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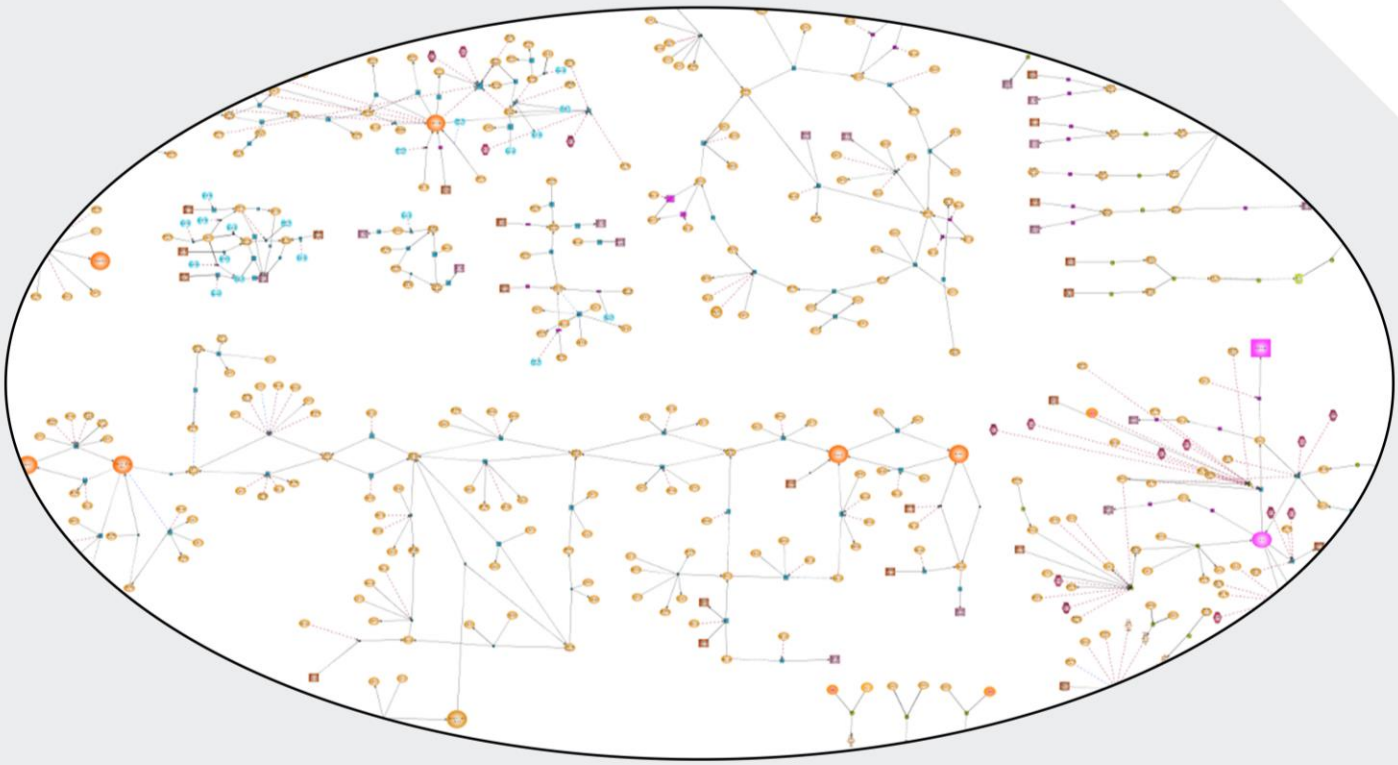
A report on Troglitazone analysis using the Heptox™ Virtual Liver platform



Introduction

Heptox - DILI prediction platform is a combination of a virtual liver model and an *in vitro* assay set that provides the mechanistic rationale behind a compound's toxicity, a prediction of the *in vivo* exposure that could lead to toxicity, and the adaptive response of the liver to the exposure. The *in silico* model outputs are equivalent to clinically observed toxic endpoints; hepatocellular necrosis, cholestasis and steatosis. This prediction system can integrate process or pathway level insights from other experimental methods to get a holistic picture of the impact of a drug or compound.

Model simulated key metabolites and fluxes are monitored to

- assess the impact of a drug on the system
- understand the major processes responsible for drug impact either directly or in an adaptive manner
- elucidate the mechanism of action behind necrosis and steatosis
- estimate cholestatic potential of the compound when transporter inhibition data is shared; we donot perform transporter assays.

In this report we have described the molecular impact of Troglitazone and analyse its impact using our prediction platform. This report includes the testing method, the results obtained and analysis.

Methods

Experimental

HepG2 cells obtained from ATCC were maintained in recommended culture media in presence of 10 % FBS. For each compound a range of concentrations were picked to determine IC50 w.r.t. cell viability. Cells were treated for three time points: 24, 48 & 72 hrs and cell viability was measured at the end of every 24 hrs. For detailed analysis, 3 concentrations are decided: 5, 2.5, 1.25 µM. Cells were treated repeatedly after every 24 hrs with fresh media and drug; harvested at the end of each treatment and various sub-cellular fractions were made following published protocols. Selected enzyme/ transporter activities were then measured using specific sub-cellular fractions or whole cell depending on the nature of the biochemical measurement.

The response of the system to the drug has two components:

1) The response of individual enzymes (present in cell extract) to a drug. Untreated cellular extract is used as the source of enzymes and drug at required concentration is added directly into the reaction mixture to estimate the direct effect on enzyme activity (f_d).

2) The adaptive response of the enzymes to the drug. Cells are treated with drugs (as described above), extracts made and enzyme activity assessed (f_a).

The following are the list of biochemical measurements used as input for *in silico* simulations:

1. Complex I specific malate pyruvate oxidation
2. Complex II specific succinate oxidation
3. Mitochondrial membrane potential
4. Fatty acid synthase (FAS)
5. Carnitine palmitoyl transferase 1 (CPT1)

6. Fatty acid influx (CD36)
7. Microsomal triglyceride transfer protein (MTP)
8. Gamma-Glutamyl cysteine synthase (γGCS)
9. Glutathione reductase (GR)
10. ROS generation

The Prediction Platform integrates *in vitro* measurements and pharmacokinetics (Table 1) to simulate *in vivo* effects.

Table 1

Compound ID	Cmax (ng/mL)	AUC (ng*h/mL)	Vd (L)	T1/2 (h)
Troglitazone	2820 (6.4 µM)	22100 (50 µM-hr)	10.5-26	16-34

Simulations

For each drug, altered activities measured experimentally are used as input to the model. Altered enzyme activity due to drug treatment expressed as fold change against normal.

The total impact of the **a drug** on **an enzyme** is given as f_d *X* f_a where f_d is the fold-change due to effect of the drug on the enzyme and f_a is the adaptive fold-change in the enzyme level.

Fold change in activity is expressed as a function of exposure (concentration of compound x time of treatment) when used for simulation. Fold change for direct effect is also expressed as a function of drug concentration which is considered as average *in vivo* plasma concentration.

All lab measured parameters are used together as input to the model. Bile transporter data, when available, is also used as input to the model. Simulations are performed to assess the impact of the drug on the liver for 5-10 days.

We typically perform 1X *(C_{max} equivalent)* - 10X exposure based prediction

- To span the effect that may be visible across a concentration-range
- To capture the effect of drug accumulation inside the tissue

The simulations estimate the

- *necrotic (depletion of ATP, GSH),*
- *steatotic (increase in cellular TG) and*
- *cholestatic (increase in serum bile and bilirubin)*

potential of a compound.

Results

IC50 w.r.t. cell viability:

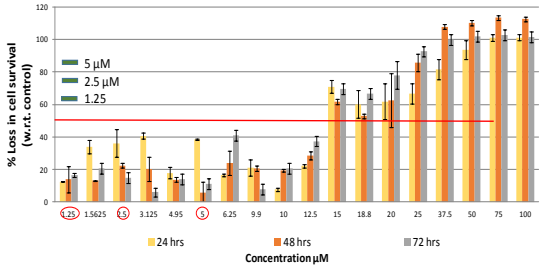


Fig.1a. Cell viability tested at 24, 48 and 72 hours with Troglitazone

50% cell death observed at 15 µM.

Troglitazone inhibits cellular energy synthesis via mitochondrial electron transport chain. 30% reduction in complex 1 specific malate pyruvate oxidation activity is obtained in indirect lab measurement (Fig. 1b); however, direct effect of drug indicates >80% reduction in complex 1 activity (Fig. 1c).

No inhibition in complex II specific succinate oxidation observed in the lab.

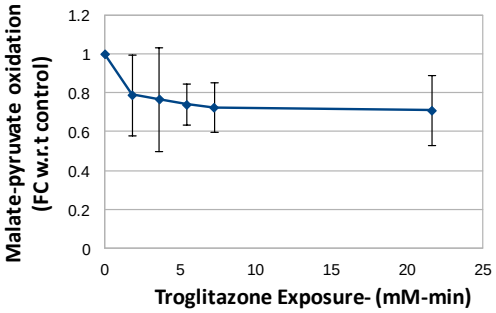


Figure 1b: 30% reduction in malate-pyruvate oxidation at longer exposure obtained in lab due to Troglitazone treatment (adaptive response)

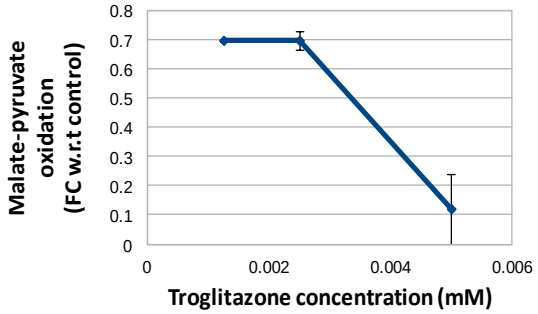


Figure 1c: Direct reduction in Complex I specific substrate oxidation at higher concentration of Troglitazone is observed in lab

Simulations are performed at 1X and 10X exposures. Reported plasma concentration of Troglitazone is 6.4 µM.

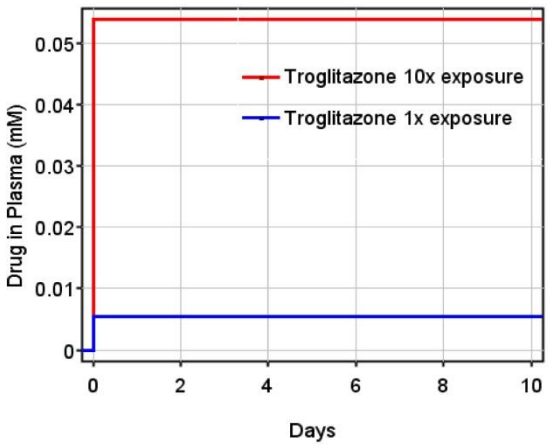


Figure 1d: Plasma profile. Simulations for Troglitazone performed at 1X and 10X exposure of average plasma drug concentration

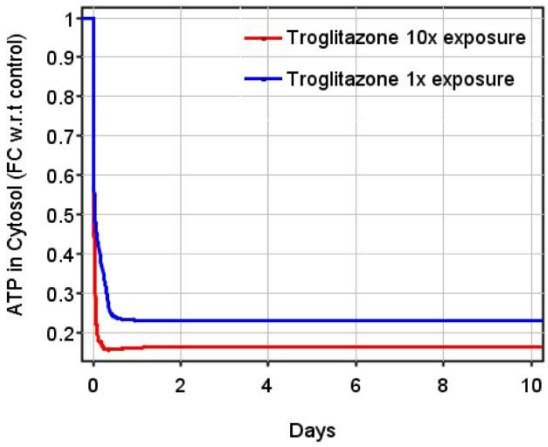
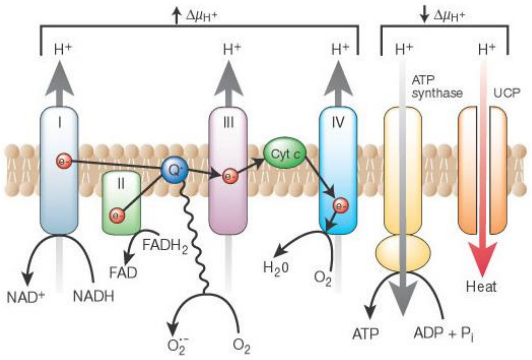


Figure 1e: Simulation predicts ~80% reduction in cellular ATP levels at both 1X and 10X exposure by Troglitazone

Simulations predict ~80% reduction is cellular ATP content (Fig. 1e) due to depolarisation of mitochondrial membrane potential (Fig.1g); inhibition in complex 1 activity in ETC causes depolarisation of mitochondrial membrane potential by the alteration in proton flow across the mitochondrial membrane.



http://www.nature.com/nature/journal/v414/n6865/fig_tab/414813a_F5.html

Figure 1f: Inhibition in complex I through IV in ETC cause reduction in mitochondrial membrane potential

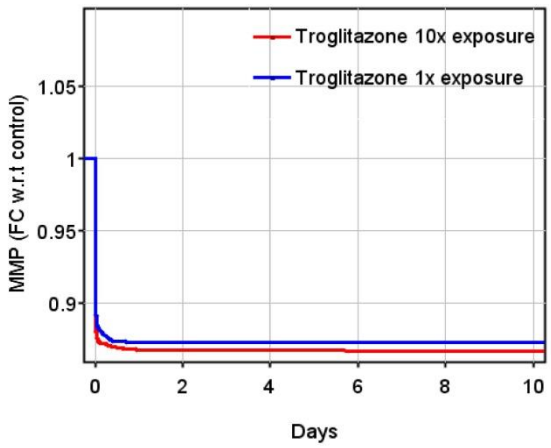


Figure 1g: Simulation predicts depolarization of mitochondrial membrane potential by Troglitazone at both 1X and 10X exposure

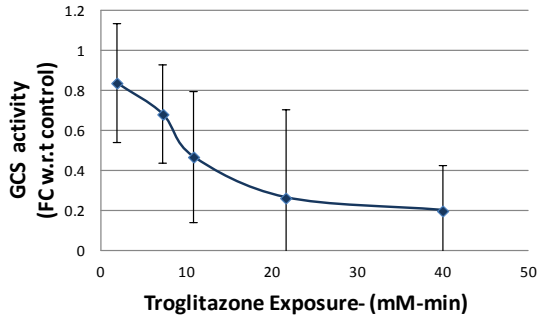


Figure 1h: 80% inhibition of γ-GCS activity at higher exposure by Troglitazone observed in the lab measurement

Enhanced reduction in GCS activity is observed in lab measured data with increase in exposure.

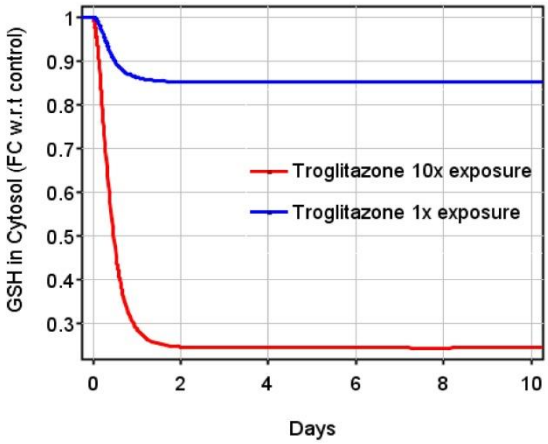


Figure 1i: Predicted 80% reduction in intracellular GSH with 10X exposure by Troglitazone

80% reduction in intracellular GSH (Fig. 1i) is predicted with 10X exposure (plasma concentration) due to inhibition in synthesis as observed in the lab (Fig. 1h) as well predicted by simulations (Fig 1j).

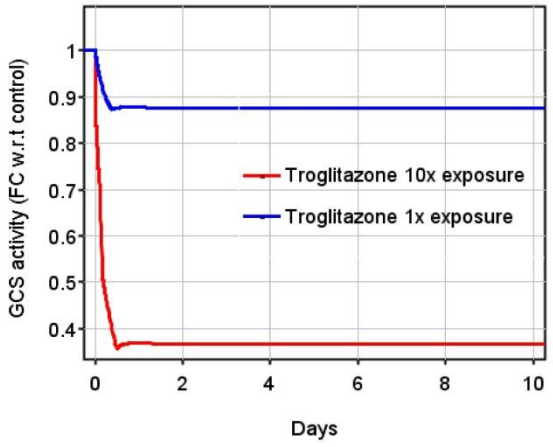


Figure 1j: Simulation predicts reduction in γ-GCS activity at 10X exposure by Troglitazone

Reduced GSH level may lead to oxidative stress in the system. Adaptive up-regulation in γ-GCS activity (Fig. 1h) by reduced GSH level is not observed by Troglitazone treatment.

Although simulations predict depletion in GSH, we have observed recovery in intracellular GSH level, from lowered level to normal, with increase in treatment concentration when cellular GSH content measured in the lab (Fig.1k).

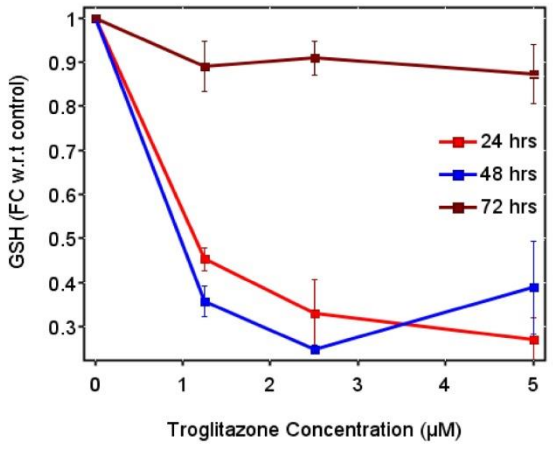


Figure 1k: Lab measured intracellular GSH level by Troglitazone

Based on both the experimental observations and model predictions we hypothesize that the compound may inhibit the transporter at later time points along with an initial inhibitory insult in GSH synthesis. Inhibition in transporter activity can block GSH/GSSG leaving the cell leading to build up of cellular GSH.

Analysis of bile transporter data for Troglitazone using Heptox model

Approach

Uptake and efflux transporter data was obtained from published literature (Table 2) for Troglitazone

Table 2

Troglitazone concentration (µM)	NTCP Activity (% inhibition)	BSEP activity (% inhibition)
1	1	20
5	5	56
10	25	72
50	50	93
100	75	97
Reference	Life Sci. 2007 Jan 30;80(8):732-40	Mol Pharmacol. 2001 Mar;59(3):627-35

Cholestatic potential of the compound is predicted at 0.5, 2.5 and 5 times exposure (Table 3).

Table 3: Simulated impact on predicted fluxes and metabolites (without considering decrease in cellular energy level due to mitochondrial alteration) for Troglitazone (given as fold change w.r.t. homeostasis)

	Transient Rise			Final Value		
Flux	**0.5X**	**3X**	**5X**	**0.5X**	**3X**	**5X**
NTCP	NC	NC	15	NC	1.75	3
OATP	NC	10.4	Very high	2.4	9.2	16
BSEP	NC	NC	NC	NC	0.63	0.5
Metabolites	**Transient Rise**			**Final Value**		
TCA cytosol	NC	9	27	1.23	4	7
TCA serum	NC	NC	Very high	~2	9	23

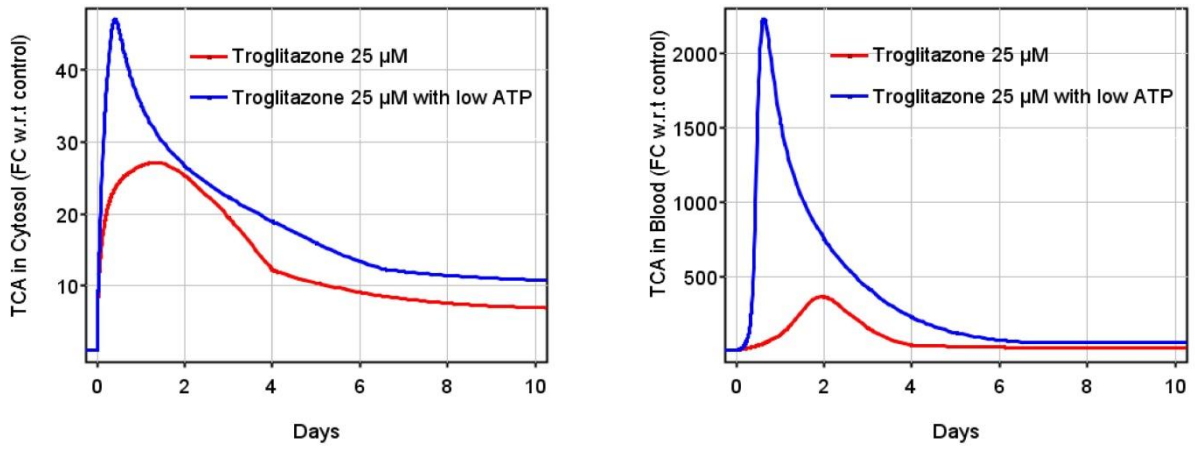


Figure 2a & b: Change in cytosolic and plasma TCA due to Troglitazone treatment; 25µM is equivalent to 5X exposure.

Does Troglitazone have an Idiosyncratic Potential?

A number of SNPs are reported to be associated with reduced expression of BSEP transporter and reduced taurocholate transport. In order to simulate the cholestatic potential of Troglitazone for individuals with reduced BSEP activity a set of virtual patients are created with variation in BSEP activity (0-90% reduction in V_{max} of BSEP transporter). Effects of Troglitazone on TCA level in plasma is simulated in these patients when treated with 0.5X and 2.5X exposure of the drug.

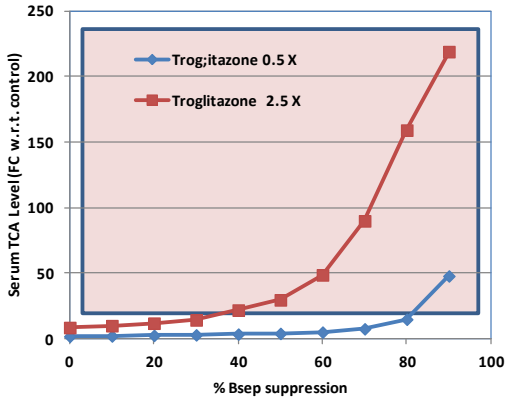


Figure 2c: BSEP suppression combines synergistically with increased Troglitazone treatment to cause cholestasis. Shaded zone indicates cholestatic levels of TCA

Simulations compared the susceptibility of individual towards Troglitazone induced cholestasis. Individuals with >40% inhibition in BSEP expression is predicted to be cholestatic by Troglitazone treatment at 2.5X Cmax exposure (Fig. 2c). Plasma TCA level above 5 fold is considered as on set of cholestatic injury.

Summary of Troglitazone: cells treated with 5, 2.5 & 1.25 µM concentration and simulations performed at 1X and 10X exposure:

- 70% ATP depletion at 1X & 10X expsoure
  - Troglitazone inhibits complex I specific substrate oxidation directly
- 80% GSH depletion at 10X plasma concentration
  - Alteration in ATP concentration and redox state is an indicator of necrotic potential of the compound
- No change in cellular TG level is predicted