Multiparametric *in vitro* toxicity approaches to understand the hepatotoxic mechanism of action of Fasiglifam (TAK-875)

Julie Eakins, Richard Maclennan, Paul Walker, Derek Angus

cyprotex AN EVOTEC COMPANY

Introduction

- TAK-875 (Fasiglifam) a GPR40 agonist, developed for the treatment of type 2 diabetes, was voluntarily withdrawn from phase III clinical trials due to adverse liver effects.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

High content imaging platform.

- HepG2 cells were plated on TC treated 96 well plates. Cryopreserved primary human hepatocytes (PHH) were seeded onto collagen coated 96 well plates. Human liver microtissues (hLiMTs) were formed using scaffold free 96-well ultra low attachment round bottom plates (Corning®).
- Following exposure to TAK-875 for 1, 24 hours (HepG2 or PHH) or 14 days (hLiMTs), cells were labelled with either Syto11 (Cell count), monochlorobimane (mBCl; GSH content), dihydroethidium (DHE; ROS formation) and MitoTracker deep red (Mitochondrial Membrane Potential: MMP) by incubation for 30 minutes.
- Alternatively cell were stained with TMRE (Tetramethylrhodamine, Ethyl Ester, Perchlorate: MMP) and Hoechst (Cell count).
- 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).

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

- HepG2 cells or cryopreserved 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 1 or 24 hours pre-incubation.
- A stress test performed according to manufactures instructions. Effects on any measured parameter within 100x C_{max} shown to have a higher potential to result in toxicity[1]

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.
- 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.

Results.

HepG2 and PHH were incubated with TAK-875 for either 1 or 24 hrs. PHH showed a decrease in MMP at both 1 and 24 hours, along with decrease in GSH at 24 hours. HepG2 showed no toxicity at 1 hour but decrease in both cell number and ATP at 24 hours (Figure 1 and Table 1).

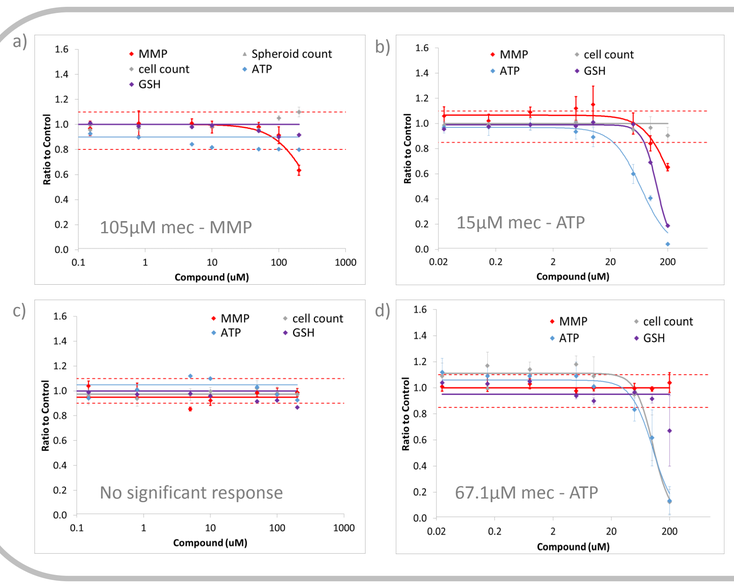

Figure 1: Effect of TAK-875 on cell health in PHH and HepG2 cells

Cryopreserved primary human hepatocytes (a and b) and HepG2 cells (c and d) were incubated with TAK-875 for 1 hour (a and c) or 24 hours (b and d) prior to assessment of cell count, MMP, ATP and GSH content.

- At 1 hour exposure only human hepatocytes exhibited a response with MMP at 105µM (minimum effective concentration: mec).
- At 24 hours exposure human hepatocytes remain the most sensitive with cellular ATP depletion observed at 15µM (mec).

- TAK-875 was shown to decrease cellular ATP in both HepG2 and PHH at 24 hours. PHH also showed decreases in MMP, suggesting impairment of mitochondrial function.
- TAK-875 inhibited mitochondrial respiration which was seen immediately following the addition of compound (0 hour), suggesting an inhibition of the electron transport chain resulting in cytotoxicity at 24 hours (Figure 2 and Table 1).
- Under standard culture conditions there was no shift in cytotoxicity observed between the glucose and galactose media (Figure 3a) however, in the absence of FBS cytotoxicity under galactose conditions was 3.67 fold greater than glucose (Figure 3b and Table 1).

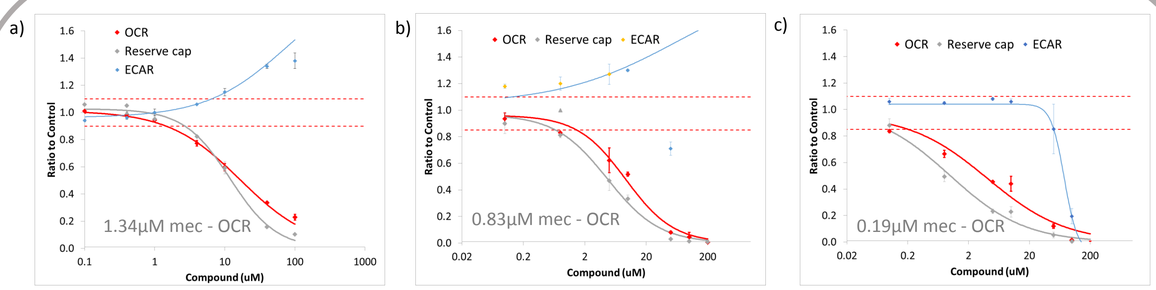

Figure 2: Effect of TAK-875 on mitochondrial respiration in HepG2

HepG2 cells were incubated for either 0 hour (a), 1 hour (b) or 24 hours (c) prior to measurement of oxygen consumption rate, reserve capacity and extracellular acidification rate. Following a 24 hour incubation Oxygen Consumption Rate (OCR) and Reserve Capacity (RC) both show a significant decreases 0.19µM and <0.1µM respectively.

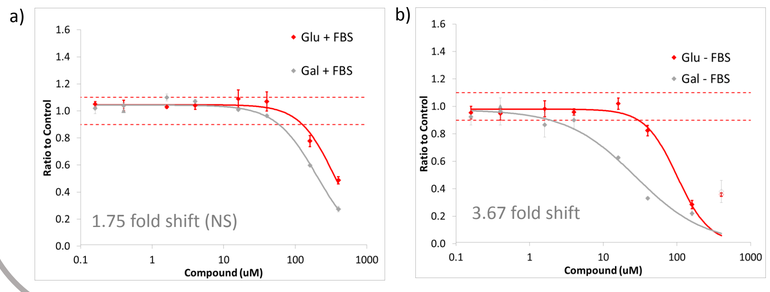

Figure 3: Effect of TAK-875 on cytotoxicity in HepG2 under glucose and galactose culture conditions

HepG2 cells were incubated for 24 hours in either high glucose or galactose containing media supplemented with either FBS (a) or without FBS (b).

A significant shift in dose response curves (3.67 fold shift) was only observed in the absence of FBS (b).

Results (continued)

- Effects on ATP depletion are dependent on the absence or presence of FBS. In HepG2 cells the presence of FBS results in depletion of ATP after 24hr incubation only, with an MEC of 67µM. In the absence of FBS this is reduced significantly to approximately 10µM, equating to the human C_{max} (Figure 4 and Table 1.

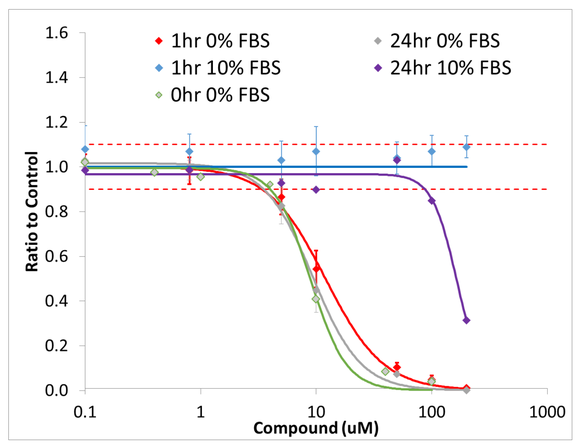

Figure 4: Effect of TAK-875 on HepG2 cellular ATP content at 1 hour and 24 hours with and without FBS

HepG2 cells were exposed to TAK-875 for either 0, 1 or 24 hours in the presence or absence of serum (FBS).

- At 1 hour exposure with FBS no response in cellular ATP was observed and following 24 hours cellular ATP was depleted with a mec of 67µM (Table 1).
- At 0, 1 and 24 hours exposure in the absence of FBS significant reduction in cellular ATP was observed with an mec of 8.93, 12 and 9.34µM respectively.
- No temporal shift was observed with cellular ATP depletion.

- Further evaluation on mitochondrial membrane potential (MMP) were performed by analyzing HepG2 cells cultured in either glucose or galactose media in the presence or absence of FBS measuring cell count, MMP using high content screening and cellular ATP.
- Absence of FBS again exhibited greater sensitivity in cellular response as can be expected from the high protein binding. For cells cultured in glucose a 2-2.3 fold shift in MEC was observed between the presence and absence of FBS for MMP, cell count and ATP. In galactose media a fold shift on MEC on cell count, MMP and ATP were 4.6, 2.7 and 7.2 fold respectively between presence and absence of FBS. Only cellular ATP showed a significant shift in glu and gal conditions in the absence of FBS (3.7 fold, figure 5).

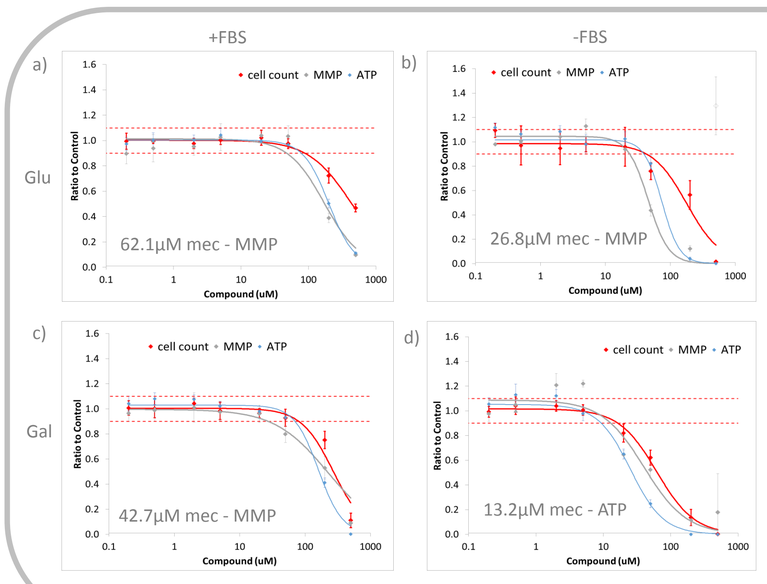

Figure 5: Effect of TAK-875 on cell count, mitochondrial potential (MMP) in HepG2 under glucose and galactose culture conditions, in the presence and absence of FBS

HepG2 cells were incubated for 24 hours in either high glucose (a and b) or galactose (c and d) containing media supplemented with either FBS (a and c) or without FBS (b and d).

- Shifts were observed in all three features, cell count (CC), mitochondrial potential (MMP) and cellular ATP, in the direction of >Glu (FBS) >Gal (FBS) >Glu (-FBS) > Gal (-FBS).

	+FBS			-FBS		
	CC	MMP	ATP	CC	MMP	ATP
Glu	160	62.1	111	73.4	26.8	49.1
Gal	125	42.7	94	26.9	16	13.2

- A variety of *in vitro* approaches have been assessed in a number of cellular models following TAK-875 exposure. Mitochondrial respiration in HepG2 cells exposed for 1 to 24 hours exhibited the lowest observed responses below 1µM.
- The data shows that TAK-875 inhibits mitochondrial function, reduced mitochondrial membrane potential and cellular ATP in serum free conditions, explained by the high protein binding characteristics of this compound.

Assay	FBS	Cell Type	Time Point	MEC (µM)	Feature
GSH, ROS, MMP	-	PHH	1hr	105	MMP
			24hr	63.7	GSH
	+	HepG2	1hr	NR	-
			24hr	70	CC
	-	hLiMTs	14d	26.1	MMP
Cellular ATP	+	HepG2	1hr	NR	-
			24hr	67.1	ATP
	-	PHH	1hr	NR	-
			24hr	15	ATP
		HepG2	0hr	8.93	ATP
			1hr	12	ATP
			24hr	9.34	ATP
		hLiMTs	14d	150	ATP
Glu/Gal	+	HepG2	24h	62.1	Glu (TMRE)
	-			42.7	Gal (TMRE)
	+			26.8	Glu (TMRE)
	-			13.2	Gal (ATP)
	+			180	Glu (MTT)
	-			90.5	Gal (MTT)
				47.7	Glu (MTT)
				5.36	Gal (MTT)
Mitochondrial respiration	-	PHH	ohr	2.75	OCR
		RH		1.72	OCR
		HepG2		1.34	OCR
				3.45	RC
			1hr	**0.83**	**OCR**
				0.95	**RC**
			24hr	**0.19**	**OCR**
				<0.1	**RC**

Table 1: Summary of effects of TAK-875 on various *in vitro* assays and cellular models

Data presented shows the minimum effective concentration (mec) for each *in vitro* assay. The mec is the earliest concentration at which the compound deviates from the vehicle control and the first response, where appropriate, is shown.

- The seahorse assay accurately identified TAK-875 as a mitochondrial toxicant, predominantly exhibiting a electron transport chain (ETC) inhibitor at 1 and 24 hours, with OCR and RC <1µM.
- Between 1 and 5µM again mitochondrial respiration was the most sensitive in HepG2 cells, primary human hepatocytes and rat hepatocytes.
- However, the Glu/Gal assay did not show a significant shift (1.75) under standard conditions.
- Mitochondrial respiration is determined in serum free media as such other in vitro approaches were utilised in the presence and absence of serum. –FBS conditions gave responses in cellular ATP and MTT between 5 to 15µM.

Conclusions

- The seahorse assay identified TAK-875 as a mitochondrial toxicant with effects at less than total plasma $C_{max,}$ (~10µM) in agreement with Otieno et al[2]. TAK-875 is highly protein bound (99.84%), giving an estimated free plasma concentration of 14nM. Here we show responses around 10x free concentrations.
- 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 (up to 7.2 fold).
- This multiparametric approach identified TAK-875 as a mitochondrial toxicant and ETC inhibitor.

References

[1] Eakins, J et al (2016): TIV (34):161-170; [2] Otieno M et al, ToxSci (2017) Feb 2017

Corporate Headquarters: Evotec AG, Manfred Eigen Campus, Essener Bogen 7, 22419 Hamburg, Germany **Email:** info@evotec.com **Web:** www.evotec.com