Identification of Major Primary and Secondary Metabolites of Selected Drugs in Dog Micropatterned Hepatocyte Co-cultures using LC-MS/MS Acquisition: Correlation with *In Vivo Human* Metabolism

Onyi Irrechukwu, Brenda Hernández-Santiago, Yvonne Schaus, Jeannemarie Gaffney, Stacy Vacher, Jared Broberg

Ascendance Biotechnology (formerly Hepregen Corporation), Medford, MA

ABSTRACT

Accurate prediction and identification of the biotransformation products of drugs/xenobiotics in preclinical studies is critical in elucidating the role of metabolites in drug safety assessment. Comparisons of the metabolites produced across species *in vitro* would enable the extrapolation of relevant data from preclinical studies to humans. Traditional *in vitro* models such as microsomes, S9 fractions and primary hepatocyte suspensions have limitations: 1) they do not express the full complement of phase I and phase II enzymes required to replicate *in vivo* drug metabolism and 2) they have short culture lifespans because of rapid decline in phenotypic function (for example, CYP450 activity), thus precluding the generation of all relevant metabolites. We have developed a novel model in which primary dog hepatocytes are seeded onto ECM-coated domains of optimized dimensions and subsequently co-cultivated with fibroblasts (i.e. micropatterned co-cultures (MPCCs)), thus retaining key biochemical functions of *in vivo* liver. We incubated selected compounds such as betaxolol, diazepam and lorazepam that represent diverse chemical structures and biotransformation pathways in the dog MPCCs. Accurate identification of drug metabolites was performed using an LC/MS/MS system for data acquisition and analysis. Dog MPCCs produced major primary and secondary metabolites of the reference compounds, matching *in vivo* human and dog metabolites. Taken together, these data highlight the superiority of a long-term, functional tissue-engineered liver model such as the MPCC platform, over traditional models in correlating *in vitro* and *in vivo* species-specific metabolites and in identifying and predicting clinically-relevant metabolites.

METHODS

Dog micropatterned co-cultures (HepatoPac®) were created using patented microfabrication tools and consist of primary hepatocytes arranged in optimized domains and surrounded by human BJ fibroblasts. In this configuration, the HepatoPac co-cultures retain *in vivo* functionality *in vitro* (Fig 1). The co-cultures were first allowed to stabilize functionally in serum-supplemented media for a 7-day period. The compounds, Betaxolol, Diazepam and Lorazepam were purchased commercially and 24-well dog hepatocyte co-culture plates were manufactured at Ascendance Biotechnology. Dog HepatoPac co-cultures were incubated with test compounds at a final concentration of 10 µM and at a final volume of 0.4 mL. At 0, 4, 48, and 168 hours post-dosing, 800 µL of acetonitrile was added directly to designated wells. The entire contents of the wells (cells and media) were collected and transferred to a collection reservoir. At the end of each treatment period, the morphology of the cultures was assessed to ensure that: 1) the HepatoPac co-cultures were phenotypically stable and 2) the compounds were not toxic to the co-cultures. Sample analysis for metabolite profiling was performed using an LC-MS/MS system comprising an Applied Biosystem/MDS Sciex QSTAR TripleTOF® system coupled with a Shimadzu VP HPLC system. Metabolite separation was achieved using a Phenomenex TARGA C-18, 5µM, 1 x 50 mm column (Phenomenex Inc, Torrance, CA). The aqueous and organic mobile phases consisted of water with 0.2% formic acid (mobile phase A) and methanol with 0.18% formic acid (mobile phase B).

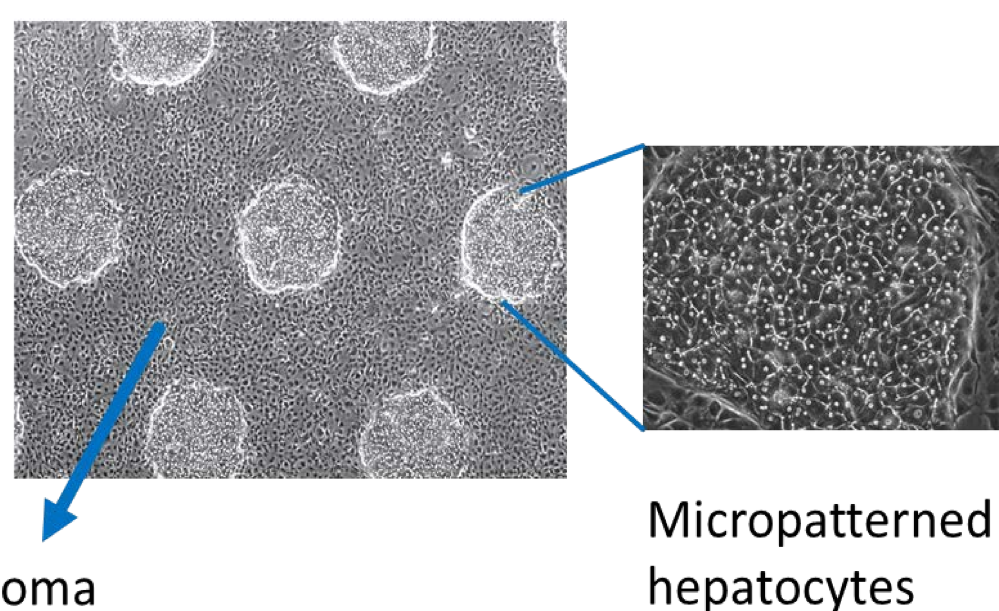


Figure 1. Dog HepatoPac Platform. HepatoPac® is created using patented microfabrication tools and consists of primary hepatocytes arranged in optimized domains and surrounded by stromal fibroblasts *(upper panel)*. The dog hepatocytes are co-cultured with specially selected BJ human stromal cells that support the highest level of performance observed in the HepatoPac micropatterned co-culture platform. Dog HepatoPac cultures retain long- term functionality for several weeks *in vitro.*

Metabolic Pathway in humans

A)

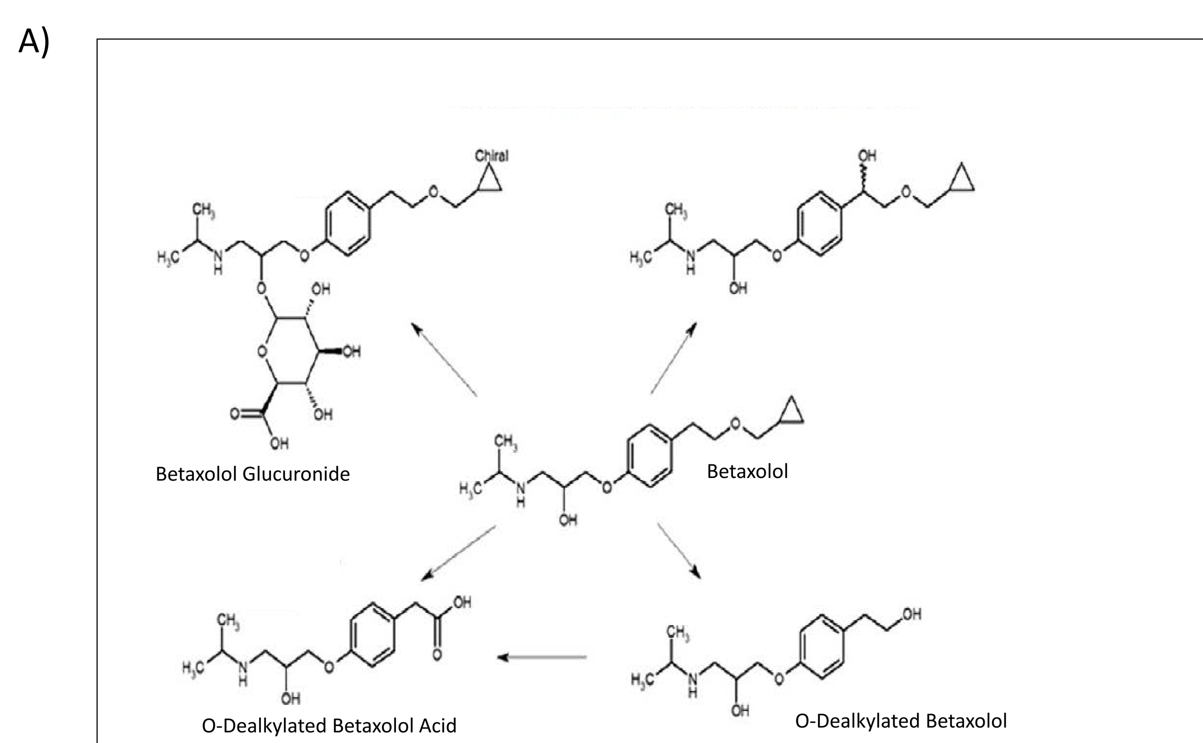


Figure 2: Metabolism of Betaxolol in humans. Betaxolol is metabolized mainly by CYP1A2 and CYP2D6 in humans (i.e. CYP1A1/2 and CYP2D15 in dogs)

Metabolic Pathway in humans

B)

C)

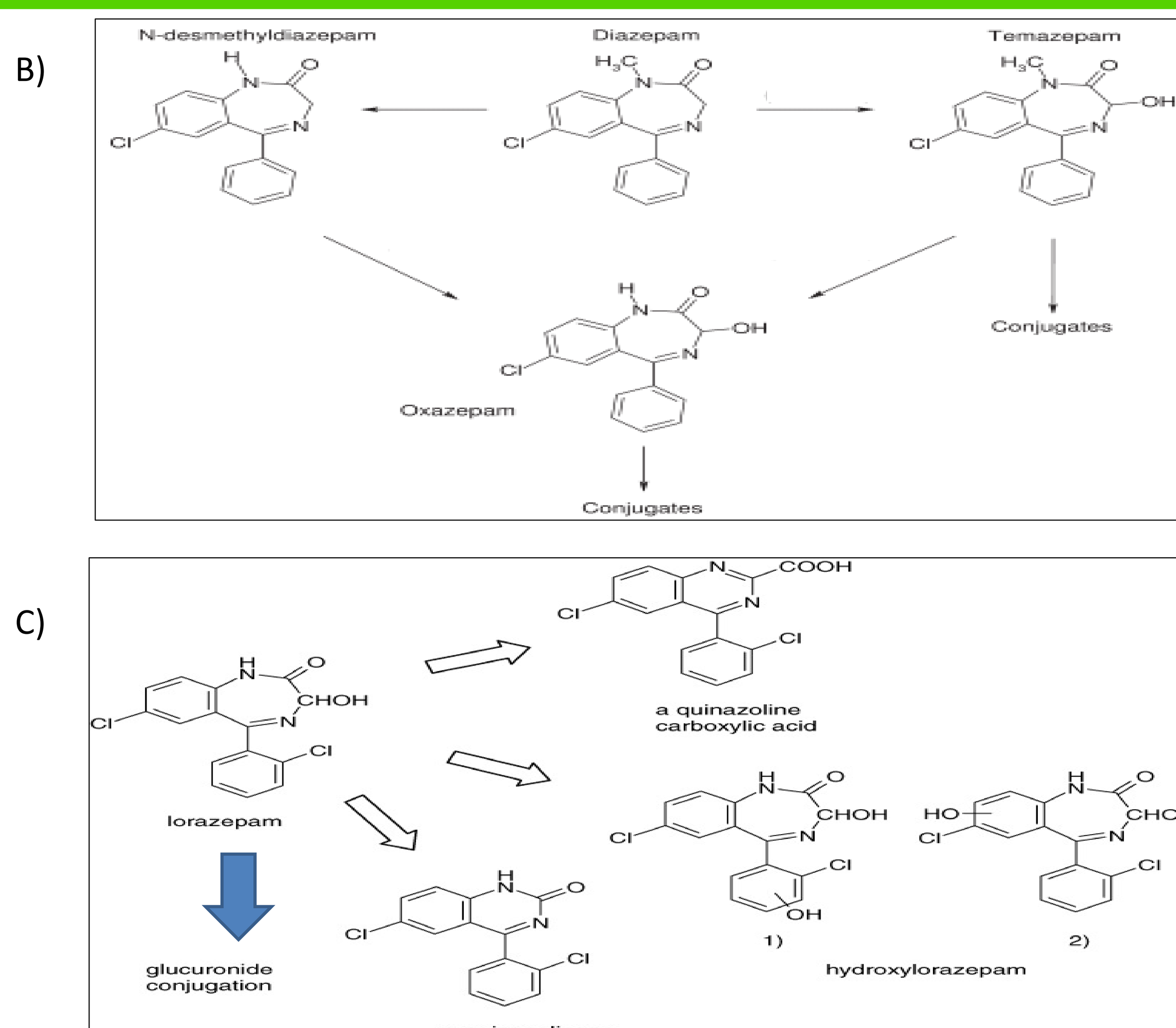
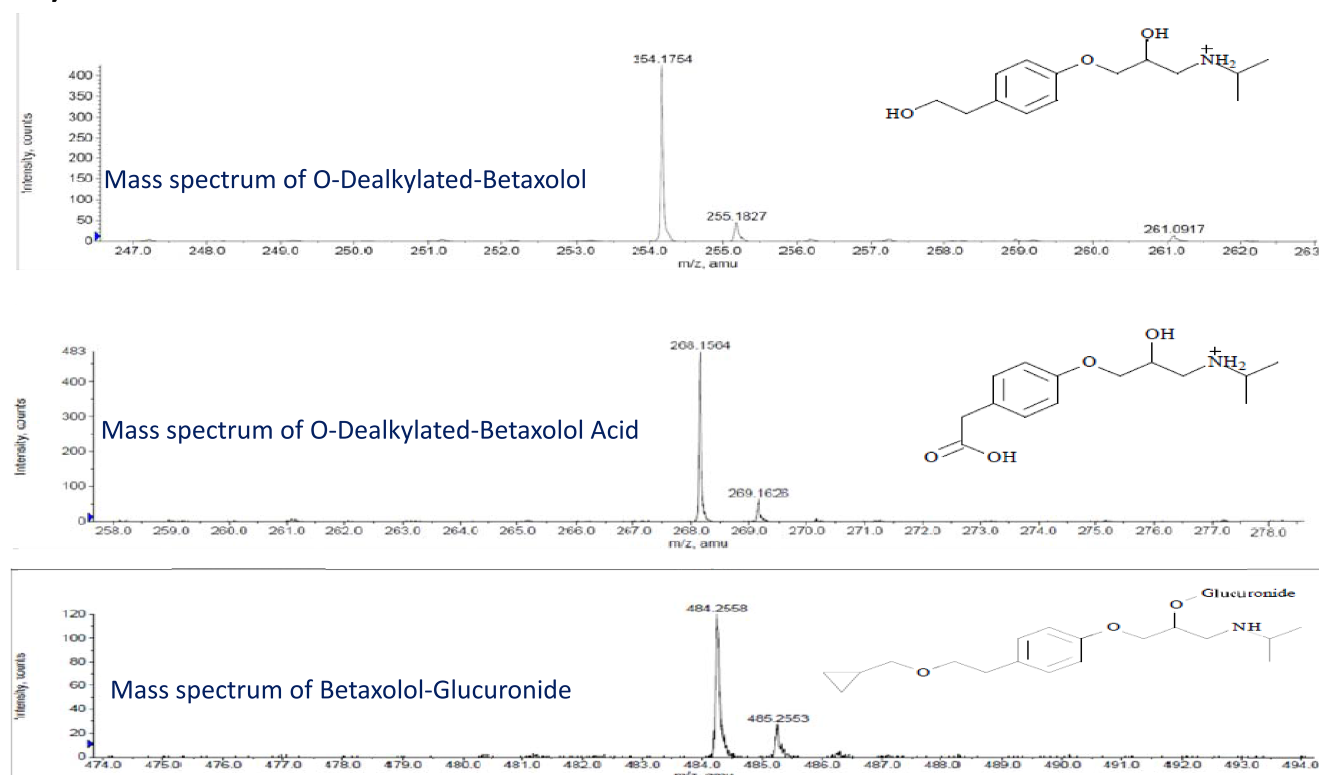


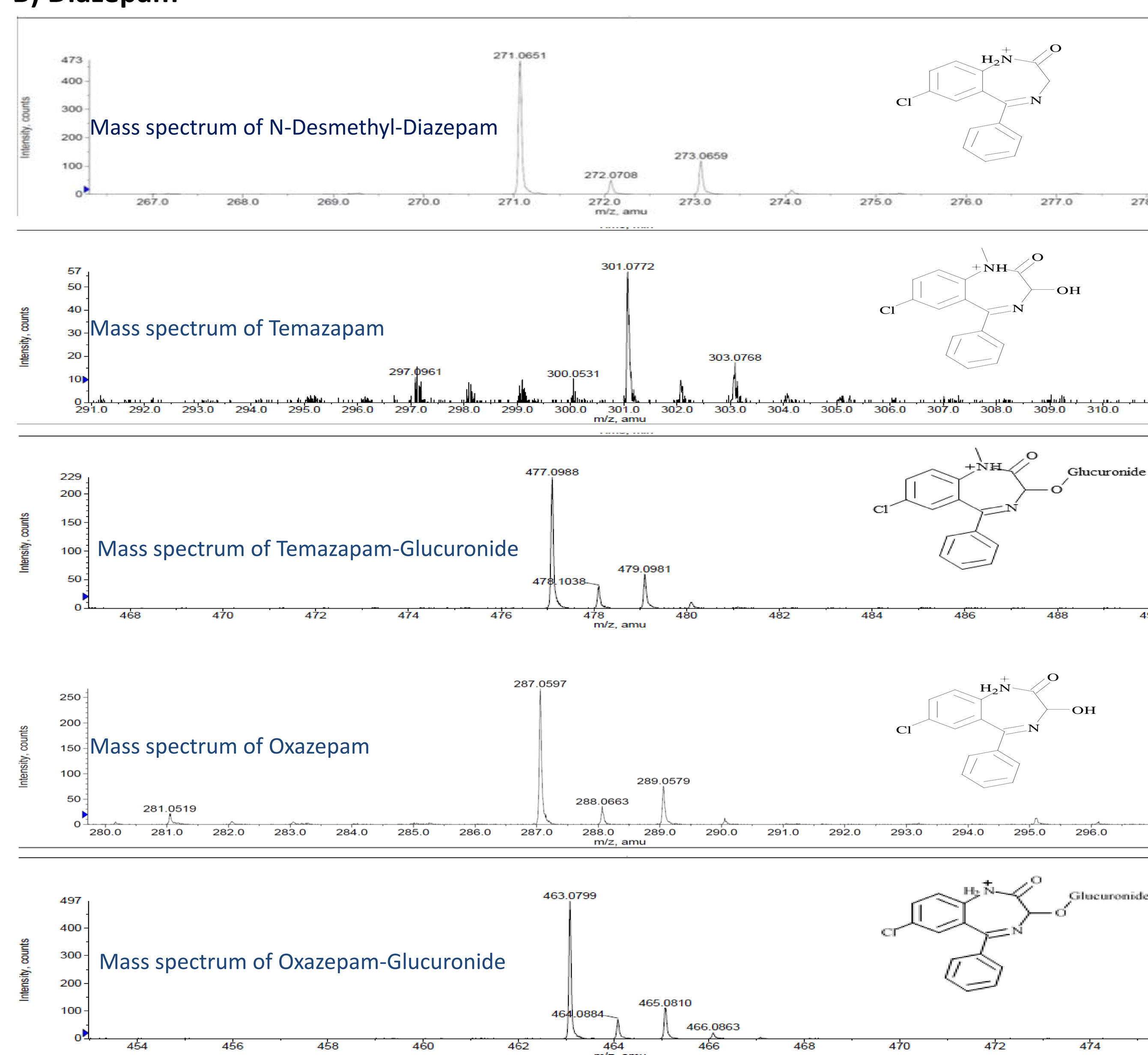
Figure 3. Metabolism of Diazepam (B), and Lorazepam (C) in humans. Diazepam is metabolized by CYP3A4 and CYP2C9/19 in humans (equivalent to CYP3A12 and CYP2C21/41 in dogs) while Lorezapam is mainly metabolized by UGT.

RESULTS

A) Betaxolol



B) Diazepam



C) Lorazepam

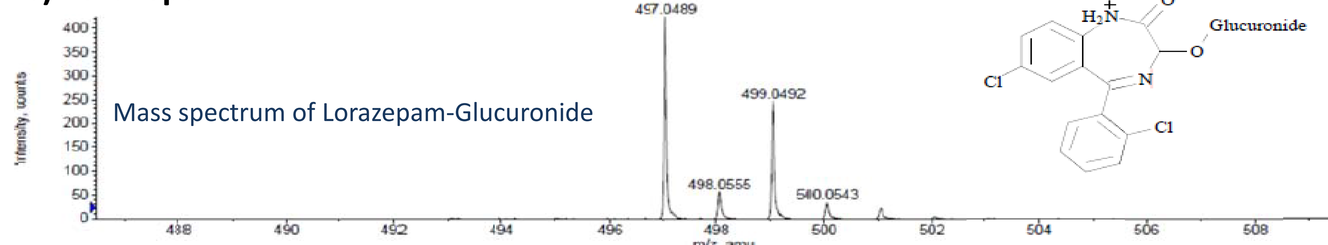
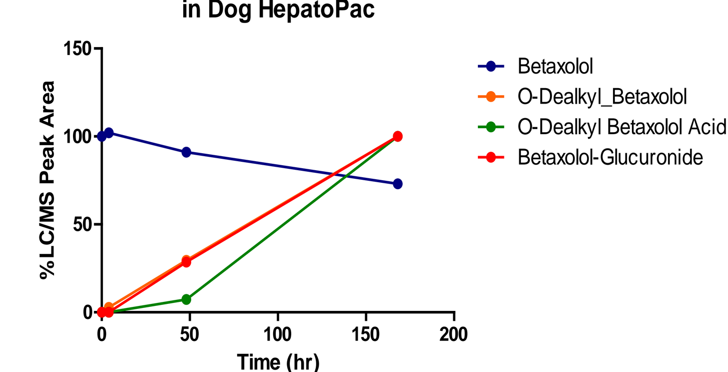


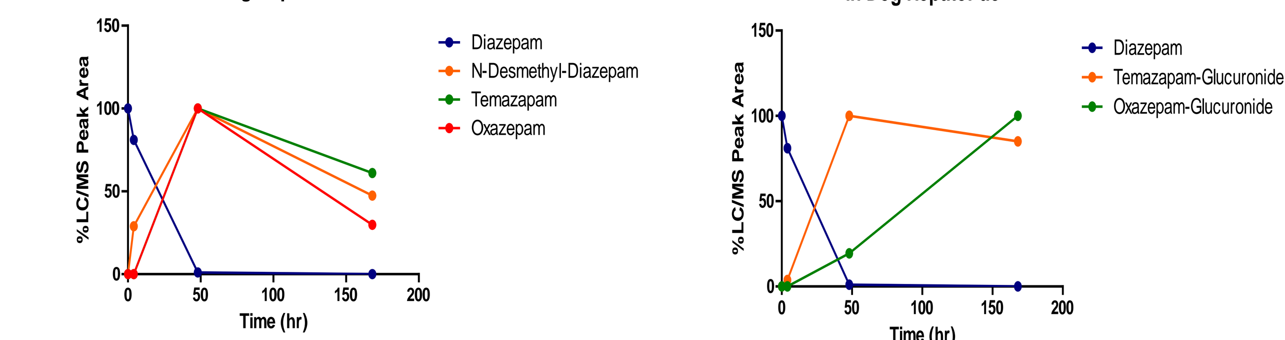
Figure 4. LC-MS/MS Spectra of Betaxolol (A), Diazepam (B), and Lorazepam (C) in Dog HepatoPac® Metabolite profiles of Betaxolol(A), Diazepam (B) and Lorazepam (C) two days post-incubation (D2) in Dog HepatoPac co-cultures.

RESULTS

A)



B)



C)

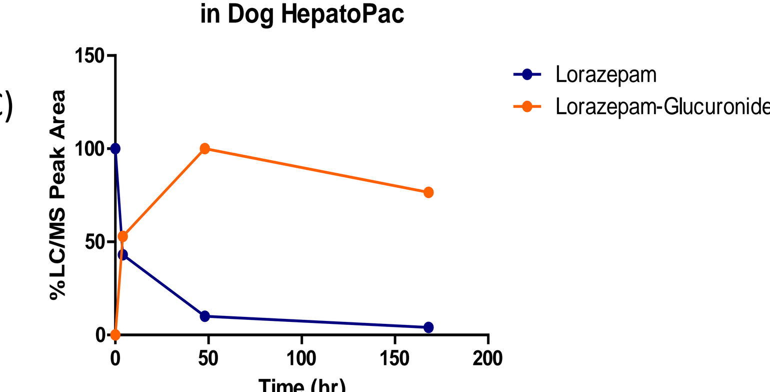


Figure 5. Metabolism of Betaxolol (A), Diazepam (B) and Lorazepam (C) in Dog HepatoPac. Dog HepatoPac cultures were treated for up to 7-days with either vehicle alone (0.1% DMSO control) or test compounds at a final concentration of 10 µM. The disappearance of each parent compound and the appearance of the major metabolites over time is shown above.

Compound	*In Vivo* Human Metabolites	Main Human enzymes involved in compound metabolism	Metabolites detected in Dog HepatoPac®		
			4hr	2 days	7 days
Betaxolol	O-Dealkyl-Betaxolol	CYP1A2, CYP2D6	Yes	Yes	Yes
	O-Dealkyl-Betaxolol Acid		ND	Yes	Yes
	Betaxolol-Glucuronide		ND	Yes	Yes
Diazepam	N-Desmethyl-Diazepam	CYP3A4, CYP2C9	Yes	Yes	Yes
	Temazepam		ND	Yes	Yes
	Temazepam-Glucuronide		Yes	Yes	Yes
	Oxazepam		ND	Yes	Yes
	Oxazepam-Glucuronide		ND	Yes	Yes
Lorazepam	Lorazepam-Glucuronide	UGT	Yes	Yes	Yes

Table 1. Dog HepatoPac® for Metabolite Identification. Relevant *in vivo* human metabolites generated in Dog HepatoPac model.

CONCLUSIONS

- Dog MPCCs incubated with betaxolol, diazepam and lorazepam, produced major primary and secondary metabolites of the reference compounds, matching *in vivo* major human metabolites.
- These data highlight the superiority of a long-term, functional tissue-engineered liver model of the Dog MPCC platform, over traditional models in correlating *in vitro* and *in vivo* species-specific metabolites and in identifying and predicting clinically-relevant metabolites.

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