Global Gene Expression Changes Induced In Primary Human Hepatocytes By Thiazolidinediones Upon Repeat Dosing of HepatoPac™ Cultures

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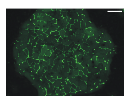
Abstract

Global gene expression changes as assessed using whole genome microarrays can provide insights into the mechanism of action of drug candidates. Furthermore, signatures of gene expression may distinguish compounds with potential to cause tissue injury with diverse mechanisms from the non-toxic compounds[1,2]. In the case of the liver, primary human hepatocytes are widely considered to be the most suitable to assess drug-induced gene expression changes *in vitro* since they contain the full repertoire of regulatory pathways; however, hepatocytes display a precipitous decline in phenotypic functions when left in suspension or cultured in a sandwich of extracellular matrix proteins. Therefore, hepatocytes in these conventional systems are generally unsuitable for assessment of gene expression changes upon chronic exposure in order to mimic clinically relevant dosing regimens. We have previously developed a microscale model of the human liver in which primary human hepatocytes are organized in colonies of empirically optimized dimensions and subsequently surrounded by supportive stromal cells (HepatoPac™). Here, we sought to discern the effects of acute (24 hours) and chronic (7 to 14 days) drug exposure on the transcriptome of primary human hepatocytes using the hepatotoxic and non-toxic drug pair, Troglitazone and Rosiglitazone, respectively. HepatoPac cultures were dosed from 24 hours to 14 days at a sub-lethal and clinically relevant C[max] dose. Messenger RNA was collected at 24 hours, 7 days, and 14 days after dosing, and hybridized to whole genome human Affymetrix GeneChips™. Subsequent analysis of gene expression data with GeneSpring software revealed a time-dependent increase in statistically significant gene expression changes induced by both Troglitazone and Rosiglitazone as compared to vehicle controls. However, Rosiglitazone significantly changed the expression of 2, 16, and 221 genes, while Troglitazone changed the expression of 17, 409 and 928 genes over 24 hours, 7 days, and 14 days, respectively. In particular Troglitazone caused up-regulation of several genes involved in cellular pathways such as oxidation-reduction, lipid metabolism and biosynthesis, and stress/wounding response. Studies are currently underway to evaluate drug-induced gene expression changes of multiple toxic/non-toxic pairs in order to evaluate common pathways. In the future, HepatoPac cultures may be utilized with both gene expression and biochemical assays to select optimal drug candidates in both drug discovery and development.

HepatoPac™

HepatoPac is a unique platform that optimizes the function and life span of plated primary hepatocytes[3]. The stability of CYP450s, Phase II enzymes and formation of bile canaliculi allows the streamlined use of one *in vitro* model for many pharmaceutical applications as compared with suspension and sandwich cultured hepatocytes, which display a limited repertoire of phenotypic functions. For metabolite identification studies, it has been shown that HepatoPac outperforms suspension hepatocytes, S9 fractions, and microsomes, offering a superior *in vitro* approach for generating major human metabolites[4]. Furthermore, HepatoPac has also shown utility for safety assessment (via biochemical and high content imaging assays), enzyme induction and inhibition, clearance predictions, and transporter-mediated uptake and canalicular efflux. HepatoPac consists of primary hepatocytes (fresh or cryopreserved) attached to micro-engineered domains of extracellular matrix coatings surrounded by supportive stromal cells in industry standard 24 and 96 well plates, maintaining higher throughput capabilities. This platform facilitates the long term function of hepatocytes *in vitro,* allowing clinically relevant chronic dosing scenarios in order to mimic toxicities that happen over weeks.



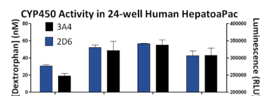
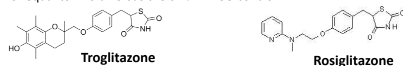


Figure 1. Bile canalicular transport of 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate on day 7 of human HepatoPac culture. CYP3A4 and CYP2D6 activities as assessed by Dextromethorphan hydroxylation and Promega's CYP3A4-Glo assay, respectively, are relatively stable for several weeks in HepatoPac.

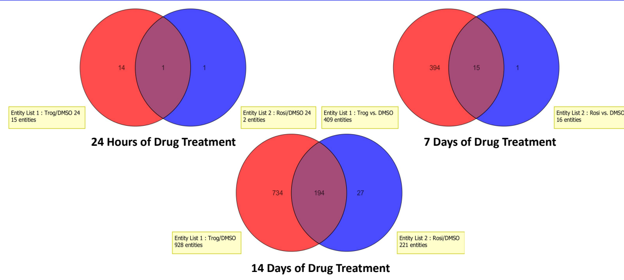
Methods

Industry standard 24-well plates were subjected to microfabrication techniques to produce a pattern of extracellular matrix-coated domains (500 um diameter, 1200 um spacing) at the bottom of each well, followed by seeding of primary human hepatocytes (plateable cryopreserved lot) onto the plates. After allowing for complete filling and adherence to the micro-patterned matrix islands, additional hepatocytes were washed away and cultures were allowed to incubate overnight. Murine embryonic fibroblasts (3T3-J2) were added to cultures the next day and allowed to adhere around the hepatocytes, thereby producing micropatterned co-cultures. Cultures were fed serum-supplemented proprietary medium every two days until initiation of dosing 8 days following initiation of hepatocyte seeding.

Cultures were treated with Troglitazone and Rosiglitazone (Cayman Chemicals) in serum-free proprietary dosing medium at their reported C[max] values of 2.82 µg/ml and 0.373 µg/ml, respectively[5]. Cultures were dosed every other day for 1, 7, and 14 total days. CYP450 3A4 activity (Promega) and Urea synthesis (Standbio Laboratory) were measured from collected supernatants. Mitochondrial activity was assessed via the cell proliferation reagent, WST-1 (Roche Diagnostics). RNA was isolated at 1, 7 and 14 day time-points using the RNeasy kit (Qiagen). RNA was pooled from three wells per condition and hybridized to Affymetrix GeneChip™ Human Genome U133 Plus 2.0 chips at the Whitehead Institute of MIT. All data was analyzed using GeneSpring GX software (Agilent). The threshold of gene expression significance was set at greater than or equal to 2-fold versus the 0.1% DMSO control.



Gene Set Analysis After 24 hr, 7 days, and 14 days of Drug Dosing at C[max]



Dosing Time course	Total Genes Changed		Shared Expression Between Troglitazone and Rosiglitazone	Unique to Drug	
	Rosiglitazone	Troglitazone		Rosiglitazone	Troglitazone
24 Hours	2	15	1	1	14
7 Days	16	409	15	1	394
14 Days	221	928	194	27	734

Figure 2. Gene expression changes occur in a time-dependent manner for both Troglitazone and Rosiglitazone; however, Troglitazone uniquely alters transcripts 1 to 2 orders of magnitude greater than Rosiglitazone at all three time-points.

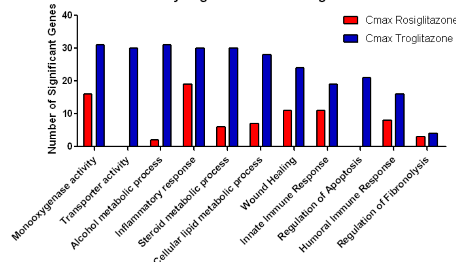


Figure 3. The effect of Rosiglitazone on different cellular pathways remains relatively unaltered following 24 hours and 7 Days of treatment (data not shown). The effects of Troglitazone versus Rosiglitazone over the time course of 14 days are represented above in terms of pathway ontology. Overall, Troglitazone induces expression of more genes in the apoptotic pathways than its structural analog, Rosiglitazone.

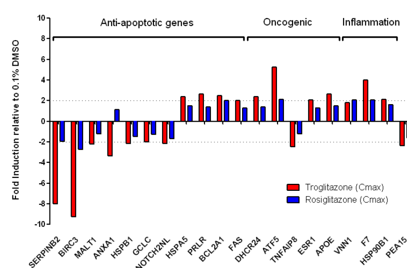


Figure 4. A brief analysis of three of the major pathways involved in hepatic toxicity reveals differences in the polarity and magnitude of gene expression between Troglitazone and Rosiglitazone. At a threshold of two-fold gene induction, only Troglitazone has an effect on the expression of these genes. Additionally, Troglitazone's ability to cause flux within important cellular survival pathways is apparent by its effect of both inducing and repressing anti-apoptotic gene expression. Early expression of these genes may be indicative of eventual drug-induced liver injury.

Functionality of Cultures During the Chronic Dosing Period of 14 Days

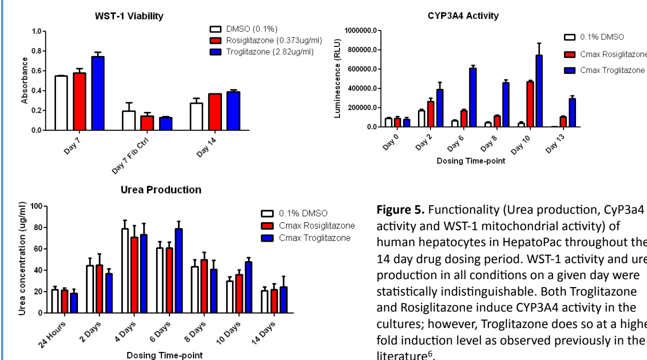


Figure 5. Functionality (Urea production, CyP3a4 activity and WST-1 mitochondrial activity) of human hepatocytes in HepatoPac throughout the 14 day drug dosing period. WST-1 activity and urea production in all conditions on a given day were statistically indistinguishable. Both Troglitazone and Rosiglitazone induce CYP3A4 activity in the cultures; however, Troglitazone does so at a higher fold induction level as observed previously in the literature[6].

Mechanistic Gene FABP4 Fold Induction		
Time	Rosiglitazone	Troglitazone
24 Hours	3.5	3.45
7 days	2.42	2.88
14 Days	4.16	4.25

Figure 6. Thiazolidinediones reduce insulin sensitivity by enhancing the activity of PPAR-gamma through the induction of FABP4. Both Troglitazone and Rosiglitazone showed approximately the same magnitude of FABP4 up-regulation (heat maps above entities are listed as control, Rosiglitazone, Troglitazone), suggesting that although their C[max] values vary, their ability to induce common mechanistic targets at these respective concentrations are the same

Taken together, the results of Figures 2 to 6 show that while Troglitazone and Rosiglitazone have similar therapeutic effects at their respective C[max] values, they induce off-target gene expression changes (i.e. apoptotic pathways) that are orders of magnitude apart. Such off-target gene expression changes may foreshadow overt drug-induced liver injury upon chronic drug administration in specific patient populations.

Conclusions and Future Directions

- HepatoPac™ technology provides a platform in which primary hepatocytes (human, rat, monkey) can be continuously dosed for several weeks *in vitro* without significant decline in basic liver-specific functionality (i.e. CYP450 activity, albumin secretion, urea synthesis, and mitochondrial activity). This allows for analysis and characterization of the human hepatocyte transcriptome at sub-lethal doses.
- Dosing of human HepatoPac at reported C[max] values of Troglitazone and Rosiglitazone produced a time-dependent increase in gene expression changes over the course of 14 days.
- Analysis of pathway ontology revealed that both Troglitazone and Rosiglitazone affect many of the same gene pathways; however, Troglitazone modulates a greater number (1-2 orders of magnitude) of unique genes than its structural analog, Rosiglitazone.
- Future experiments will seek to elucidate functional/phenotypic consequences of key gene expression changes in HepatoPac in order correlate gene expression with hepatic injury.
- Additional toxic/non-toxic drug pairs with clinical histories are being selected from the literature for further experimentation in HepatoPac with the goal of elucidating potential common gene expression patterns upon chronic dosing of drugs from different therapeutic classes.

References

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