Fasiglifam (TAK-875): Investigating drug induced liver injury utilising *in-vitro* mechanistic assays

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INTRODUCTION

- TAK-875 (Fasiglifam) a GPR40 agonist, developed for the treatment of type 2 diabetes and was voluntarily withdrawn from phase III clinical trials due to adverse liver effects.
- Early preclinical data failed to identify hepatotoxicity.
- TAK-875 is highly protein bound (99.84%), with a human C_{max} value of 10µM (free plasma ~14 nM).

METHODS

Glucose/galactose cytotoxicity assay (Glu/Gal)

- HepG2 cells were seeded in 96 well flat bottom plates and allowed to adhere for 24 hours.
- Media was exchanged to DMEM containing either 10mM galactose or 25mM glucose prior to the assay in either the presence or absence of 10% serum.
- Cells were exposed to compounds for 24 hours, and cytotoxicity assessed using the MTT assay. Compounds were classified as positive if a 3 fold shift in sensitivity was observed in the galactose conditions compared to that in glucose.

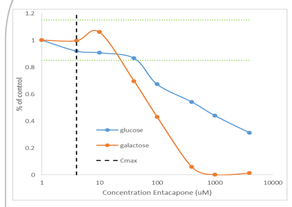

Figure 1: Graphical representation of a mitochondrial toxicant.
Shift in galactose Glu/Gal assay showing a 10 fold sensitivity with entacapone (Gal conditions).

Measurement of mitochondrial membrane potential.

- HepG2 cells were plated on TC treated 96 well plates. Cryopreserved primary human hepatocytes (PHH) were seeded onto collagen coated 96 well plates.
- Following exposure to TAK-875 for 24 hours cells were labelled with either Rhodamine 123, TMRE (Tetramethylrhodamine, Ethyl Ester, Perchlorate) or MitoTracker deep red (Mitochondrial Membrane Potential: MMP) and Hoechst (Cell count), by incubation for 30 minutes.
- Alternatively the dyes were pre-loaded into the cells for 30 minutes prior to dosing.
- Fluorescent images were acquired using the confocal mode of an ArrayScan™ XTI HCS reader (ThermoScientific) following with cellular ATP, which was measured using 3D CellTiter-Glo (Promega).

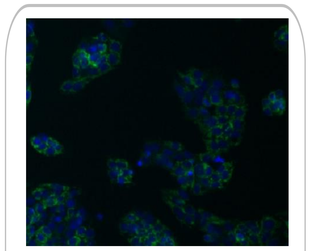

Figure 2: Representative data showing Rhodamine 123 staining (green) of mitochondrial membrane potential in HepG2 cells. Nuclear material (Blue).

Mitochondrial function (Agilent Seahorse XF^{e}96 flux analyser)

- HepG2 cells, rat hepatocytes or cryopreserved human hepatocytes (data not shown) were plated on XF^{e}96 seahorse plates which were pre-coated with collagen for the cryopreserved human and rat hepatocytes.
- Cells were either dosed followed by immediate measurements (acute 0hr) or following either by 1 or 24 hour pre-incubation with test compound prior to analysis of ECAR, OCR and reserve capacity.
- A stress test was performed according to manufactures instructions. Effects on any measured parameter within 100x C_{max} was previously shown to have a higher potential to result in mitochondrial toxicity[1]

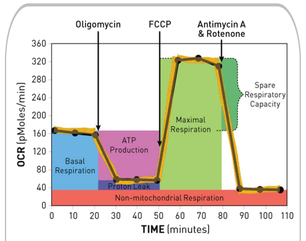

Figure 3: Schematic diagram of the mitochondrial stress test, using the selective inhibitors: oligommycin, FCCP, antimycin A and rotenone.

RESULTS

TAK-875 demonstrates a potential mitochondrial toxicity liability

Glucose/galactose cytotoxicity assay (Glu/Gal)

HepG2 cells were incubated with TAK-875 for 24 hours in glucose or galactose containing media in either the presence or absence of serum. In the absence of serum, TAK-875 was identified as a mitochondrial toxicant with a 3.59-fold shift in toxicity.

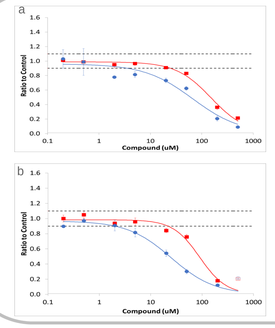

Figure 4: Effect of TAK-875 on cytotoxicity in HepG2 cells in glucose compared to galactose containing media
HepG2 cells were incubated with TAK-875 for 24 hours in either glucose (squares) or galactose (circles) containing media. These were cultured in either the presence (a) or absence (b) of serum.

- In the presence of serum there was no mitochondrial toxicity identified, with only a 2.26-fold increase in cytotoxicity in galactose containing media compared to glucose. The AC50 values were 66.7 compared to 151uM respectively.
- In the absence of serum there was a marked shift in toxicity in the galactose containing media of 3.59-fold compared to glucose. The respective AC50 values were 24.9 and 89.4uM.
- In the absence of serum TAK-875 was identified as a mitochondrial toxicant.

TAK-875 shows an increase in toxicity in the absence of serum

HepG2 cells were incubated with TAK-875 for 24 hours in glucose or galactose containing media in either the presence or absence of 10% serum, following which cell count and cellular ATP were determined. Reduction in both cell count and cellular ATP was seen in all conditions, but was to be most sensitive in serum free galactose containing media.

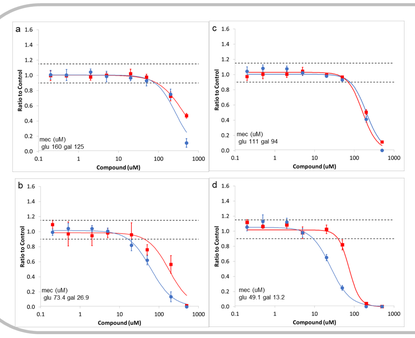

Figure 5: Effect of TAK-875 on cell health in HepG2 cells in glucose compared to galactose containing media
HepG2 cells were incubated with TAK-875 for 24 hours in either glucose (squares) or galactose (circles) containing media. These were cultured in either the presence (a and c) or absence (b and d) of serum.

- Cell count (a and b) was affected under all assay conditions, but was most evident in serum free galactose media (c).
- Cellular ATP (c and d) was also reduced under all conditions, again cells cultured in serum free galactose media appeared to be the most sensitive.
- Effects on cellular ATP was seen before cell loss

TAK-875 inhibits mitochondrial membrane potential

HepG2 cells were incubated with TAK-875 for 24 hours in serum free media. Mitochondrial membrane potential (MMP) was measured using TMRE, Rhodamine 123 or Mitotracker Deep red. Dyes were added for 30 minutes, either pre or post compound dosing.

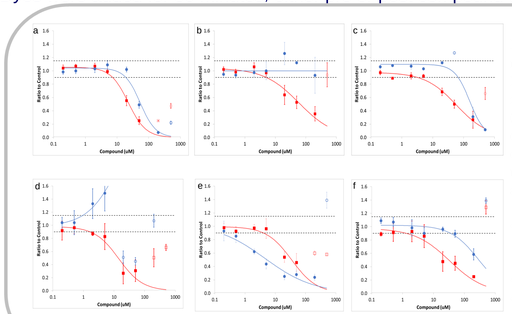

Figure 6: Effect of TAK-875 on mitochondrial membrane potential (MMP) and cell number
HepG2 cells were incubated with TAK-875 for 24 hours and cell count (squares) and MMP (circles) were determined. Dye was added either before (a,b and c) or post (d, e and f) exposure to compound. MMP was measured using either TMRE (a and d), rhodamine (b and e) or Mitotracker Deep Red (c and f).

- Pre-incubation with TMRE shows an increase at lower concentrations but no decrease in potential.
- Rhodamine shows clear decrease in MMP.
- Mitrotracker is less sensitive than cell count.

TAK-875 inhibits mitochondrial respiration

Mitochondrial Function (Agilent Seahorse)

The effects of TAK-875 on mitochondrial function was measured using the Agilent Seahorse Flux Analyser. HepG2 cells were incubated with TAK-875 for 0, 1 or 24hr. Inhibition of mitochondrial respiration was seen immediately following the addition of compound to the cells.

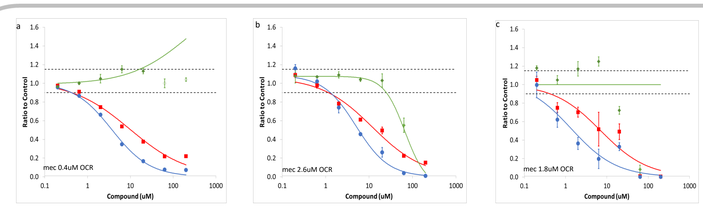

Figure 7: Effect of TAK-875 on mitochondrial function in HepG2 cells
HepG2 cells were incubated with TAK-875 for 0 (a) , 1 (b) or 24 (c) hours in serum free media prior to the assay using the seahorse flux analyser. The immediate effects of compound on mitochondrial function was determined by injecting the compound onto the cells whilst in the machine and measuring effects immediately. Oxygen consumption (OCR; squares), reserve capacity (circles) and extracellular acidification (ECAR: diamonds) were measured.

- Inhibition of OCR was seen immediately following the addition of TAK-875 to HepG2 cells. This was accompanied by an increase in ECAR, suggesting an inhibition of the electron transport chain.
- Following either 1 or 24hr the effects on OCR were accompanied by decreases in ECAR suggesting the mitochondrial toxicity is the initiating mechanism for toxicity. There was no temporal shift in toxicity with the increased incubation period.

RESULTS

- Several standard *in vitro* approaches use assay conditions containing serum. It is important to consider protein binding in these assays as is shown for TAK-875 were serum can mask potential assay outcome and sensitivity.
- Due to the highly protein bound nature of TAK-875, we assessed a number of routine cell health end-points under serum and serum free assay conditions. This resulted in a marked shift in cellular ATP (cytotoxicity) and interestingly it was also identified as a potential mitochondrial toxicant using the Glu/Gal assay (serum free only).
- Mitochondrial toxicity was confirmed using the seahorse assay, in which TAK-875 was shown to be an ETC inhibitor at less than total plasma C_{max} concentrations (~10µM) in agreement with Otieno et al[2].
- It was also shown that Mitochondrial Membrane Potential was disrupted by TAK-875 using high content imaging and MMP dyes, TMRE and Rhodamine, prior to dosing with TAK-875 show changes in MMP at lower concentrations than observed cell loss.

SUMMARY/CONCLUSIONS

- Taking into account the high protein binding of this compound, serum free conditions were utilized in a variety of *in vitro* assays, exhibiting clear increases in sensitivity.
- Using a multi-parametric approach we have established that TAK-875 interferes with mitochondrial respiration and membrane potential which is accompanied with reduced cellular ATP.
- These findings are seen before there is cell loss, suggesting this is a plausible mechanism for TAK-875 induced toxicity.
- We recommend that protein binding is taken into account when designing appropriate assays.

REFERENCES

[1] Eakins, J.A et al, (2016): Toxicology In Vitro (34):161-170;
[2] Otieno M.A et al, (2017) Toxicological Sciences, 16 Feb 2017.

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