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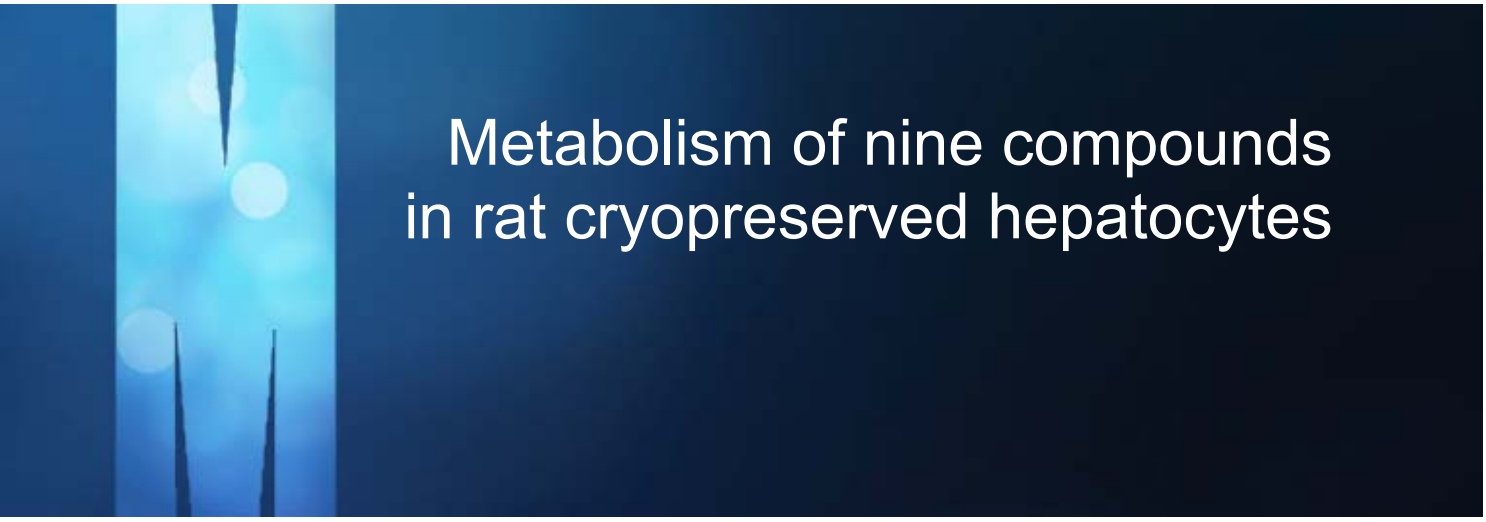

Metabolism of nine compounds in rat cryopreserved hepatocytes

MMV_OSDD

Report #: CDCO_MMV_OSDD_18_002

21 June, 2018

Quality Statement:

This non-GLP study was conducted using established techniques in accordance with the relevant guidelines and standard operating procedures (SOPs) of the Centre for Drug Candidate Optimisation, Monash University. This report accurately reflects the raw data obtained during the performance of this study.

The results described represent part of an on-going lead optimisation/drug discovery program. The study has been conducted to provide preliminary information on the *in vitro* metabolic characteristics of the candidate compound(s) under standard incubation conditions. A complete kinetic analysis has not been conducted.

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Study number(s):	MMV_OSDD_18_002

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cdco CENTRE FOR DRUG CANDIDATE OPTIMISATION

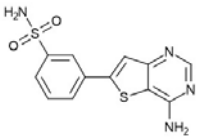
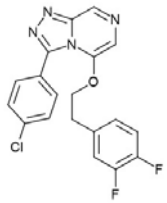
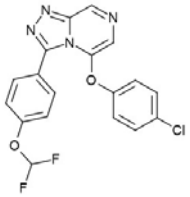
A. Study Objective

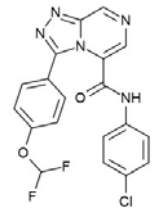
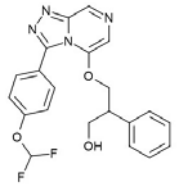
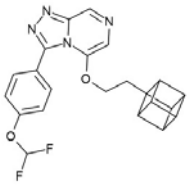
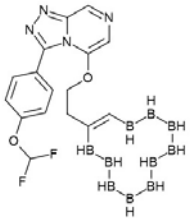
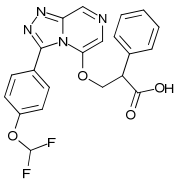
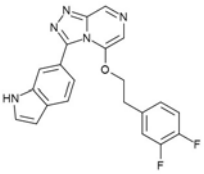
To determine the *in vitro* metabolic stability of nine compounds in rat cryopreserved hepatocytes.

B. Results and Discussion

- MMV025100 exhibited a very low rate of clearance in rat cryopreserved hepatocytes, below the limit of sensitivity for the assay (see Table 1 and Experimental Methods).
- MMV669784, MMV670246, MMV1576784 and MMV1579336 exhibited low rates of clearance and MMV639565, MMV693155, MMV897700 and MMV1579341 exhibited moderate rates of clearance in rat cryopreserved hepatocytes (Table 1).
- Note that the impact of binding to hepatocyte and plasma proteins has not been taken into account in the prediction calculations.

Table 1: Metabolic stability parameters for nine compounds incubated at a concentration of 0.5 µM in rat cryopreserved hepatocytes.

Compound (Batch)	**Degradation half-life** (min)	***In vitro* CL_{int}** (µL/min/ 10^6 cells)	**Predicted CL_{int}** (mL/min/kg)	**Predicted CL_{blood}** (mL/min/kg)	**Hepatocyte Predicted E_H**	**Degradation Rate Classification**
 **MMV025100** (MNB6-7)	> 375	< 1	< 5	< 5	< 0.07	low
 **MMV639565** (AEW 302-1)	52	7	34	22	0.33	intermediate
 **MMV669784** (EGT 302-1)	362	1	5	4	0.07	low

 **MMV670246** (EGT 95-3)	199	2	9	8	0.11	low
 **MMV693155** (EGT 198-4)	29	13	61	32	0.47	intermediate
 **MMV897700** (EGT 65-1)	26	14	67	34	0.50	intermediate
 **MMV1576784** (EGT 257-1)	85	4	21	16	0.23	low
 **MMV1579336** (EGT 199-5)	224	2	8	7	0.10	low
 **MMV1579341** (MK035-1)	34	11	52	29	0.43	intermediate

C. Experimental Methods

Incubation methods:

The hepatocyte stability assay was conducted by incubating each compound at a concentration of 0.5 µM with suspensions of rat cryopreserved hepatocytes (Xenotech Lot # 1710067) in Krebs-Henseleit buffer (KHB) at 37°C at a cell density of 1 - 2 x10^6 viable cells per mL. Cell viability was determined by the Trypan Blue exclusion method (in the absence of test compound) and the average viable cell counts determined over the incubation period were included in the calculations. At various time points over a 90 minute incubation period, samples were immediately quenched by adding acetonitrile containing diazepam as internal standard.

Analytical conditions:

Instrument:	Metabolic stability: Waters Xevo G2 QTOF MS coupled to a Waters Acquity UPLC
Detection:	Positive electrospray ionisation under MS^E mode
Cone Voltage:	30 V
Column:	Ascentis Express C8 column (50 x 2.1 mm, 2.7 µm)
LC conditions:	Gradient cycle time: 4 minutes; Injection volume: 5 µL; Flow rate: 0.4 mL/min
Mobile phase:	Acetonitrile-water gradient with 0.05% formic acid

Calculations:

Test compound concentration versus time data in hepatocytes were fitted to an exponential decay function to determine the first-order rate constant for substrate depletion. In cases where clear deviation from first-order kinetics was evident, only the initial linear portion of the profile was utilised to determine the degradation rate constant (k). The substrate depletion rate constant was used to calculate a degradation half-life [1]. The analogous hepatocyte $CL_{int,\ in\ vitro}$ and hepatocyte predicted CL_{int} values were calculated by taking into account hepatocellularity and liver mass for rats according to equations [2] and [3] below.

[1] $$t_{1/2} = \frac{\ln(2)}{k}$$

[2] $$\text{Hepatocyte } CL_{int,\ in\ vitro} = \frac{k}{\text{hepatocyte cell number } (10^6 \text{ viable cells/mL})}$$

[3] $$\text{Hepatocyte } CL_{int} = CL_{int,\ in\ vitro} \times \frac{\text{liver mass (g)}}{\text{body weight (kg)}} \times \frac{\text{hepatocellularity } (10^6)}{\text{g liver mass}}$$

The following scaling parameters were assumed in the above calculations [a]:

Species	Viable cell count in the assay (10^6 cells/mL)	Liver mass (g liver/kg body weight)	Hepatocellularity ($x10^6$ cells/g liver mass)	Hepatic blood flow (Q) (mL/minute/kg body weight)
Rat	1.9	36.6	128	67.6

The hepatocyte predicted *in vivo* blood clearance values (CL_{blood}) [4] and predicted *in vivo* hepatic extraction ratios (E_H) [5] were subsequently calculated using the hepatic blood flow values (Q) for each species described above.

[4] $$CL_{blood} = \frac{Q \times CL_{int}}{Q + CL_{int}}$$

[5] $$E_H = \frac{CL_{blood}}{Q} = \frac{CL_{int}}{Q + CL_{int}}$$

Predicted hepatic extraction ratios (E_H) based on the relative rates of compound degradation *in vitro,* classify compounds as potentially being subject to low (<0.3), intermediate (0.3 - 0.7), high (0.7 - 0.95) or very high (> 0.95) *in vivo* hepatic extraction.

The assumptions underlying the calculation of the *in vivo* hepatic intrinsic clearance based on the rate of degradation in hepatocytes are stated below.

ASSUMPTIONS:
Calculations of intrinsic clearance are based on the "*in vitro* $T_{1/2}$ method" [b] which assumes:

1) The substrate concentration employed is well below the apparent K_M for substrate turnover; and,
2) There is no significant product inhibition, nor is there any mechanism-based inactivation of enzyme.

The use of hepatocytes in the prediction of the *in vivo* hepatic clearance and extraction ratio assumes that hepatic metabolic clearance is the major clearance mechanism for compounds *in vivo*;

Data should be considered within these terms of reference.

The limit of sensitivity of the hepatocyte assay corresponds to 15% loss of compound over the assay duration. For compounds showing < 15% loss over 90 min (i.e. degradation half-life > 375 min) metabolic stability parameters are reported as below:

Species	*In vitro* CL_{int} (µL/min/10^6 cells)	Hepatocyte-Predicted Values		
		CL_{int} (mL/min/kg)	CL_{blood} (mL/min/kg)	E_H
Rat	< 1	< 5	< 5	< 0.07

References

[a] Ring B.J. et al. PhRMA CPCDC Initiative on predictive models of human pharmacokinetics, part 3: Comparative assessment of prediction methods of human clearance. *J. Pharm. Sci.*, 2011, 100:4090-4110.

[b] Obach R.S. Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: An examination of *in vitro* half-life approach and nonspecific binding to microsomes, *Drug Metab. Dispos*., 1999, 27:1350-1359.