A report on TAK-875 analysis using the Heptox™ Virtual Liver Platform
# EXECUTIVE SUMMARY

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW</th>
<th>Simulated exposures based on average drug plasma concentration (DPC)</th>
<th>In vitro test concentrations (µM)</th>
<th>Treatment duration for HepG2 (hour)</th>
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</thead>
<tbody>
<tr>
<td>TAK 875</td>
<td>524.625</td>
<td>10 µM (1<em>DPC or 1X), 20 µM (2</em>DPC or 2X), 40µM (4<em>DPC or 4X), 50µM (5</em>DPC or 5X), 100µM (10*DPC or 10X)</td>
<td>0.5, 5, 25, 50</td>
<td>24, 48, 72</td>
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## Necrotic Potential

1. Perturbation in mitochondrial ETC by TAK-875 causes depletion in cellular ATP due to depolarization of mitochondrial membrane resulting in potential for necrotic insult at 5X and higher.

## Steatotic Assessment

1. Tak-875 is predicted to be a direct inhibitor of fatty acid transport across the cell membrane that may lead to fatty acid build up in plasma; liver may use carbohydrate to provide cellular energy.
2. Inhibition in beta oxidation of fatty acid is caused directly by the drug and indirectly by reduced availability of substrate (due to inhibited fatty acid transport).
3. Reduction in ketone bodies formation by liver may lead to hypoketonemia in the system.

## Oxidative Stress Induction

1. 30% reduction in cellular GSH is predicted at 10X exposure.

### Model simulated output

- **ATP**: Simulation predicts greater than 80% cellular ATP depletion at 5X & 10X exposure.
- **TG**: Simulation predicts ~15% decrease in cytosolic TG at all 3 drug exposure by TAK-875.
- **GSH**: Simulation predicts 30% decrease in intracellular GSH at 4X & 10X exposures, but not at 1X exposure by TAK-875.
- **Depolarization of mitochondrial membrane is predicted with increase in exposure.**
- **Simulation predicts exposure dependent enhanced reduction in ketone body level (mitochondrial acetoacetate).**
Introduction

Heptox™ - DILI prediction platform is a combination of a virtual liver (in silico) 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.

Key model simulated metabolites and fluxes are monitored to

- assess the impact of 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

We can estimate cholestatic potential of a compound when transporter inhibition data is shared; we do not perform transporter assays.

In this report we have studied the molecular effect of TAK-875 and analysed its impact using our prediction platform. This report includes the testing method, the results obtained and analysis.

Methods

Experimental

HepG2 cells obtained from ATCC are maintained in recommended culture media in presence of 10 % FBS. For each compound a range of concentrations are picked to determine IC$_{50}$ as a measure of cell viability. Cells are treated daily over a period of 3 days and cell viability is estimated at the end of every 24 hrs. For detailed analysis, 3 non-cytotoxic concentrations (<50% inhibition) are selected for each compound. Cells are treated daily upto 72 hrs; harvested at the end of every 24 hrs and whole cell extracts made following published protocols. Selected enzyme and transporter activities (listed below) are then measured using specific subcellular fractions and whole cells depending on the nature of the measurement.

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

1) Direct response - The response of individual enzyme (present in cell extract) to drug: untreated cellular extract is used as source of enzyme and drug is added directly into the reaction mixture at specified concentration to estimate the direct effect on enzyme activity ($f_d$).

2) Indirect/adaptive response - The adaptive response of the enzyme to the drug: cells are treated with the drug (as described above), extracts made and enzyme activity assessed ($f_a$) at different time points.
The following are the list of biochemical measurements used as input for \textit{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
11. Hepatic transporters (if data is provided by sponsors)

The prediction platform integrates \textit{in vitro} measurements and pharmacokinetics to simulate \textit{in vivo} effects. The PK information of Tak-875 are used in the simulation to predict \textit{in vivo} effects.

**Simulations**

For each drug treatment, altered activities measured experimentally are divided by the untreated value to obtain a “fold-change” (FC) and used as input to the model.

The total impact of a \textbf{drug} on an \textbf{enzyme} is given as $f_d \times f_a$ 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.

$f_d$ is the enzyme inhibition caused by the presence of the drug and is a function of the drug concentration. $f_a$ is the adaptive change in the enzyme activity which is a function of drug exposure determined as concentration of compound x time of treatment (e.g. 10 μM x 1440 min [24h]).

All lab measured parameters are used together as input to the model. Hepatic 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 prediction in the exposure range of 1X – 10X of normal exposure (average plasma drug concentration) to understand the therapeutic window and to account for drug accumulation (if any) in the tissue.

The simulations estimates the potential of a compound to cause

- necrosis (depletion of ATP, GSH),
- steatosis (increase in cellular TG) and
- cholestasis (increase in serum bile and bilirubin).
Results

Cell viability is assayed using a range of concentration for 3 different time points.

Figure 1: Cell viability test at 24, 48 & 72 hrs with TAK-875; 0.5, 5, 25 & 50 µM concentrations are used for further analysis

We aim to understand the mechanistic rationale behind the progression of toxicity. Hence, concentrations spanning less than 50% inhibition (i.e. non cytotoxic) are selected for detailed analysis.

40% cell death observed with 50 µM concentration upon 48 & 72 hrs treatments (Fig.2).

Lab measurements are plotted as measurement (FC w.r.t. control) vs Concentration (of TAK-875) and model simulated outputs are plotted as simulated value vs Days.

Estimation of necrotic potential

TAK-875 indicates alteration of various mitochondrial targets. Both complex 1 and complex 2 specific substrate oxidations are reduced by 80% at highest treatment concentration as observed in both indirect and direct measurement (Fig 2-5).

Figure 2: Greater than 70% inhibition in malate pyruvate oxidation mediated Complex I activity by TAK-875 at higher treatment concentrations

Figure 3: 90% inhibition in succinate oxidation mediated Complex II activity by TAK-875 at higher treatment concentration
With the available PK information simulations are performed at various exposure levels. Reported average plasma concentration of TAK-875 is ~10 µM.

Model simulation predicted exposure dependent depolarisation of mitochondrial membrane (Fig. 6).

We observed time and dose dependent reduction in rhodamine uptake by intact mitochondria (Fig. 7), a measure of mitochondrial membrane potential, corroborating the model simulated outcome.
Simulation predicted TAK-875 induced mitochondrial alteration leading to reduction in cellular energy content. At 5X exposure and higher, cellular ATP content drops to ~ 10% of normal (Fig. 8).

**Estimation of steatotic potential**

Fatty acid transport across cell membrane is inhibited in a dose and time-dependent manner in both the adaptive and direct measurements.

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**Figure 8**: Simulation predicts greater than 80% cellular ATP depletion at highest exposure by TAK-875

**Figure 10**: Direct effect of various concentrations on fatty acid transport by TAK-875 indicates a dose dependent inhibition - >80% at 30µM concentration

*We hypothesize that TAK-875 may be a direct inhibitor of fatty acid entry and alteration in indirect measurement at later time points is an adaptive response.*

We have observed reduction in FAS enzyme activity in indirect measurement (Fig 11); FAS is the rate limiting enzyme in *de novo* fatty acid synthesis. Direct inhibition is not observed (data not shown).

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**Figure 9**: Down regulation of fatty acid transport by TAK-875. Inhibition is maximum at 48 hours of treatment but recovers by 72 hours.

**Figure 11**: *In vitro* measurement indicates enhanced lowering of FAS activity at 48 and 72 hrs treatments by TAK-875.
The rate limiting enzyme for beta oxidation of fatty acid, CPT1 activity is inhibited significantly in direct measurement (Fig 12) more than indirect (not shown).

Figure 12: In vitro direct measurement indicates lowered CPT1 activity by TAK-875 with increase in treatment concentration.

TAK-875 treatment perturbs fatty acid metabolism network at different points – 1) decrease in fatty acid entry, 2) decrease in beta oxidation of fatty acids & 3) decrease in fatty acid synthase activity.

More than 50% reduction in CPT1 flux is also predicted at 1X and higher exposure by TAK-875 (Fig. 13).

Figure 13: Simulation predicted lowering in CPT1 flux by TAK-875 at higher exposure

Inhibition in FAS flux as predicted in Fig. 14 leads to a build up of cytosolic MalonylCoA (MCoA) content (Fig.15) inhibiting CPT1 further.

Figure 14: Simulation predicted exposure based enhanced reduction in FAS flux due by TAK-875.

Figure 15: Simulation predicted increase in MCoA by TAK-875

10x of TAK-875 is ~70-100 μM and the highest concentration used in the lab is 50 μM. Effect at the higher concentration was extrapolated from the concentration-effect curve and used as an input for the simulations.
The downstream impact of changes in fatty acid metabolism by TAK-875 was compared at various exposure levels by monitoring the simulated changes in mitochondrial acetoacetate (AcAc) and beta-hydroxybutyrate (BHB). Both AcAc and BHB together are referred to as ketone bodies.

**Estimation of oxidative stress**

Lab measurement indicates direct inhibition on GCS activity by Tak-875 (Fig. 18); GCS is the rate limiting enzyme in GSH synthesis process. ~20-25% inhibition in GSH synthesis is predicted by model simulation higher exposure (Fig. 19).

The significant inhibition on fatty acid transport and CPT1 flux is simulated to cause a reduction in mitochondrial AcAc and BHB due to Tak-875 treatment. 40-60% exposure based reduction in AcAc and BHB is predicted (Figure 16 & Figure 17).
Conclusion

HepG2 cells are treated with various concentrations of TAK-875 and model simulations are performed at various exposure levels.

- Perturbation in mitochondrial ETC causes depletion of cellular ATP due to depolarisation of mitochondrial membrane resulting in potential for necrotic cell death at higher exposure
- Minor change in cellular GSH is predicted
- Tak-875 is predicted to be a direct inhibitor of fatty acid transport into the cell which may lead to fatty acid build up in plasma and may push liver to use more carbohydrate to provide the required energy. The inhibition of fatty acid beta oxidation is caused by directly by the effect of drug as well as indirectly by reduced availability of substrate
- Reduction in formation of ketone bodies by liver may lead to hypoketonemia in the system (Curr Opin Gen Surg. 1993:78-84, Bulletin of the Osaka Medical College 49 1, 211-16, 2003), making the system vulnerable to toxicity. Ketone bodies, produced from the liver, are transported to other tissues for energy production via mitochondrial TCA cycle. Under adverse condition brain gets a portion of its energy from ketone bodies.