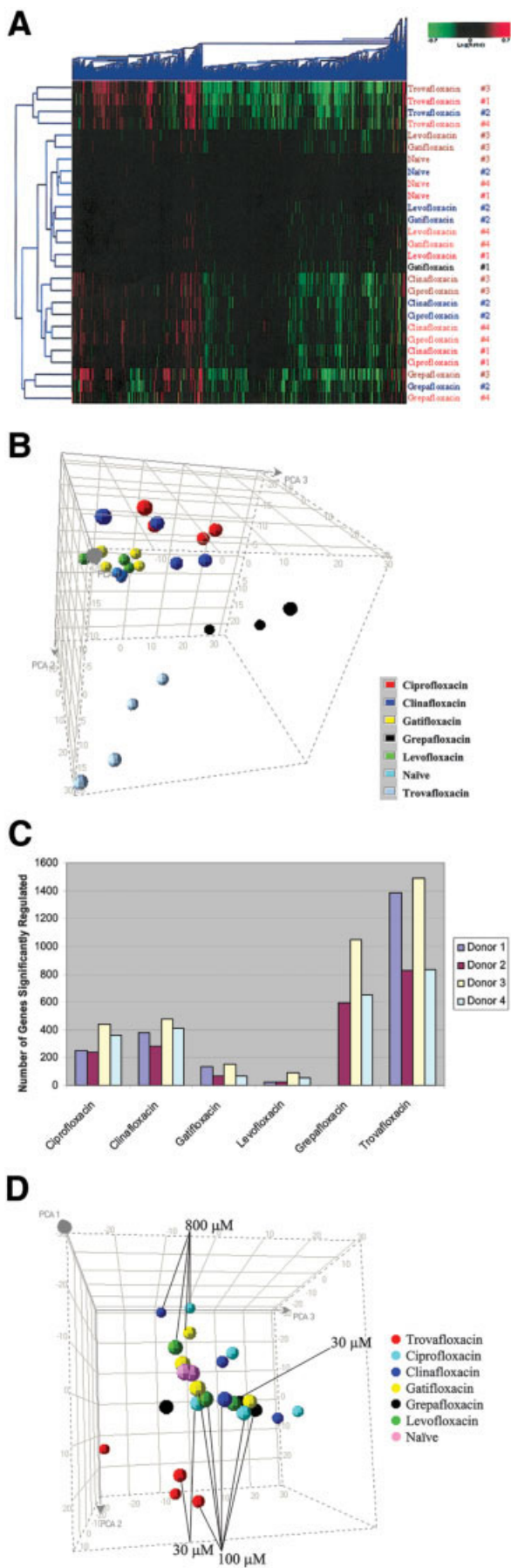
the treatment and not according to the hepatocyte isolation (Fig. 2A-B). For all donors, treatment with trovafloxacin resulted in a higher number of gene expression changes (Fig. 2C). In addition, treatment with trovafloxacin resulted in an expression profile that was distinct from the other quinolone profiles (Fig. 2A-B). This is further demonstrated in Table 2, which shows the correlation of the expression profiles for all of the quinolones from treated hepatocytes isolated from donor 2. We explored the possibility that higher concentrations of the other quinolones could give an expression profile similar to that seen with trovafloxacin. Isolated human hepatocytes therefore were treated with higher concentrations of the other quinolones, and these profiles were compared with trovafloxacin at 400 μM . In addition, we compared an expression profile from a lower dose of trovafloxacin with expression profiles from the other quinolone agents. Figure 2D shows a principal component analysis of expression profiles from hepatocytes treated with 30, 100, 400, or 800 μM of the different quinolones. The results show that, regardless of dose level, hepatocytes treated with trovafloxacin result in an expression profile that is distinct from the other quinolones tested.

Mitochondrial toxicity has been hypothesized to be a mechanism underlying idiosyncratic toxicity.^{8,10} We investigated whether genes involved in mitochondrial function were regulated specifically by trovafloxacin. Figure 3A shows a heat map of genes involved in mitochondrial function. Some of these genes, such as heme oxygenase, thioredoxin reductase 1, p21 cyclin-dependent kinase inhibitor, and topoisomerase 2A, have been shown to be involved in oxidative stress.²⁸ Although all of the quinolones regulate these genes to a certain extent, trovafloxacin

regulates these genes the most consistently, even at lower doses.

To provide functional verification of the microarray results, the quinolone agents were subjected to a full battery of *in vitro* toxicology assays in HepG2 cells. One of the assays measures the total intracellular GSH level, which is associated with oxidative stress.²⁹ The GSH assay clearly distinguished trovafloxacin from the other quinolones, resulting in a TC50 of 19 μM , significantly less than any of the other quinolones (Table 3). Grepafloxacin had the next lowest TC50, at a level of 82 μM . TC50 is the concentration that resulted in depletion of 50% of the GSH.

To examine further whether trovafloxacin induced oxidative stress in HepG2 cells, timecourse studies were performed measuring GSH levels in HepG2 cells treated with trovafloxacin at 2 and 6 hours. The results showed that at 6 hours, cells treated with trovafloxacin at 30 μM resulted in a reduction in GSH levels, whereas the cell viability, measured by MTT and cell number, were not affected (Fig. 3B). In addition, reactive oxygen species production was measured in HepG2 cells treated with trovafloxacin for 6 hours using DCFDA. The results showed that the levels of fluorescent DCFDA increased with 10 μM of trovafloxacin treatment and continued to do so in a dose-responsive manner (Fig. 3B). HepG2 cells treated with trovafloxacin for 2 hours did not show a measurable reduction in GSH; however, the levels of fluorescent DCFDA did show a slight dose-dependent increase with trovafloxacin treatment (Fig. 3B). The results from the DCFDA assay clearly show that trovafloxacin is inducing oxidative stress in HepG2 cells. Because the cells seem to be undergoing oxidative stress at 2 hours, with no



measurable loss of GSH at this timepoint (data not shown), the possibility exists that the reactive oxygen species generation is the cause of GSH depletion.

Finally, the expression profile from isolated human hepatocytes treated with trovafloxacin was compared with the expression profile from diethylmaleate, a well-characterized glutathione-depletion agent and inducer of oxidative stress.³⁰ The results showed that treatment with trovafloxacin and diethylmaleate resulted in mutual gene expression changes involved in oxidative stress, such as glutathione-S-transferase, acyl-CoA oxidase, and the oxidative stress responsive 1 gene (Fig. 4). Thus, the microarray findings, in combination with the GSH and DCFDA results, suggest that trovafloxacin may be an inducer of oxidative stress, which could result in a hepatotoxic reaction.

To identify other potential mechanisms for the idiosyncratic toxicity associated with trovafloxacin, we attempted to identify genes that were regulated specifically by trovafloxacin. To achieve this, we used strict criteria that specified that a gene must be regulated significantly by trovafloxacin in all donors and could not be regulated significantly by another quinolone in any of the donors. Using these criteria, we identified 142 genes specifically

Fig. 2. (A) Cluster analysis of quinolone agents across all isolations. Trovafloxacin yields a unique gene expression profile as revealed by agglomerative cluster analysis for drug-treated human hepatocytes after approximately 24-hour exposure to quinolones for donors 1 through 4. Analysis of variance in Rosetta Resolver software (Rosetta Inpharmatics, Kirkland, WA) was used to combine duplicate patterns for each donor and drug. Genes represented have a *P* value of .01 or less and are regulated at least \pm two-fold. Drug labels are color coded to match donor number. Results reveal a similar expression pattern for quinolones between the different donors. All drugs were dosed at 400 μ M. Grepafloxacin treatment was not included for donor 1. (B) Principle component analysis of expression profiles resulting from treatment with quinolone agents. Principle component analysis for the quinolones investigated verified that the trovafloxacin gene expression pattern is unique. Microarray data from each treatment were combined for replicate treatments using Rosetta Resolver's ANOVA function. Three principal components were generated and plotted for each drug and donor using Spotfire software (Spotfire, Somerville, MA). A different color point represents each drug treatment, with each individual point representing a distinct human hepatocyte donor. (C) Graph showing the number of genes significantly regulated by the different quinolone agents across donors. A gene was considered significantly regulated with a change of twofold or more and a *P* value of .01 or lower. The values are the average of duplicate cell treatments in each donor. Grepafloxacin was not included in the study using cells from donor 1. (D) Principle component analysis of dose-response studies. Principle component analysis for the quinolones investigated verified that the trovafloxacin gene expression pattern is unique, regardless of dose level. Microarray data from each treatment were combined for replicate treatments using Rosetta Resolver's ANOVA function. Three principal components were generated and plotted for each drug and dose level using Spotfire software. Quinolone dose level points of 30, 100, and 800 μ M are labeled, whereas 400- μ M dose points remain unlabeled.

Table 2. Correlation of Expression Profiles Resulting From Treatment With Quinolone Agents in Hepatocytes From Donor 2

	Levofloxacin	Ciprofloxacin	Clinafloxacin	Gatifloxacin	Grepafloxacin	Trovafoxacin
Levofloxacin	1.00	0.88	0.86	0.89	0.70	0.71
Ciprofloxacin	0.88	1.00	0.94	0.91	0.79	0.68
Clinafloxacin	0.86	0.94	1.00	0.93	0.75	0.79
Gatifloxacin	0.89	0.91	0.93	1.00	0.70	0.71
Grepafloxacin	0.70	0.79	0.75	0.70	1.00	0.66
Trovafoxacin	0.71	0.68	0.79	0.71	0.66	1.00

and consistently regulated only by trovafoxacin (Supplementary Fig. 1). Importantly, none of the other quinolones specifically regulated genes using these criteria. A number of these genes are involved in functions that have been ascribed previously to be a potential cause for idiosyncratic toxicity, such as mitochondrial function or inflammation. In addition, many of these genes were involved in RNA transcription from polymerase II. To confirm that trovafoxacin regulates genes involved in RNA transcription, expression patterns from isolated human hepatocytes treated with trovafoxacin were compared with the expression pattern from hepatocytes treated with etoposide, a topoisomerase II inhibitor.³¹

Microarray analysis revealed that treatment with both etoposide and trovafoxacin resulted in a number of gene changes involved in RNA transcription, such as RNA polymerase I and II, transcription elongation factor B, and topoisomerase II β (Fig. 4).

The unique gene expression profile induced by trovafoxacin in human hepatocytes could be the result of species differences or to *in vivo* or *in vitro* differences. To address this, isolated rat hepatocytes were treated with the same six quinolones used in the human hepatocyte experiments, and microarray analysis was performed on the samples. The results showed that, similar to isolated human hepatocytes, trovafoxacin regulated more genes than

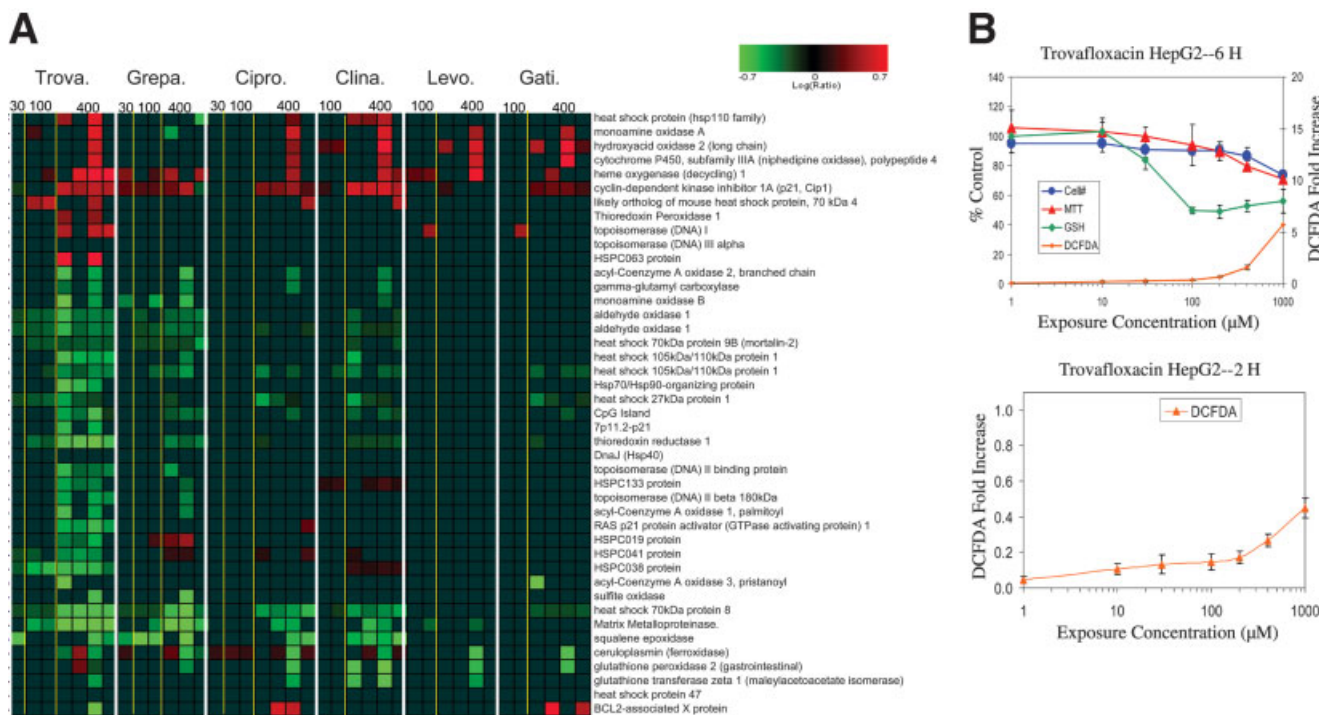


Fig. 3. (A) Mitochondrial-related genes regulated by trovafoxacin. Many genes that are associated with the mitochondria are regulated uniquely by trovafoxacin on treatment of human hepatocytes. Experimental replicates were combined using Rosetta Resolver's ANOVA function (Rosetta Inpharmatics, Kirkland, WA). Each vertical column represents a distinct donor, whereas each yellow border represents a distinct dose level (30, 100, or 400 μ M). The white borders subdivide the quinolone treatments. The experimental design included grepafloxacin (Grepa.) treatments for three donors, whereas the all other compounds were treated for four donors. (B) Cell viability, glutathione (GSH), and DCFDA results after 2 and 6 hours of trovafoxacin treatment in HepG2 cells. Cells were treated with several levels of trovafoxacin and after 2 or 6 hours, and several readouts were used to gain understanding of potential oxidative stress events that may be occurring upon drug treatment. After trovafoxacin treatment, results reveal a decrease in total cellular GSH and an increase in levels of fluorescent DCFDA, a marker of oxidative stress. % Control, cell number, MTT, and GSH curves; DCFDA Fold Increase, DCFDA curve; Trova., trovafoxacin; Levo., levofloxacin; Gati., gatifloxacin; Cipro., ciprofloxacin; Clina., clinafloxacin.

Table 3. Calculated TC50 Values for Glutathione Depletion of Different Quinolone Agents in HepG2 Cells

Compound Name	TC50 (μM)
Trovafoxacin	19
Grepafloxacin	82
Ciinafloxacin	225
Ciprofloxacin	189
Gatifloxacin	215
Levofloxacin	361

Abbreviation: TC50, concentration that resulted in a depletion of 50% of the glutathione.

the other quinolones in rat hepatocytes, although the gene expression changes were quite different between rat and human (Fig. 5A-B). A few of the genes identified as being uniquely regulated by trovafloxacin in human hepatocytes also were regulated in rat hepatocytes. These genes are highlighted in red (Supplementary Fig. 1).

Discussion

Isolated human hepatocytes have proven to be a valuable tool for studying hepatic toxicity.^{32,33} Cultured hepatocytes have been used to examine drug metabolism, genotoxicity, and the relevance of animal toxicology findings to humans. Examples in the literature of human hepatocytes used for toxicogenomic studies, however, are

sparse. This is partially the result of the cost and difficulty in obtaining human hepatocytes. However, potentially a greater reason for the lack of microarray studies in human hepatocytes is the concern over interindividual variability.^{20,27} Unlike laboratory animals, which are more genetically defined, a great deal of genetic diversity exists from one human to another. In addition, lifestyle differences are expected to play a role in gene expression changes seen from one donor to another. From the four donors used in our hepatocyte isolation studies, variability existed for alcohol use, smoking, medications used, weight, age, and cause of death, all of which could affect gene expression profiles. Indeed, a direct comparison of the microarray results from naive cells from donor 1 compared with those from donor 2, for instance, showed approximately 750 gene changes at the twofold upregulation or downregulation, with a *P* value less than .01 (data not shown).

It therefore was a concern with these studies that gene expression changes induced by the different quinolone agents would not replicate from one donor to another and that the gene expression profiles would cluster by donor, not by quinolone agent. However, the gene expression changes induced by the quinolone agents were extremely robust and reproducible across the different donors (Fig. 2A). Thus, although the different hepatocyte isolations

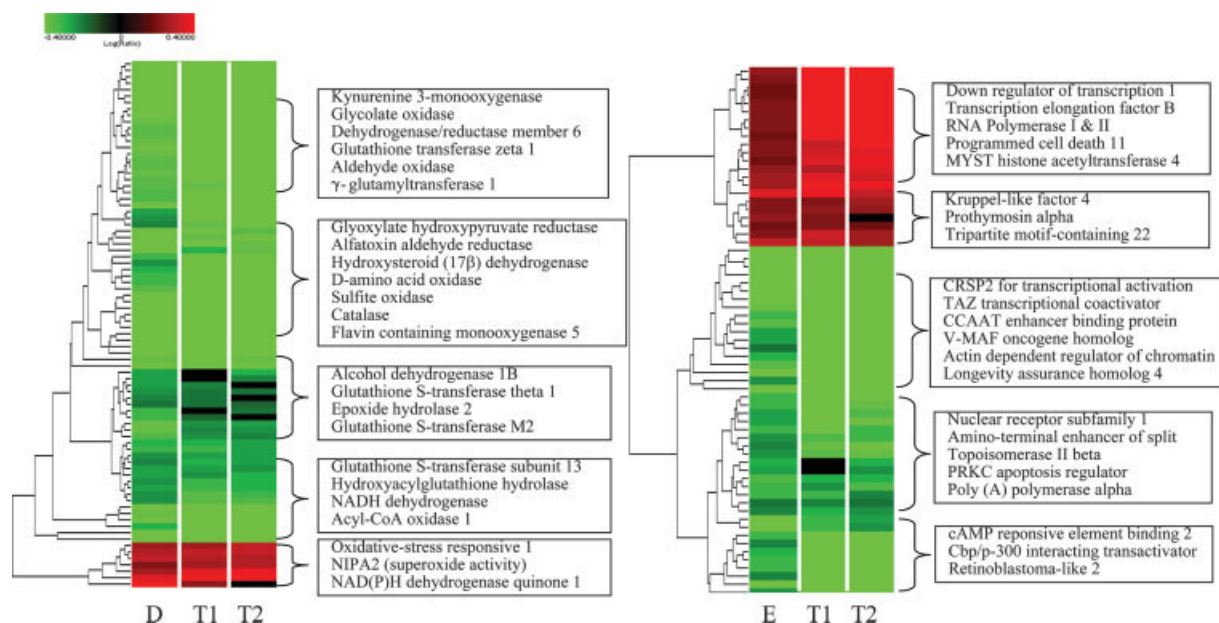


Fig. 4. Glutathione/oxidoreductase genes and transcription genes regulated in common between diethylmaleate/etoposide and trovafloxacin for human hepatocytes in culture. Several transcripts associated with glutathione metabolism and redox activity were regulated in common between DEM, a known glutathione depletion reagent, and trovafloxacin. In addition, many genes involved with cellular transcription were regulated commonly between the topoisomerase inhibitor etoposide and trovafloxacin. Genes visualized were regulated with a *P* value of .05 or less. D, 1.0 mM diethylmaleate; E, 176 μM etoposide; T1, 400 μM trovafloxacin 1; T2, 400 μM trovafloxacin 2; NADH, nicotinamide adenine dinucleotide; NIPA, nonimprinted in Prader-Willi Syndrome/Angelman Syndrome 2; NAD(P)H, nicotinamide adenine dinucleotide phosphate; MYST, monocytic leukemia histone acetyltransferase 4; CRSP2, cofactor required for Sp1 transcriptional activation, subunit 2, 150 kd; TAZ, transcriptional co-activator with PDZ-binding motif; V-MAF, musculoaponeurotic fibrosarcoma oncogene homolog; PRKC, protein kinase C; cAMP, cyclic AMP.

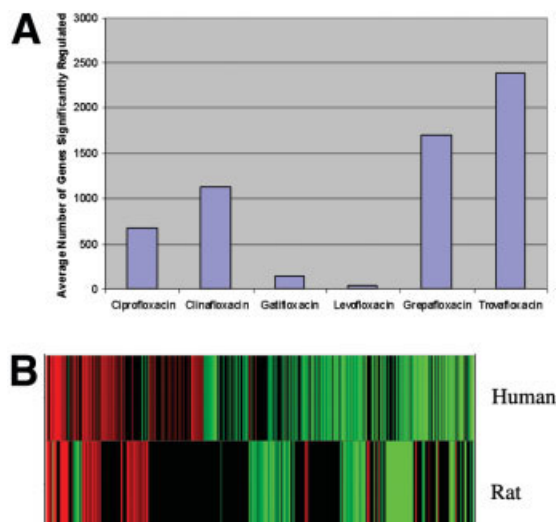


Fig. 5. (A) Graph showing the number of genes significantly regulated by the different quinolone agents in isolated rat hepatocytes. A gene was considered significantly regulated with a change of twofold or more and a P value of .01 or less. The values are the average of duplicate cell treatments. (B) Comparison of gene expression changes induced by trovafloxacin in isolated rat and human hepatocytes. Rat and human gene sequences were compared based on Unigene identification.

showed considerable variability in gene expression changes when comparing naive samples, they all responded in a similar fashion to the different quinolones. Therefore, our studies show that isolated human hepatocytes can be used for toxicogenomic studies, supporting the further use of this system for microarray studies.

Across all donors, treatment with trovafloxacin resulted in more gene expression changes than the other, less hepatotoxic quinolone agents. This is in agreement with the findings of Kier et al.,³⁴ who showed that treatment isolated human hepatocytes showed more gene expression changes with the idiosyncratic hepatotoxin troglitazone than pioglitazone or rosiglitazone. A number of genes specifically regulated by trovafloxacin fall into functional categories that previously were ascribed to mechanisms for idiosyncratic toxicity. For instance, a number of mitochondrial genes specifically were regulated by trovafloxacin, including several mitochondrial ribosome proteins and mitofusin-1. Eukaryotic mitochondrial ribosomes have been shown to be a possible off-target site contributing to the toxicity of a number of antibacterial agents.³⁵ Mitofusin-1, which was downregulated by trovafloxacin, is essential for maintaining mitochondrial morphological features, and suppression of this gene causes fragmentation of mitochondria.^{36,37} Along with mitofusin-1, another gene that has been shown to be involved in maintaining mitochondrial morphology is *bax*.³⁸ *Bax* also was downregulated by trovafloxacin (Fig. 3A). Regulation of these genes by trovafloxacin could result in compromised mitochondrial function, which, in combination with other factors, could lead to hepatotoxicity.^{8,10}

Mitochondrial toxicity induced by trovafloxacin also was indicated by the regulation of genes associated with oxidative stress and by the results of the glutathione depletion studies in HepG2 cells. Although all of the quinolones affected oxidative stress to a certain extent, trovafloxacin seemed to be the most potent inducer, based on both gene expression analysis and glutathione depletion. Also, HepG2 cells treated with trovafloxacin showed an increase in fluorescent DCFDA, an indicator of oxidative stress. In addition, treatment of isolated human hepatocytes with trovafloxacin and diethylmaleate, a well-characterized glutathione-depletion agent, resulted in a number of similar gene changes involved in oxidative stress. Interestingly, rats treated with trovafloxacin showed no obvious increase in GSH/oxidized glutathione levels, suggesting either that this effect may be species specific or that the changes may be subtle.

Trovafloxacin also regulated genes involved in RNA processing and regulation of transcription. Interestingly, studies have shown that although quinolone agents target bacterial topoisomerase and gyrase, some quinolones also seem to interact with eukaryotic type II topoisomerase.^{39,40} For example, ciprofloxacin has been shown to be a modest enhancer of DNA cleavage mediated by eukaryotic topoisomerase II.⁴⁰ Based on this, some quinolones have been tested for their efficacy as anticancer agents. Inhibition of topoisomerase II would result in impairment of transcription, because topoisomerase II has been shown to be essential for transcription by RNA polymerase II.^{41,42} Microarray studies comparing etoposide with trovafloxacin showed similar gene expression changes occurring for topoisomerase II, RNA polymerase I and II, and other genes involved in RNA processing and transcription (Fig. 4). The fact that trovafloxacin specifically downregulated genes involved in transcription and RNA processing may suggest that it has a higher cross-reactivity with the eukaryotic polymerase II system than the other quinolone agents tested. This effect, coupled with other factors such as lifestyle or environment, could result in a hepatotoxic reaction.

An underlying question with the studies in isolated human hepatocytes is whether the unique gene expression changes induced by trovafloxacin were the result of using a human cell system or whether they were the result of differences between *in vitro* and *in vivo* conditions. However, the gene expression changes seen with trovafloxacin treatment in isolated rat and human hepatocytes were quite different. For instance, as opposed to what was seen in human hepatocytes, most of the genes uniquely regulated by trovafloxacin in rat hepatocytes were involved in intracellular transport, protein synthesis, and cytoplasm

organization (data not shown). A few genes identified as being uniquely regulated in human hepatocytes by trovafloxacin also were regulated in rat hepatocytes, such as genes involved in phosphorylation and organogenesis (Supplementary Fig. 1). Thus, if rats are not sensitive to trovafloxacin-induced hepatotoxicity, it may be that these genes are not involved in the mechanism of hepatotoxicity.

In conclusion, we have shown that cultured human hepatocytes, coupled with microarray analysis, can be a valuable tool for investigating molecular mechanisms of toxicity. The gene expression changes induced by the quinolones were reproducible both within and between donors. Our results clearly distinguish trovafloxacin from other marketed quinolone agents and identify gene changes that may suggest a mechanism for the hepatotoxicity induced by this agent. This work establishes the basis for future microarray analysis of new compounds to determine the presence of these expression changes and their usefulness in predicting idiosyncratic hepatotoxicity.

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