Use of the CYP3A4 Selective Inhibitor CYP3cide in CYP3A5 Genotyped Cryopreserved Human Hepatocytes to Explore the Individual Contribution of CYP3A4 and CYP3A5 in Drug Metabolism

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Abstract

The human cytochrome P450(CYP) 3A family is of critical importance to drug discovery and development due to its involvement in the metabolism of the majority of drugs on the market. Of the four isozymes within the human CYP3A family, CYP3A4 is the most abundant and well studied, however, the polymorphic member CYP3A5 can contribute significantly to the metabolism of many drugs, such as midazolam. The expression levels of CYP3A5 varies greatly in the population but has been reported to contribute between 2 and 60% of the CYP3A activity.[1] Understanding the contribution of each CYP in individual donors is important for gauging the impact of CYP3A5 expressers on safety and efficacy of CYP3A substrates. CYP3cide (PF-4981517; 1-methyl-3-[1-methyl-5-(4-methylphenyl)-1H-pyrazol-4-yl]-4-[(3S)-3-piperidin-1-ylpyrrolidin-1-yl]-1H-pyrazolo[3,4-d]pyrimidine) has been shown to be a potent, efficient, and specific time-dependent inactivator of human CYP3A4 in recombinant CYPs (rCYP) and liver microsomes (HLM).[2] We tested the ability of this compound to function in intact cryopreserved human hepatocytes and allow the exploration of compound behavior in the absence of CYP3A4 activity. Human cryopreserved hepatocytes were genotyped using a realtime single nucleotide polymorphism assay (SNP) assay for CYP3A5*3 which encodes an early stop codon generating a non-functional protein. Time dependent inhibition experiments were performed measuring metabolism of midazolam to determine inactivation kinetic parameters. We utilized an 8-point dilution series over 6 time points to determine K_{inact} and K_I. Human hepatocyte lots with CYP3A5 *3/*3, and *1/*3 genotypes were screened to determine optimized inhibition conditions to determine the inhibition of midazolam and testosterone metabolism. Human hepatocyte lots with CYP3A5 *3/*3, *1/*3 and *1/*1 genotypes were screened using optimized inhibition conditions to determine the inhibition of midazolam metabolism. Remaining activity of 1-OH-midazolam formation in the CYP3A5 *3/*3, *1/*3 and *1/*1 groups ranged from 37-93%, 47-84% and 11-25% respectively. CYP3cide allows for the investigation of the impact of CYP3A5 expression on drug metabolism, in intact genotyped cryopreserved human hepatocytes.

Introduction

The identification of genetic polymorphisms in the cytochrome P450 enzymes and their impact on drug-drug interaction(DDI) liability has posed new challenges in the development of new compounds. The CYP3A family has been of high importance in drug metabolism and DDIs due to the high expression of CYP3A4 in the liver and its role in the metabolism of a high proportion of drugs. The CYP3A5 family member's expression is highly dependent on a single common polymorphism, CYP3A5*3. This change creates a cryptic splice site in intron 3, which changes the reading frame and results in a premature termination codon and a non-functional protein.[3] Those individuals who possessed a 3A5*1 allele showed a three fold increase in their total 3A content.[3] CYP3A5*1,the expressed isoform, is prevalent in only 5-15% of Caucasians, but in 40-60% of African and African Decent.[3,4] This combination has the potential to cause errors in predicting clearance and DDI. Walsky et al, found a compound, named CYP3cide to be a potent and specific CYP3A4 Inhibitor.[2] This group characterized CYP3cide in recombinant CYPs and human liver microsomes, however it's utility in hepatocytes has not been explored.

Hepatocytes have been suggested as a better model to assess drug metabolism of a new chemical entity (NCE) containing a complete array of phase I and II enzymes as well as membrane transporter proteins. [5,6] Groups have reported with compounds that interact with drug transporters can have significant differences either higher or lower in their inhibitory properties. Clinical predictions of changes in pharmacokinetics have been shown to be improved using hepatocytes over HLM when TDI is involved.[6]

Methods

Materials

Chemical reagents (unless otherwise specified) including CYP3cide were obtained from Sigma Aldrich(St. Louis, MO). Substrates stock solutions were made in acetonitrile. CYP3cide was resuspended in DMSO. Cryopreserved Human Hepatocyte and media were obtained from BioreclamationIVT (Baltimore, MD).

Genotyping

Single donor cryopreserved human hepatocytes (BioreclamationIVT) were genotyped for CYP3A5*3 (rs776746) using extracted genomic DNA in a realtime PCR SNP assay (cat# C__26201809_30) (Applied Biosystems) on an ABI7500 running SDS v1.3.

Genotype	Phenotype
CYP3A5*3/*3	non-expressers of CYP3A5
CYP3A5*1/*3	expressing one functional copy of CYP3A5
CYP3A5*1/*1	expressing two copies of CYP3A5

Inhibition Assays

Cryopreserved Human Hepatocytes were thawed in InVitroGro™ CP medium(Bioreclamation IVT) , following instructions for use as prescribed by BioreclamationIVT. Cells were pelleted and resuspended in Krebs Henseleit Buffer(KHB) to a final concentration of 4x10^6 cells/ml. Inhibitor stock solutions were made by serial dilution in KHB with maintaining 0.1% solute concentration. Cells and inhibitors solutions were warmed for 10min at 37C and combined for indicated times. Each reaction contained 100,000 cells in 25ul and was combined with 25ul of inhibitor solution in a deep well 96-well plate. Incubations were done in a 37C incubator with shaking. After elapsed time, 1ml (20 volumes) of KHB was added and the well and the plate was spun at 100xg for 5min. Supernatant was aspirated to remove inhibitor and cells were resuspended in 100ul of substrate at concentrations indicated in the individual experiments. Reactions were terminated with the addition of an equal volume of ice cold methanol and metabolite formation was measured by LC/MS/MS.

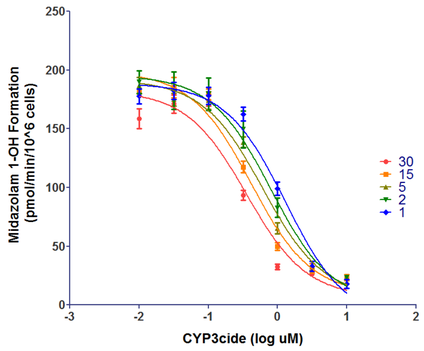
Data Analysis

Data analysis were performed using Prizm 5.02 software (San Diego, CA). Data points were generated in triplicate except where indicated.

References

1. Williams JA, Cook J and Hurst SI.(2003) A Significant Drug-Metabolizing Role For CYP3A5?. Drug Metab Dispos 31:1526-1530
2. Walsky RL, Obach RS, Hyland R, Kang P, Zhou S, West M, Geoghegan KF, Helal CJ, Walker GS, Goosen TC, and Zientek MA. (2012) Selective Mechanism-Based Inactivation of CYP3A4 by CYP3cide (PF-04981517) and Its Utility as an In Vitro Tool for Delineating the Relative Roles of CYP3A4 versus CYP3A5 in the Metabolism of Drugs. Drug Metab Dispos 40:1686–1697
3. Kuehl P, Zhang J, Lin Y, Lamba J, Assem M, Schuetz J, Watkins PB, Daly A, Wrighton SA, Hall SD, Maurel P, Relling M, Brimer C, Yasuda K, Venkataramanan R, Strom S, Thummel K, Boguski MS and Schuetz E. (2001) Sequence diversity in *CYP3A promoters* and characterization of the genetic basisof polymorphic CYP3A5 expression. Nature Genetics 27: 383-391
4. Daly AK. (2006) Significance of the minor cytochrome P450 3A isoforms. Clinical Pharmacokinetics 41: 13-31.
5. Brown HS, Wilby AJ, Alder J, Houston BJ. (2010) Comparative Use of Isolated Hepatocytes and Hepatic Microsomes for Cytochrome P450 Inhibition Studies: Transporter-Enzyme Interplay. Drug Metab Dispo 38: 2139-2146.
6. Chen Y, Liu L, Monshouwer M, Fretland AJ. (2011) Determination of Time-Dependent Inactivation of CYP3A4 in Cryopreserved Human Hepatocytes and Assessment of Human Drug-Drug Interactions. Drug Metab Dispo 39: 2085-2092.

Results

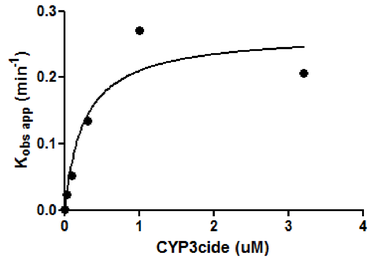


IC50 with incubation time				
30 min	15 min	5 min	2 min	1 min
0.3551 uM	0.4128	0.6112	0.8011	1.302

Time and Concentration Dependent Inhibition

Methods: Range finding experiments were performed to determine optimum conditions for determining inhibition kinetics and explore the potency of the IC50 shift. A genotyped CYP3A5 *3/*3 donor was used to exclude the presence of CYP3A5. Incubations were run with 7uM midazolam, (experimentally derived K_m, data not shown). Incubation time and concentration were varied with times as indicated and concentrations between 0.01 and 10uM CYP3cide in half log intervals. Graph and IC50 Determinations were generated using log(inhibitor) vs. response (three parameters) in Prizm v.5.02 with a hill slope=-1.

Results: CYP3cide showed dose dependent inhibition of metabolite formation with the CYP3A4 substrate Midazolam. The inhibition increased modestly with pre-incubation time.



Calculated Inhibition Constants in Hepatocytes			
Substrate	K_I (uM)	K_{inact} (min-1)	K_{inact}/K_I (ml*min^{-1}/umol^{-1})
Midazolam	0.2660	0.2661	1000

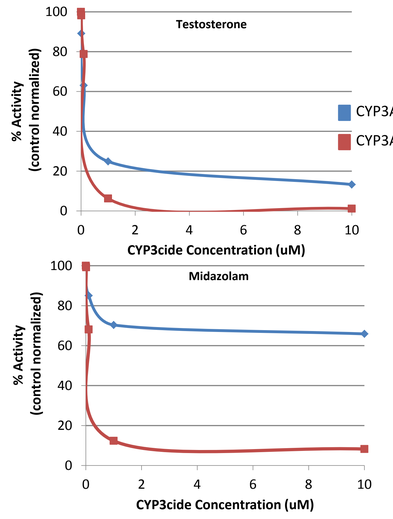
Inhibition Kinetics Determination

Methods: Incubations were performed with a genotyped CYP3A5 *3/*3 to exclude the presence of CYP3A5 activity. Pre-incubations were performed at concentration from 0.01 to 3.2M CYP3cide in half log intervals. Samples were washed as indicated in methods and incubated with midazolam at 10X K_m for 15min. $K_{obs\ app}$ is the negative slope of the natural log of the percent activity remaining plotted against time pre-incubated. Inhibition constants were generated by using a nonlinear regression fitting of the data to the following:

$$K_{obs\ app} = K_{obs\ app\ [I]=0} + \frac{K_{inact} \times [I]}{K_I + [I]}$$

. Fitting was performed by Prizm v.5.02 .

Results: CYP3cide in hepatocytes showed time dependent inhibitory properties. The K_I generated was similar but lower than that reported in HLM 0.266uM in hepatocytes to 0.42uM for midazolam 1-OH formation in HLM.[2] The K_{inact} was lower in hepatocytes compared to HLM, 0.2661 to 1.6min^{-1} . This generated a K_{inact}/K_I ratio of 1000 ml*min^{-1}/umol^{-1}, lower than that seen in HLM but indicating an efficient inhibitor.



CYP3cide Inhibition in Gentoyped Hepatocytes

Methods: Genotyped hepatocyte lots of CYP3A5 expresser (CYP3A5 *1/*3) and non-expresser (CYP3A5 *3/*3) were incubated with CYP3cide for 15min with concentrations from 0.01-10uM in log increments and KHB only. After pre-incubation 15uM midazolam or 50uM testosterone was added in an equal volume and incubated for 20min. The percentage remaining activity was calculated by normalizing metabolite formation of midazolam 1-OH or testosterone 6-β-OH with inhibitor to that at 0um CYP3cide.

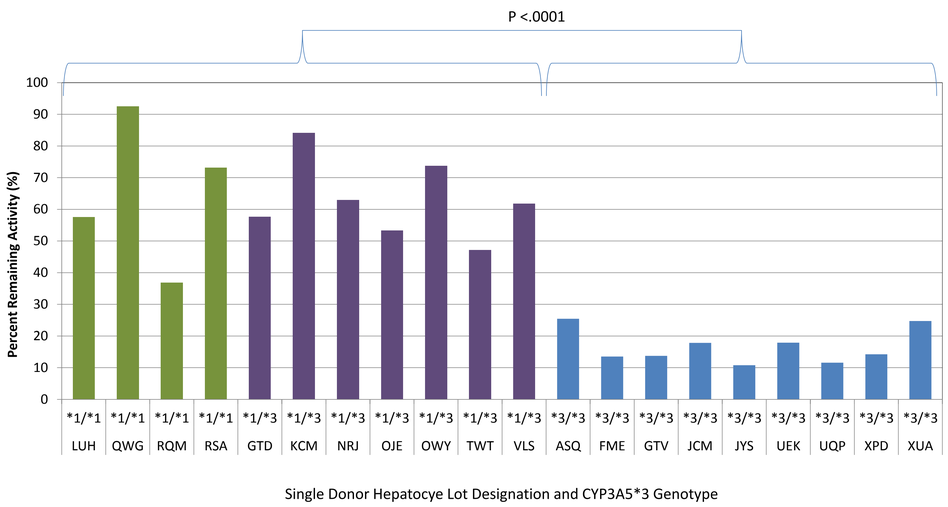
Results: CYP3A5 non-expressers metabolism saw a marked reduction in metabolism of both CYP3A specific substrates, midazolam and testosterone, with exposure to CYP3cide. The CYP3A5 expresser saw a significant reduction in testosterone metabolism in accordance with the majority of metabolism due to CYP3A4. The 3A5 expresser retained ~70% of the activity of control showing the significant contribution of CYP3A5 to its overall metabolism. For substrates with preferential metabolism by CYP3A5, genetic background can play a significant role.

Inhibition in Genotyped Cryopreserved Heptocytes

Methods: CYP3A5*3 genotyped cryopreserved human hepatocyte lots containing homozygous and heterozygous CYP3A5 expresser (CYP3A5 *1/*1 and *1/*3) and non-expresser (CYP3A5 *3/*3) were incubated with CYP3cide for 15min with 1uM CYP3cide. After pre-incubation with inhibitor and wash step, 15uM midazolam was added and incubated for 20min. LC/MS/MS was used to determine midazolam 1-OH formation which was normalized to 0uM CYP3cide for each lot to generate percentage activity remaining. Means were generated for each group and standard deviation. An unpaired two tailed t-test was performed to generate P value.

Results: Human hepatocyte lots with a functional copy of CYP3A5 showed higher retention of midazolam metabolic capacity than those without. There was high variability within groups which is typical of individual donors. There was no significant difference in the mean between the CYP3A5 *1/*1 and *1/*3 groups, possible indicating a regulatory mechanisms in controlling 3A5 levels beyond gene dosing. There was a significant difference ($p < 0.0001$) between the mean of the 3A5 *1 containing lots and the 3A5 *3/*3 lots. In comparison to published HLM data a higher concentration of CYP3cide and longer incubation was needed for optimal inactivation 0.56uM vs 1uM and 5min vs 20min for HLM and hepatocytes respectively.[2] CYP3cide provides a reagent which allows for the elucidation of CYP3A4 contribution to metabolism using an intact hepatocyte.

Genotype	Remaining Activity Range	Average Remaining Activity (Mean±SD)
*1/*1	36.8 - 92.5%	65.0% ± 23.5
*1/*3	47.1 – 84.1%	61.9% ± 12.5
*3/*3	10.8 – 25.4%	16.6% ± 5.4



Conclusions

- **CYP3cide is a potent time dependent CYP3A4 inhibitor which functions in cryopreserved hepatocytes with similar but less robust activity compared to HLM**
- **CYP3cide in single donor cryopreserved human hepatocytes provides a useful reagent to explore the individual contributions of CYP3A4 and CYP3A5 to the metabolism of CYP3A substrates**
- **Genotyped single donor cryopreserved hepatocytes show significant variation in activity levels between donors of CYP3A5 *1/*1 and CYP3A5*1/*3 , but little difference between the two genotypes**