



# High-Throughput Mass Spectrometry-Based Screening to Identify Selective CYP3A5 Inhibitors

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## ABSTRACT

Cytochrome P450 3A4 (CYP3A4) is often regarded as the most important P450 responsible for drug metabolism. In addition to CYP3A4, CYP3A5 represents at least 50% of the total hepatic CYP3A content in those polymorphically expressing CYP3A5, and may be the most important genetic contributor to interindividual and interracial differences in CYP3A dependent drug clearance.<sup>1</sup> The availability of a CYP3A5 selective substrate or inhibitor would enable greatly our understanding of its implications in drug metabolism. We developed a 384-well plate-based screening method to rapidly evaluate potential compounds as selective CYP3A5 inhibitors. Although high-throughput methods using fluorogenic probe substrates to assess CYP inhibition have been described, these often do not translate to inhibition studies conducted with drug-like molecules.<sup>2</sup> Our approach applied a drug-like probe substrate (midazolam) with mass spectrometry-based detection. Recombinant human CYP3A4 and CYP3A5 were incubated for 4 min at 37°C with midazolam (8 concentrations 3A4; 0.3-38  $\mu$ M and 9 concentrations 3A5; 0.1-19  $\mu$ M) to determine the  $K_m$  for each CYP isoform. Reactions were stopped with 92/5/3 (Acetonitrile/Water/Formic Acid) containing 1 $\mu$ M deuterated (d4)-1-OH midazolam as internal standard. High throughput sample preparation, chromatography, and detection of 1-OH and (d4)-1-OH midazolam was developed using the Biotrove Rapidfire technology interfaced to an AP4000 triple quadrupole mass spectrometer. Potential inhibitors of CYP3A4 and CYP3A5 were incubated at 1 $\mu$ M; ketoconazole was used as a positive control. Optimization of sample preparation and chromatography/detection resulted in 8 sec/sample analysis times. The  $K_m$  values of midazolam for CYP3A4 and CYP3A5 were determined to be 1.0  $\mu$ M and 0.87  $\mu$ M, respectively. 11,500 compounds were screened to assess inhibition potential against CYP3A4/CYP3A5. Criteria for selecting hits were based on percent inhibition and selectivity for CYP3A5. Confirmed hits from the assay will be followed up with determinations of  $IC_{50}$  values against each CYP isoform. The data suggests that the developed approach using plate-based high-throughput mass spectrometry technology is rapid and effective for determining selective inhibitors of CYP3A5.

## INTRODUCTION

### Background

- CYP450 enzyme subfamily CYP3A contributes to metabolism of ~ 50% of marketed drugs<sup>3</sup>
- CYP3A subfamily comprised of three forms, 3A4, 3A5 and 3A7, generally having similar substrate inhibition specificities<sup>4</sup>
- Distinguishing between the contribution of CYP3A4 and CYP3A5 *in vivo* is difficult<sup>5</sup>
- CYP3A5 is more frequently expressed in livers of African Americans (60%) than in those of Caucasians (33%)<sup>1</sup>
- CYP3A5 represents at least 50% of the total hepatic CYP3A content in those polymorphically expressing CYP3A5, and may be the most important genetic contributor to inter-individual and interracial differences in CYP3A dependent drug clearance<sup>1</sup>

### Rationale

- The lack of a CYP3A5 selective inhibitor currently hampers efforts to ascertain its implications in drug metabolism
- Identifying a selective CYP3A5 inhibitor could provide a tool to distinguish relative contribution of this enzyme in the metabolism of novel compounds in drug discovery and development
- Assays developed that utilize fluorescent probes often do not translate to inhibition studies with drug-like molecules
- Interfaced to ESI-MS/MS detection, Biotrove HTMS technology does not require the use of tagged or labeled molecules
- Biotrove high-throughput sample analysis and column switching results in <10 second cycle times per sample
- Biotrove can deliver direct, rapid quantification of product formed and/or parent substrate remaining, and internal standard



Figure 1. Biotrove screening technology platform consisting of Biotrove system interfaced to Applied Biosystems API4000 triple quadrupole mass spectrometer

## MATERIALS AND METHODS

### Reagents

- Midazolam and ketoconazole were obtained from Sigma Chemicals (St. Louis, MO); [d4]1'-hydroxymidazolam was synthesized in house and used as an internal standard
- All other chemicals were of HPLC grade or better
- Recombinant human CYP3A4 and CYP3A5 were obtained from Invitrogen (Carlsbad, CA); reported protein concentrations were 10.5 mg/ml and 5.7 mg/ml, respectively
- [d4]1'-hydroxymidazolam (1  $\mu$ M) was prepared in 92/5/3 acetonitrile/water/formic acid (stop solution)
- 13 mM NADPH solution was prepared fresh daily in 100 mM phosphate buffer at pH 7.4

### rCYP3A4 and rCYP3A5 Assay Development

- Recombinant enzyme (rCYP3A4 or rCYP3A5) was preincubated for 10 min with midazolam (+/- ketoconazole or test compound) at 37°C in 384-well plates
- Reactions were initiated by adding NADPH
- Incubations were stopped at 4 min by addition of the stop solution (40  $\mu$ L) containing deuterated internal standard
- Ketoconazole (1  $\mu$ M) was used as a positive control for inhibition of CYP3A activity
- Formation of 1'-hydroxymidazolam from concentrations of midazolam spanning the anticipated  $K_m$  for CYP3A4 (0.3-38  $\mu$ M) and CYP3A5 (0.1-19  $\mu$ M) was determined

### Analytical Procedure (Biotrove)

- Frozen 384-well plates were thawed and centrifuged prior to analysis for 5 min at 3000 rpm
- Five  $\mu$ L of each sample was loaded onto a proprietary HPLC column (Biotrove column "B")
- Analytes were trapped on column "B", washed with loading buffer (H<sub>2</sub>O), and eluted from the column with 80/20 ACN/H<sub>2</sub>O into the ESI source of the mass spectrometer
- MRM transitions, MS voltage and MS gas settings were optimized to detect 1'-hydroxymidazolam (m/z 342→324) and [d4]1'-hydroxymidazolam (m/z 346→328) simultaneously in each sample
- Chromatography conditions were optimized to yield ~8 second cycle times per injection

### Screening Set

- 11,500 proprietary Pfizer compounds that represented diverse chemical space were screened against both rCYP enzymes at 1  $\mu$ M
- Midazolam (3  $\mu$ M) was incubated slightly above the determined  $K_m$  for both CYP3A4 and CYP3A5 to reduce the potentially large number of hits for each rCYP
- Ketoconazole (1  $\mu$ M) was used in each plate as a positive control for inhibition of CYP activity
- Z' scores were determined to ensure the robustness of each analytical plate

## RESULTS

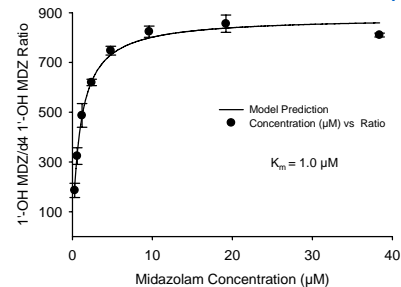


Figure 2a. Substrate saturation curve for rCYP3A4

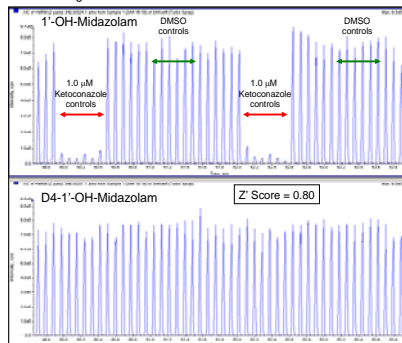


Figure 3a. Representative chromatograms (plate 17) of product formed (top) and internal standard (bottom) from 3  $\mu$ M midazolam incubation with rCYP3A4. Positive controls (ketoconazole) depicted by the red arrows and negative controls (DMSO) by the green arrows.

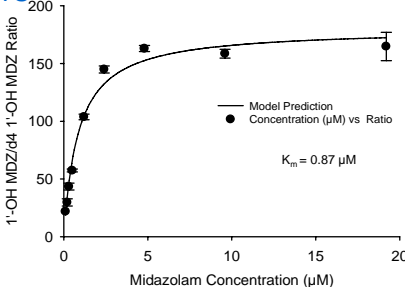


Figure 2b. Substrate saturation curve for rCYP3A5

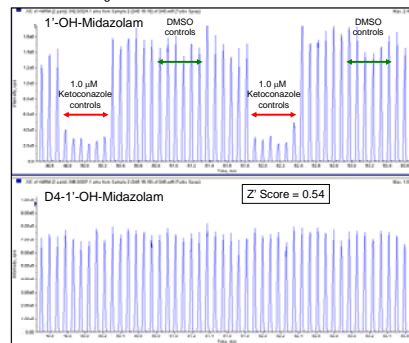


Figure 3b. Representative chromatograms (plate 17) of product formed (top) and internal standard (bottom) from 3  $\mu$ M midazolam incubation with rCYP3A5. Positive controls (ketoconazole) depicted by the red arrows and negative controls (DMSO) by the green arrows.

## RESULTS

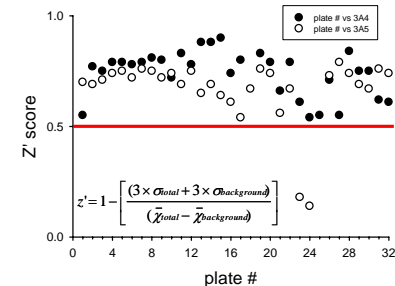


Figure 4. Z' score values for both CYP3A4 and CYP3A5 for all 32 plates comprising 11500 Pfizer compounds.

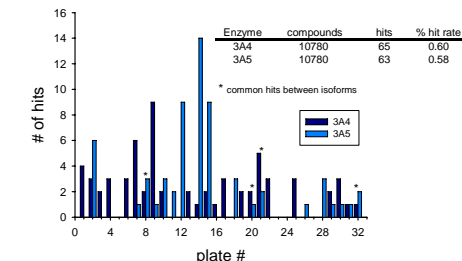


Figure 5. "Hits" from screening of 10780 Pfizer compounds at 1 $\mu$ M. Hit criteria were established as >=30% inhibition of CYP3A4 or CYP3A5.

## CONCLUSIONS

- Biotrove HTMS technology provides a platform for rapid, robust determination of CYP-mediated metabolites
- $K_m$  values for 1'-OH midazolam formation by CYP3A4 and CYP3A5 were similar to values previously reported
- As expected, ketoconazole at 1  $\mu$ M inhibited both CYP3A4 and CYP3A5 mediated metabolism
- Unexpectedly, inhibitors identified for CYP3A4 had minimal overlap with those identified as CYP3A5 inhibitors
- Plates that did not meet the Z' criteria will be rerun
- Inhibitors identified will be evaluated over a concentration range to determine  $IC_{50}$  values for each CYP isoform

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