# A MULTI-ENDPOINT ASSAY UTILIZING THE ALL-HUMAN HEPATOCYTE TRI-CULTURE MODEL TRUVIVO® FOR ANALYSIS OF HEPATIC CLEARANCE AND HEPATOTOXICITY PROFILES

inotiv
analyze. answer. advance.

Kevin Johnson*[1], Toni Steiner[2], Heather Sexton[2], Michele Wich[2], Haley Roeder[1], Lauren Denny[1], Edward LeCluyse[3], Jeanne Rumsey[1], Matthew Hutzler[1], and David Thompson[2]
[1]Biotransformation, [2]Mechanistic Pharmacology & Toxicology, Inotiv, Maryland Heights, MO 63043
[3]Research and Development, LifeSciences, LifeNet Health, Research Triangle Park, NC 27709
*Presenting author(s)

## Introduction

Many in vitro models for hepatotoxicity have been explored over the past decade including novel alternative methods (NAMs) such as hepatic spheroids, organoids and liver on chip models. While these platforms provide improved physiological models of the tissue, they are often limited by throughput cost and the need for specialized equipment. While initially used for drug metabolism and PK modeling, hepatocyte co-cultures have reemerged as useful models for studying pharmacological pathways, generation of in vitro disease models, and investigative tools for hepatotoxicity. We offer utilization of an all-human hepatocyte co-culture model (TruVivo®) to assess hepatic metabolism and facilitate assessment of hepatotoxicity risk potential in the same assay. The all-human hepatocyte co-culture assay was used to model the hepatic clearance and metabolite profile for various compounds with a range in metabolic clearance and metabolic pathways of low clearance drugs. Liquid chromatography and high-resolution mass spectrometry (LC-HRMS) facilitate measurement of hepatic clearance and metabolite formation from the same samples and HRMS raw data. This capability allows investigation of metabolite profiles in which traditional suspended hepatocytes may be inadequate. Coincubation of selective inhibitors and/or inducers is useful for investigation of enzyme phenotyping and DDI potential of the low clearance compounds.

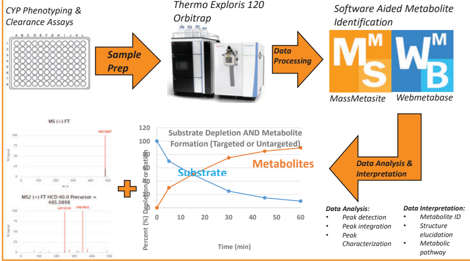
In addition, we were able to assess reactive metabolite and hepatotoxicity risks of Alpidem (a known hepatotoxic drug) and its analog Zolpidem (not hepatotoxic) using cultures from the same donor. This included cell viability, glutathione (GSH) depletion, generation of reactive oxygen species (ROS) and mitochondrial membrane potential (MMP). Alpidem binds to the peripheral benzodiazepine receptor (TSPO) located on the outer mitochondrial membrane (thought to be a contributor to mitochondrial injury). The metabolite profile of both compounds were consistent with historical data, yet assessment in a human hepatocyte tri-culture provided additional dimension for metabolite analysis. Inclusion of ABT (broad CYP inactivator) inhibited metabolite formation of alpidem. This included a known GSH conjugate and related cysteine-glycine and cysteine adduct. The multi-end point in vitro assay was able to detect time- and concentration-dependent loss of cell viability and intracellular GSH by alpidem, consistent with reactive metabolite formation and/or ROS production. Interestingly, while ABT completely abolished the formation of oxidative metabolites, it did not prevent loss of cell viability. No such signs of in vitro hepatotoxicity were observed for zolpidem, and consistent with previous reports no reactive metabolites were detected. The assay may provide insight into possible mechanisms of toxicity for compounds such as reactive metabolite formation with GSH depletion, mitochondrial dysfunction, and ROS generation. The Truvivo® kit enables the simultaneous assessment of hepatic metabolism of reactive or low clearance compounds, and hepatotoxicity risk in the same assay. The human co-culture allows the flexibility of repeat dosing or extended time courses out to 72-168 hours for low clearance compounds. In summary, the more cost-effective assay appears to be a useful tool to study the drug metabolism and hepatotoxicity risk of drug candidates.

## Methods

**Low Clearance Compound Assessment (CLint, MetID, DDI).** Cells and media were purchased from Thermofisher and LifeNet Health. Cells were seeded and cultured according to instructions provided with the kits. Stromal cells were seeded first followed by Primary hepatocytes (Donor 8450 ThermoFisher) at 30,000 cells per well in 96-well collagen-coated plates. Media was changed daily (except on weekends) until initiation of treatment on Day 7 in culture. For the DDI plate, cells were treated for 48 hours. After 47 hours, a cocktail of substrates (1A2, 2B6, 3A4, 2C8, 2C9, and 2C19) was added to wells to measure activity. Media was removed following 1 hour, extracted with ACN and supernatants were analyzed on a Thermo Exploris 120 Orbitrap. The remaining hepatocytes in each well were lysed with 350 µL of MR1 lysis buffer containing TCEP (tris(2-carboxyethyl)phosphine) as a reducing agent (Machery-Nagel, Allentown, PA). The lysates were centrifuged, and supernatants were subsequently transferred to deep well plates. NucleoMag beads and MR2 Buffer were added to each of the samples which were then loaded onto a KingFisher Flex instrument (Thermo Fisher Scientific) for isolation of total RNA using NucleoMag RNA kits (Macherey-Nagel). Finally, isolated RNA samples were quantified using a Qubit Flex Fluorometer (Thermo Fisher Scientific). For CLint and MetID plates, substrates were incubated for 7 days in presence or absence of inducer/inhibitor. CLint samples were cocktailed, and time points (supernatant) were collected once a day. MetID samples included a single final timepoint at 7 days. Supernatants were extracted with ACN and supernatants were analyzed on a Thermo Exploris 120 Orbitrap.

**Alpidem and Zolpidem In Vitro Tox and Metabolite Assessment**. Cells and media were provided by LifeNet Health. Cells were seeded and cultured according to instructions provided with the kits. Stromal cells were seeded first followed by hepatocytes at 30,000 cells per well in 96-well collagen-coated plates. Following seven days of culture, test compounds were added at three different concentrations and cells were further incubated for up to 24, 48 and 72 hours. DMSO (0.5% final concentration) served as the vehicle control. Four different endpoints were assessed at each time point. The endpoints included cell viability, glutathione (GSH) depletion, generation of reactive oxygen species (ROS) and mitochondrial membrane potential (MMP). MMP was assessed by fluorescence using the cationic dye JC-10 (Abcam) while the other endpoints were assessed using chemiluminescent kits from Promega. All cell-based measurements were made on a Cytation 5 Multimode plate reader (BioTek). In addition, media was changed daily with replenishment of test compounds. For Metabolite identification studies, at the end of 24-hour incubations, media samples were collected and analyzed for parent compound, major metabolites, and GSH conjugates by LC-MS/MS (cells were not extracted).

**Figure 1: Metabolic Clearance and Metabolite ID Workflow Using LC-HRMS**




## Results

**Figure 2. Metabolic Clearance and Metabolite Formation of Alprazolam**

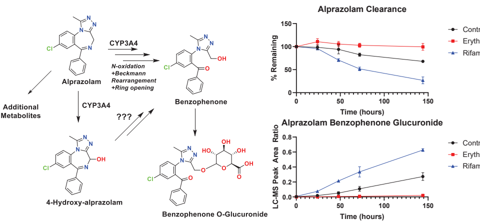



**Figure 3 Metabolic Clearance and Metabolite Formation of XK469**

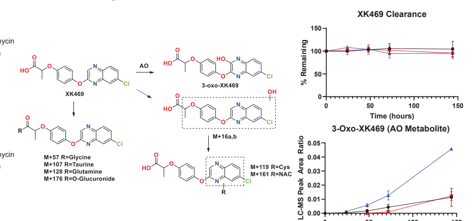



**Figure 4. Metabolic Clearance and Metabolite Formation of Meloxicam**

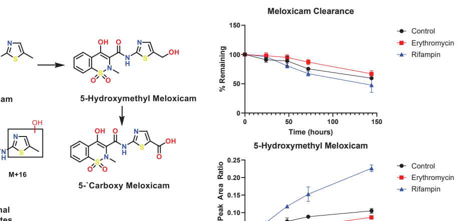



**Figure 5. Metabolic Clearance and Metabolite Formation of Carbamazepine**

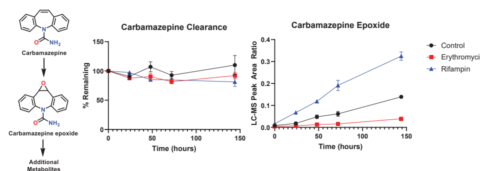



**Table 1. Substrate/Inhibitor/Inducer Concentrations**

| Substrate | Conc (µM)* | Inhibitor/Inducer | Conc (µM) |
|---|---|---|---|
| Alprazolam | 1 or 10 | Erythromycin | 30 |
| Meloxicam | 1 or 10 | Rifampin | 20 |
| XK-469 | 1 or 10 | | |
| Carbamazepine | 1 or 10 | | |

*1uM incubations used for CLint and MetID analysis; 10uM incubations for additional MetID assessment (where applicable)
**Coincubation with Fluoxetine (10µM) had minimal effect on CLint and Metabolite formation rate (data not shown)
***XY-plots exclude first 24 hour incubation period to ensure system reached equilibrium

**Table 2. Intrinsic Clearance (CLint, mL/min/kg)**

| Compound | Control | | | +Erythromycin | | | +Rifampin | | |
|---|---|---|---|---|---|---|---|---|---|
| Alprazolam | **0.487** | +/- | 0.053 | <0.417 | +/- | NC | **1.58** | +/- | 0.31 |
| Meloxicam | **0.618** | +/- | 0.055 | **0.495** | +/- | 0.082 | **0.949** | +/- | 0.129 |
| XK-469 | <0.417 | +/- | NC | <0.417 | +/- | NC | <0.417 | +/- | NC |
| Carbamazepine | <0.417 | +/- | NC | <0.417 | +/- | NC | <0.417 | +/- | NC |

**Table 3. Metabolite Formation* (E6/min) (LC-MS Peak Area Ratio)**

| Compound | Control | | | +Erythromycin | | | +Rifampin | | |
|---|---|---|---|---|---|---|---|---|---|
| Alprazolam | **36.2** | +/- | 6.2 | **2.63** | +/- | 0.35 | **75.2** | +/- | 2.8 |
| Meloxicam | **29.8** | +/- | 2.0 | **32.7** | +/- | 1.4 | **48.3** | +/- | 4.1 |
| XK-469 | **1.66** | +/- | 1.00 | **2.25** | +/- | 0.57 | **7.10** | +/- | 0.10 |
| Carbamazepine | **15.5** | +/- | 0.4 | **4.12** | +/- | 0.4 | **36.1** | +/- | 1.8 |

**Figure 6. CYP Induction using Truvivo® (single donor)**

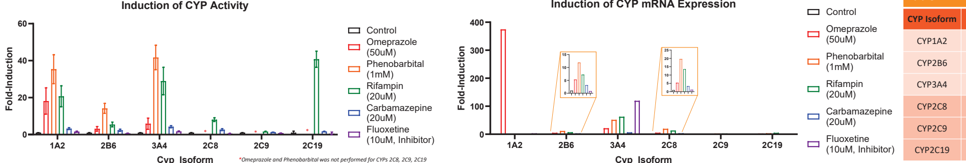



**Table 4. DDI Probe Substrate Concentrations**

| CYP Isoform | Substrate | Conc (µM) | Cocktail |
|---|---|---|---|
| CYP1A2 | Phenacetin | 100 | Substrate Cocktail 1 |
| CYP2B6 | Bupropion | 250 | |
| CYP3A4 | Testosterone | 200 | |
| CYP2C8 | Amodiaquine | 20 | Substrate Cocktail 2 |
| CYP2C9 | Diclofenac | 20 | |
| CYP2C19 | S-Mephenytoin | 100 | |

**Figure 7. Alpidem and Zolpidem In Vitro Metabolism using Truvivo®**

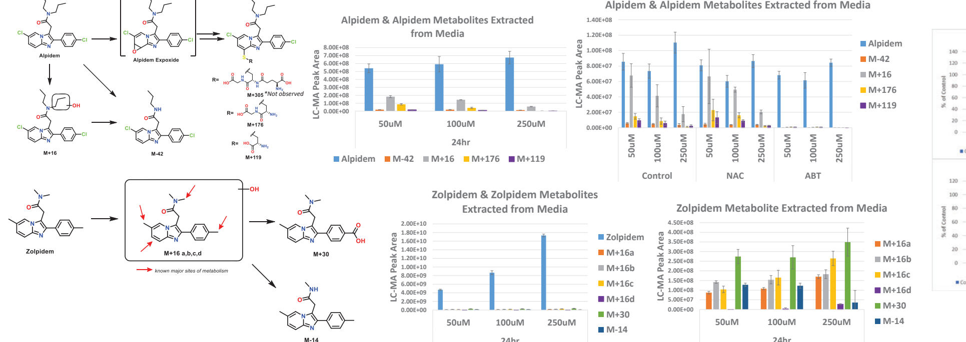



**Figure 8. Alpidem and Zolpidem In Vitro Hepatotoxicity using Truvivo®**

Cell Viability

ROS

MMP

## Summary of Conclusions

- The TruVivo® hepatocyte co-culture system was useful to perform metabolic clearance and metabolite ID (metabolite formation) of low clearance drugs in the same samples/raw data.
- The same TruVivo® hepatocyte co-culture (donor) was used to measure induction and inhibition (DDI potential) of select compounds.
- Enzyme phenotyping can be performed co-incubating selective inhibitors with substrates in the TruVivo® hepatocyte co-culture system.
- The TruVivo® hepatocyte co-culture allows flexibility to choose plateable hepatocyte lots/sources using all human cells in house.
- The TruVivo® hepatocyte co-culture system was able to distinguish the hepatotoxicity from two drugs (Alpidem and Zolpidem), that passed through pre-clinical and clinical studies with an acceptable safety profile. Unlike Alpidem, which was removed from the market, the in vitro assay did not detect signs of hepatotoxicity with zolpidem, a close structural analog of alpidem with no known liver toxicity.
- Results provided insight into possible mechanisms of toxicity for both alpidem such as reactive intermediate formation with GSH depletion, mitochondrial dysfunction and ROS generation. The extended culture time of the co-cultures afforded an opportunity for repeat dosing out to 10 days and potentially longer.
- In summary, TruVivo® appears to be a useful tool to study the clearance, metabolite profiles, and hepatotoxicity of problematic and/or stable drugs.

## References

1. Chan, T.S., et al. Evaluation of Erythromycin as a Tool to Assess CYP3A Contribution of Low Clearance Compounds in a Long-Term Hepatocyte Culture. Drug Metab. and Disp. 2020, 48 (8), 690-697
2. Allqvist, A., et al. Inhibition of CYP3A4 and CYP3A5 catalyzed metabolism of alprazolam and quinine by ketoconazole as racemate and four different enantiomers. Eur J Clin Pharmacol. 2007, 63, 173–179
3. Fraser, A.D. Bryan, W., Isner, A.F. Urinary screening for alprazolam and its major metabolites by the Abbott ADx and TDx analyzers with confirmation by GC/MS. J. Anal. Toxicol. 1991, 15(1), 25-29
4. Hutzler J.M., et al. Characterization of aldehyde oxidase enzyme activity in cryopreserved human hepatocytes. Drug Metab Dispos. 2012, 40(2), 267-75.
5. Burton R.D., et al. Assessment of the Biotransformation of Low-Turnover Drugs in the HµREL Human Hepatocyte Coculture Model. Drug Metab Dispos. 2018, 46 (11), 1617-1625.
6. Chesné C, et al. Metabolism of Meloxicam in human liver involves cytochromes P4502C9 and 3A4. Xenobiotica. 1998, 28(1), 1-13
7. Berson, A., et al. Toxicity of alpidem, a peripheral benzodiazepine receptor ligand, but not zolpidem, in rat hepatocytes: role of mitochondrial permeability transition and metabolic activation. J Pharmacol Exp Ther. 2001, 299 (2): 793-800
8. https://go.drugbank.com/drugs/
9. https://public-mzcloud.cmdprod.thermofisher.com/

## Acknowledgements

*We would like to thank the LifeNet Health team for the continued partnership and donation of the Truvivo® cells for the hepatotoxicity work.*