



ZUCKERMAN SPAEDER LLP

1800 M STREET, NW WASHINGTON, DC 20036-5802
202.778.1800 202.822.8106 fax www.zuckerman.com

William B. Schultz
Partner
(202) 778-1820
wschultz@zuckerman.com

May 17, 2007

VIA FACSIMILE

Ms. Jennie Butler
Director
Division of Dockets Management
U.S. Food and Drug Management
5630 Fishers Lane, Room 1061 (HFA-305)
Rockville, MD 20857

Re: Comments of Mutual Pharmaceutical Co., Inc. to the Supplemental Submission of
King Pharmaceuticals, Inc. in Support of Citizen Petition and Petition for Stay
Docket Nos. 2004P-0140/CPI and 2004P-0140/PSAI

Dear Ms. Butler:

As you requested, this will confirm in writing that Mutual Pharmaceutical Co. understands that all the attachments submitted in connection with the above submission will be available to the public once they are filed in the docket. Thank you for your assistance and please give me a call if you have any further questions.

Sincerely yours,

William B. Schultz



***In Vitro* Assessment of Reaction Phenotyping (Enzyme Identification) for Human Cytochrome
P450 Enzymes by Metaxalone**

Report

Issue Date: January 2007
Study Number: 2400-0603-1800
Sponsor Study Number: SP046306

Sponsor

Mutual Pharmaceutical Company, Inc.

**1100 Orthodox St.
Philadelphia, PA 19124**

Prepared by

***In Vitro* Services
CellzDirect, Inc.
480 Hillsboro St., Suite 130
Pittsboro, NC 27312
1624 Headway Circle, Suite 100
Austin, Texas 78754
Stephen Ferguson, Ph.D.
Tel: 512-623-3032
Fax: 512-623-3001
Email: stephenf@cellzdirect.com**

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Report: Reaction Phenotyping for Human CYP450 by Metaxalone

ABBREVIATIONS USED

ATM – Abbreviated test method

CON – Control

CYP450 – Cytochrome P450

NADPH – β -Nicotinamide adenine dinucleotide 2' phosphate (reduced form)

QC – Quality control

FDA – Food & Drug Administration

LLOQ - Lower Limit of Quantitation

SUMMARY

Title

In Vitro Assessment of Reaction Phenotyping (Enzyme Identification) for Human Cytochrome P450 Enzymes by Metaxalone.

Objective

The aim of these studies is to identify the cytochrome P450 enzymes involved in the *in vitro* biotransformation of metaxalone in support of drug discovery and development efforts at URL Mutual, in care of Salamandra, LLC. This drug is known to be metabolized in humans; however, the enzymes involved are not known.

Methods

LC-MS/MS methods were developed to measure metaxalone turnover and to establish a linear range for quantitation by monitoring metaxalone disappearance. Preliminary incubations were performed with human liver microsomes to establish optimal time and protein conditions to measure metaxalone metabolic turnover. A concentration of 1 mg/mL and incubation time of 60 minutes were determined to be the most suitable for further incubations. To evaluate the involvement of CYP 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 in the disappearance of 0.75 and 7.5 μ M metaxalone, two complementary methods were used: (i) incubations using pooled human liver microsomes and specific chemical inhibitors and (ii) incubations using single cDNA expressed CYP450s.

Results and Conclusions

Metaxalone showed CYP-mediated metabolic turnover of approximately 29.8% at 0.75 μ M concentrations and 24.6% for 7.5 μ M concentrations of metaxalone following incubation with human microsomes (1 mg/mL) for 60 minutes at 37°C. These results are consistent with literature reports indicating that metaxalone is metabolized *in vivo*. Co-incubation of metaxalone and selective CYP450 chemical inhibitors with human liver microsomes showed substantial inhibition of metaxalone turnover with selective inhibitors for CYP3A4, CYP2E1, and CYP1A2. Additionally, inhibitors for CYP2D6, CYP2C19, CYP2C8, and CYP2C9 also markedly inhibited turnover of metaxalone in human liver microsomal incubations. Incubation of metaxalone with recombinant CYP450 enzymes showed relative metaxalone turnover in the following order: CYP3A4 > CYP1A2 > CYP2E1, > CYP2D6 = CYP2C19. CYP2C9 and CYP2C8 also appeared to turnover metaxalone at the higher concentration of metaxalone examined (7.5 μ M).

Taken together, these data indicate that several CYP450 enzymes are likely involved in the NADPH-dependent metabolism of metaxalone, however CYP1A2, CYP2E1, CYP2D6, and CYP3A4 appear to be the most important CYP450 enzymes examined. CYP2C19, CYP2C9, and CYP2C8 also appear capable of metabolizing metaxalone.

In Vitro Assessment of Reaction Phenotyping (Enzyme Identification) for Human Cytochrome P450 Enzymes by Metaxalone.

Study Number: 2400-0603-1800

APPROVAL SECTION

CellzDirect:

Study Director:

Stephen Ferguson, Ph.D.
CellzDirect, Inc.

Date

Study Monitor:

J. Michael Morgan Ph.D. DABT
Salamandra, LLC

Date

Sponsor:

Matthew W. Davis M.D. R.Ph.
Mutual Pharmaceuticals, Inc.

Date

1.0 INTRODUCTION

Biotransformation is the major route of elimination for a majority of drugs and metabolism by Phase I oxidative cytochrome P450 enzymes (P450) is the most common metabolic pathway. To characterize the metabolic clearance of a drug candidate, the relative contribution of P450 enzymes to the overall elimination process is required along with identification of the enzymes responsible for the oxidative reactions. Reaction phenotyping (enzyme identification) studies are conducted to identify the specific enzymes responsible for the metabolism of a drug. Identification of cytochrome P450 enzymes *in vitro* can prove useful in predicting the potential for drug interactions, polymorphic impact on drug disposition and formation of toxic or active metabolites, and is recommended when P450 enzymes contribute to greater than 25% of a drug's total clearance (1-3). Alternatively, evidence that certain metabolic pathways are not important via *in vitro* studies may preclude the need for further clinical investigations, or help focus the design of clinical drug-drug interaction studies (4-6). While this study is limited to P450 enzymes, other Phase I (e.g. flavin-containing monooxygenase (FMO), monoamine oxidase, epoxide hydrolase) and Phase II (e.g. UDP-glucuronyl transferase, glutathione S-transferase, sulfotransferase, methyltransferase, and N-acetyltransferase) drug metabolizing enzymes may also contribute to metabolism and should be considered.

The goal in reaction phenotyping is to determine the metabolic profile of a drug and the relative contribution of specific enzymes to overall metabolic clearance, potentially followed by systematic characterization of the specific enzymes involved in formation of each major metabolite. To do this successfully, more than one approach should be used. Microsomal preparations containing individual recombinant human CYP450 isoforms in liver-like distributions are a valuable tool for evaluating the intrinsic ability CYP450s to metabolize a drug candidate (7). If the test compound is metabolized by multiple enzymes, further studies with highly selective chemical inhibitors and/or antibodies can be conducted in human hepatic microsomes to address the relative contribution (1). Chemical inhibitors for which there is prior clinical data should be used to aid in valid *in vitro/in vivo* correlations. The disadvantage of this system is that few highly specific chemical inhibitors are available. Potent inhibitory monoclonal antibodies against various human P450 isoforms are more specific inhibitors. A fourth approach is more time intensive and involves mechanistic determinations (K_m and V_m) of the test compound with each active recombinant P450 enzyme, to enable calculations of intrinsic clearance (V_m/K_m). Combined with knowledge of the relative abundance of each P450 isoform in the pool of human liver microsomes being used, the relative contribution of different P450 enzymes in the metabolism of the compound of interest in human liver microsomes can be evaluated (7, 8).

The Food and Drug Administration recommends at least two experimental approaches be used to determine the liver-derived enzymes that are involved in the metabolism of the investigational drug (10). As such, this study was designed to include the use of CYP450-specific inhibitors and

recombinant enzymes to discern which hepatic CYP450(s) may be involved in the metabolism of metaxalone. Knowledge of which CYP450s are involved in the metabolism of metaxalone aids in the understanding of potential clinical drug interactions and help to predict intra-individual variations in test article clearance due to genetic polymorphisms in CYP2C9, CYP2C19 and CYP2D6.

In the current study, metaxalone will be examined in a reaction phenotyping study to characterize the metabolism of this drug. Metaxalone has previously been described by Bruce and coworkers to be metabolized in man (9). To further evaluate metaxalone metabolism in vitro with modern methodologies, the NADPH-dependent turnover of metaxalone was assessed in pooled human liver microsomes and with recombinant expressed enzymes to identify specific CYP450s that may be important for metaxalone metabolism.

2.0 MATERIALS AND METHODS

These studies were conducted by CellzDirect in accordance with CellzDirect Protocol 4200-0603-1800 (Appendix 2) and CellzDirect Standard Operating Procedures. Assay-specific SOPs include, but are not limited to, IV-001, IV-002, IV-010, IV-013, IV-014, IV-015, IV-016, IV-017, IV-021, IV-024, IV-060, IV-072, and IV-082. In addition, all assays described in this document were conducted following CellzDirect Abbreviated Test Methods (ATMs).

2.1 Test Article

Metaxalone was provided by the sponsor (>100 mg of metaxalone, MW = 221.26, Lot # P-1055-116A1, purity = 99.8%). A certificate of analysis is provided in Appendix 2. Stock solutions at 1.875 mM were prepared in methanol and stored at -20°C. Stocks were diluted daily in the appropriate buffers such that the final organic solvent concentration was <1%.

2.2 Chemicals, Reagents, and Solvents

Potassium phosphate monobasic, potassium phosphate dibasic, NADPH tetrasodium salts, and other reagents were purchased from Sigma Chemical Co. or equivalent vendors. Methanol (HPLC grade), water (HPLC grade), ethyl acetate, and other solvents were purchased from Fisher, Burdick & Jackson, J. T. Baker, Mallinckrodt, or equivalent vendors. All inhibitors were of the highest purity available. Individual suppliers are as follows: furafylline, pilocarpine, thio-TEPA, quercetin, sulfaphenazole, ticlopidine, quinidine, clomethiazole, and ketoconazole were obtained from Sigma Chemical Co.

All recombinant P450s were obtained from BD Gentest. CellzDirect's human liver microsomes (Lot No: PL020) pooled from 15 individuals (male and female) were utilized in this study. The microsomes were previously characterized for the major cytochrome P450 enzyme activities by CellzDirect's In Vitro Products and Services Division (Appendix 3).

2.3 Experimental Design

The primary objective of this study is to determine which human liver CYP450 isoforms may be involved in the *in vitro* biotransformation of the test compound. The scope of work to be performed for this is outlined in the following tasks: Task 1) Set-up of Analytical Methodology and Preliminary Incubations; Task 2) CYP450 Selective Inhibition Studies; Task 3) Recombinant CYP450 Studies; and Task 4) Report. Metaxalone concentrations were chosen in conjunction with the Sponsor to be 0.75 and 7.5 μM (approximately 1x and 0.1x C_{max}) based on estimated clinical plasma concentrations of metaxalone. (Currently approved Package Insert for Skelaxin[®] (11).

2.3.1 Task 1: Set-up of Analytical Methodology and Preliminary Incubations

Analytical method: An isocratic LC-MS/MS was developed to allow for chromatographic resolution and quantitation of the parent test article contained within an incubation matrix. Set-up of the analytical method involved determining the parent and daughter ions for the compound by direct infusion. Secondly, the compound was injected in mobile phase onto an isocratic LC-MS/MS chromatographic system to assess peak shape, retention times, and signal to noise ratio of a known amount on column. A standard curve, comprised of at least 8 calibration standards was prepared and assessed. The following LC-MS/MS method was used for metaxalone quantitation:

Metaxalone	
Substrate:Metabolite:	Metaxalone
Standard Metabolite Range:	0.078 to 10.0 μM
Mobile Phase A	2% Methanol, 98% Water, and 0.1% Acetic Acid
Mobile Phase B	80% Methanol, 20% Water, and 0.1% Acetic Acid
Gradient	0.400 mL/min, initial: 50% Mobile Phase B, 0.01 min: 50% Mobile Phase B, 2.00 min: 80% Mobile Phase B, 2.10min: 50% Mobile Phase B, and 4.00min: Stop
Detection Method:	LC-MS/MS
HPLC Column:	WATERS AQ12 4x23mm
Flow rate (approx.):	0.400 mL/min
Source:	Electrospray (positive ion)
Run Time (approx.):	4.0 minutes
MRM (Sulfamethoxazole):	222.14 \rightarrow 160.83
Quantitation:	Least Squares Regression $1/X^2$ Weighting

Method Validation: A one day validation was performed to establish the analytical method using pooled human liver microsomes. To this end, a triplicate standard curve and six QCs (at three different concentration levels) were analyzed. The validation will aim to address the following:

- Intra-Day Accuracy

- Intra-Day Precision (ruggedness)
- Linearity (Correlation Coefficient)
- Specificity (blank matrix)

CellzDirect considered the validation acceptable if standard and QC samples meet criteria set forth in CellzDirect SOPs. Specifically, a run is considered acceptable if the back-calculated values for each calibration standard are within $\pm 20\%$ ($\pm 25\%$ at the LLOQ) of the theoretical value and 50% of the back-calculated QC samples at each concentration level and $2/3^{\text{rds}}$ of the back-calculated QC samples overall are within 20% of their theoretical values.

Preliminary Incubations (Time and Protein Dependence): Pilot microsomal incubations were performed in order to establish appropriate protein concentration and time points for linear reaction conditions for metaxalone turnover. Metaxalone at 0.75 and 7.5 μM was incubated with 0.1, 0.25, 0.5, and 1.0 mg/mL microsomal protein for 60 minutes at 37°C, and incubations with 0.5 mg/mL were incubated for 0, 15, 30, 45, and 60 minutes at 37°C. The reactions, in 0.1 M phosphate buffer, pH 7.4, were initiated by addition of 1 mM NADPH. Negative controls (no NADPH and heat-treated microsomes) were included to account for any non-enzymatic or non-NADPH dependent reactions. The reactions were terminated at the appropriate time points by addition of 1 volume (relative to total reaction volume) of methanol. The samples were centrifuged at approximately 3000 rpm and the clear supernatant was transferred to a clean tube and analyzed by the LC-MS/MS method described above. A concentration of 1 mg/mL microsomal protein and incubation time of 60 minutes were determined to be the most suitable for further incubations.

2.3.2 Task 2: Incubations with Selective CYP Inhibitors

The objective of these experiments was to identify CYP450 isoform(s) involved in the potential metabolism of the parent test article in a microsomal system (which approximates the *in vivo* distribution of hepatic enzymes) and selective CYP chemical inhibitors. The specific chemical inhibitors and concentrations used are detailed in Table 1 below.

Table 1. Protocol for in vitro Assessment of Chemical Inhibition in Human Liver Microsomes.

P450 Isoform	specific chemical inhibitor	concentration
CYP1A2	Furafylline	50 μM
CYP2A6	Pilocarpine	100 μM
CYP2B6	Thio-TEPA	75 μM
CYP2C8	Quercetin	10 μM

CYP2C9	Sulfaphenazole	20 µM
CYP2C19	Ticlopidine	1 µM
CYP2D6	Quinidine	10 µM
CYP2E1	Clomethiazole	100 µM
CYP3A4	Ketoconazole	1 µM

The inhibitors or vehicle control were incubated with the pooled human liver microsomes (1 mg/mL) and β-NADPH (1 mM) in 0.1 M phosphate buffer, pH 7.4. The reaction was initiated by addition of 0.75 or 7.5 µM metaxalone and incubated for 60 minutes. Incubations were conducted in triplicate and terminated by addition of organic solvent. Samples were extracted and analyzed by LC-MS/MS as described in Section 2.3.1. The rates of test article depletion were compared to controls without inhibitors.

2.3.3 Task 3: Studies with Recombinant Cytochrome P450 Enzymes

In order to determine which specific CYP450s are capable of metabolizing metaxalone, individual CYP450 recombinant enzymes were incubated with 0.75 and 7.5 µM metaxalone. CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 expressed enzymes at 5 and 20 pmol protein were incubated in 0.1 M phosphate buffer, pH 7.4 with β-NADPH (1 mM) and with metaxalone for 60 minutes at 37°C. The incubations, performed in triplicate, were terminated by addition of 1 volume (relative to total reaction volume) of methanol. The samples were analyzed as described in Section 2.3.1. The rates of test article depletion were compared with control (microsomes expressing no recombinant CYP450 enzymes). Additional controls included incubation with each isoform with the universal CYP substrate, phenanthrene. Turnover of phenanthrene was monitored fluorometrically at 254 nm (excitation) and 378 nm (emission).

3.0 DATA HANDLING

3.1 Data Analyses

Micromass Masslynx software (version 3.4, Manchester, UK) was used to collect and process chromatographic data. Data were graphed with the software program Microsoft Excel® 2003 (Redmond, WA). Reaction velocities were calculated using the following equation where TA=Test Article:

$$\text{Percent Turnover} = 100 - \left\{ \frac{[\text{TA}(\text{final})]}{[\text{TA}(0 \text{ min})]} \right\} \times 100$$

$$\text{Percent Inhibition of Turnover} = 1 - \frac{[\text{TA}(0 \text{ min})] - [\text{TA}(\text{final-inhibitor})]}{[\text{TA}(0 \text{ min})] - [\text{TA}(\text{final-no inhibitor})]} \times 100$$

3.2 Data Storage

All raw data and supporting documents will be stored by the Archivist at CellzDirect. The data will be transferred to archives according to CellzDirect Standard Operating Procedures after submission of the final report to Mutual Pharmaceutical Company, Inc.

3.3 Regulatory Compliance

This study was conducted in compliance with the appropriate CellzDirect Standard Operating Procedures. All analytical runs were evaluated based on the acceptance criteria set forth in CellzDirect SOP IV-002. More specifically, analytical runs were accepted only if at least 2/3 of the quality control samples ($\geq 50\%$ at each level) were within $\pm 20\%$ of the nominal values. Any deviations from the acceptance criteria were clearly documented.

4.0 RESULTS AND DISCUSSION

4.1 Metaxalone Method Validation and Preliminary Incubations

LC-MS/MS-based methods were developed for metaxalone quantification and were validated for linearity and reproducibility within the appropriate concentration range. Preliminary incubations of metaxalone with pooled human liver microsomes (using 0.1, 0.25, 0.5, and 1 mg/mL microsomal protein in 60 min incubations, and 0, 15, 30, 45, and 60 minute incubations with 0.5 mg/mL microsomal protein). Linear conditions for metaxalone disappearance were observed with both time- and protein- dependence as shown in Figures 1 and 2, Table 2). Maximum turnover of 38.0% at 60 minutes with 1 mg/mL protein. This turnover was NADPH-dependent, indicating that disappearance of metaxalone under these conditions was likely a metabolic process involving CYP450 enzymes. Based on these results, the microsomal protein concentration used for all subsequent assays was 1 mg/mL with an incubation time of 60 minutes to maximize the dynamic range of substrate turnover. These incubations were conducted with 0.75 and 7.5 μM metaxalone (approximately 1x and 0.1x C_{max}) as substrate.

4.2 Selective CYP Inhibitors

Selective chemical inhibitors were used to evaluate the effects of individual CYP450s in human liver microsomes on the metabolism of metaxalone (Figure 3, Tables 3 and 4). Metaxalone disappearance was observed at 24.6% at 7.5 μM vs. 29.8% at 0.75 μM at the conditions examined.

These results are consistent with the data produced *in vivo* by Bruce and coworkers showing 27% of metaxalone was converted to a carboxylic acid derivative that appears to be subsequently glucuronidated to an additional metabolite (9). Inhibition of metaxalone disappearance in human liver microsomes with either 0.75 and 7.5 μM metaxalone was observed by the addition of the CYP1A2 selective inhibitor furafylline (80.6 - 111%), CYP3A4 inhibitor ketoconazole (126-130%), CYP2E1 inhibitor clomethiazole (98.6-113%), and CYP2D6 inhibitor quinidine (98.7-59.5%) (Figure 3). In addition, marked inhibition of metaxalone turnover was observed with CYP2C19 inhibitor ticlopidine (72.6-93.1%), CYP2C9 inhibitor sulfaphenazole (81.4-52.8%), and CYP2C8 inhibitor quercetin (77.2-84.7%). Significantly smaller inhibition of turnover was observed with CYP2A6 and CYP2B6 inhibitors, however these responses were not supported by the chemical inhibitor data discussed below. Therefore, CYP2A6 and CYP2B6 likely do not play a significant role in the CYP450-mediated metabolism of metaxalone. It is noteworthy that higher concentrations of metaxalone led to lower CYP450-dependent turnover (24.6% at 7.5 μM vs. 29.8% at 0.75 μM) as well as a stronger contribution from multiple CYP450s. Overall, these data indicate that CYP3A4, CYP1A2, CYP2E1, and CYP2D6 are the major CYP450 enzymes involved in metaxalone metabolism (as monitored by metaxalone disappearance) in human liver microsomes, with a smaller contribution from CYP2C19, CYP2C8, and CYP2C9. No published reports on metaxalone metabolic phenotyping were found.

4.3 Recombinant CYP450s

Individual human recombinant CYP450 enzymes (Supersomes[®]) were used to evaluate the effect of individual CYP450s on the metabolism of metaxalone. The universal CYP450 positive control substrate phenanthrene, examined with each recombinant enzyme to ensure proper activity, confirmed that each recombinant enzyme microsomal preparation was active. Metaxalone disappearance at either 0.75 or 7.5 μM was evaluated in incubations containing either 5 or 20 pmol of recombinant CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 or CYP3A4 (Figures 4 and 5, Tables 5 - 8). Several CYP450s at both 5 and 20 pmoles were shown to be capable of metabolizing metaxalone at 0.75 μM concentrations including CYP3A4, CYP2E1, CYP2D6, CYP2C19, and CYP1A2 which showed percent turnover of 17.9, 11.3, 9.9, 6.9 and 9.2% at 5 pmol CYP450, and 34.0, 16.2, 19.5, 12.5, and 34.8% at 20 pmol, respectively. At 7.5 μM concentrations of metaxalone CYP3A4, CYP2E1, CYP2D6, CYP2C19, and CYP1A2 were most effective with percent turnovers of 16.6, 18.4, 12.6, 6.3, and 10.0% at 5 pmol CYP450, and 32.4, 17.2, 24.9, 18.7, and 30.4% at 20 pmol CYP450, respectively. CYP2C8 and CYP2C9 also moderately metabolized 7.5 μM metaxalone (ranging from 3.0% to 9.3% turnover) but were not effective in metabolizing metaxalone at lower concentrations (0.75 μM). These data demonstrate that

multiple CYP450s can contribute to the metabolism of metaxalone, however CYP1A2, CYP3A4, CYP2E1 and CYP2D6 appear to be the most efficacious in metabolizing metaxalone.

5.0 CONCLUSIONS

Metaxalone (0.75 and 7.5 μM) appears to be metabolized by CYP450s in pooled human liver microsomes with a percent turnovers ranging from 23.9-31.5% under the conditions examined. Analysis of metaxalone metabolism by reaction phenotyping using both chemical inhibitor and recombinant CYP450 approaches revealed that CYP1A2, CYP2D6, CYP2E1, and CYP3A4 are all capable of metabolizing metaxalone, and are likely the major enzymes governing the CYP450-mediated metabolism of metaxalone in humans. Some possible contributions from CYP2C19, CYP2C8, and CYP2C9 were also observed.

6.0 REFERENCES

- (1) Lu, A., Wang, R. and Lin, J. (2003) Cytochrome P450 in vitro reaction phenotyping: a re-evaluation of approaches used for P450 isoform identification. *Drug Metab Dispos* 31, 345-350.
- (2) Williams, J., Bauman, J. N., Cai, H., Conlon, K., Hansel, S., Hurst, S., Sadagopan, N., Tugnait, M., Zhang, L. and Sahi, J. (2005) In vitro ADME phenotyping in drug discovery: current challenges and future solutions. *Current Opinion Drug Discovery Development* 8, 78-88.
- (3) Williams, J., Hurst, S., Bauman, J., Jones, B., Hyland, R., Gibbs, J., Obach, R. and Ball, S. (2003) Reaction phenotyping in drug discovery: moving forward with confidence? *Curr Drug Metab* 4, 527-534.
- (4) Venkatakrishnan, K., Von Moltke, L. and Greenblatt, D. (2001) Human drug metabolism and the cytochromes P450: application and relevance of in vitro models. *J Clin Pharmacol* 41, 1149-1179.
- (5) Streetman, D., Bleakley, J., Kim, J., Nafziger, A., Leeder, J., Gaedigk, A., Gotschall, R., Kearns, G. and Jr, B. J. (2000) Combined phenotypic assessment of CYP1A2, CYP2C19, CYP2D6, CYP3A, N-acetyltransferase-2, and xanthine oxidase with the "Cooperstown cocktail". *Clin Pharmacol Ther* 68, 375-383.
- (6) Williams, J., Hyland, R., Jones, B., Smith, D. A., Hurst, S., Goosen, T., Peterkin, V., Koup, J. and Ball, S. (2004) Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUCI/AUC) ratios. *DMD* 32, 1201-1208.
- (7) Crespi, C. L. and Miller, V. P. (1999) The use of heterologously expressed drug metabolizing enzymes--state of the art and prospects for the future. *Pharmacol Ther* 84, 121-131.
- (8) Rodrigues, A. D. (1999) Integrated cytochrome P450 reaction phenotyping: attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. *Biochem Pharmacol* 57, 465-480.
- (9) Bruce RB, Turnbull L, Newman J, Pitts J. (1966) Metabolism of metaxalone. *J Med Chem.* 9, 286-288.
- (10) Food and Drug Administration (2006) Guidance for industry Drug Interaction Studies - Study Design, Data Analysis, and Implications for Dosing and Labeling.
- (11) Metaxalone Sulfate Approved Label 2005 (<http://www.fda.gov/cder/foi/label/2005/021799lbl.pdf>).

7.0 FIGURES

Figure 1: Time Dependence on Metaxalone Metabolic Turnover

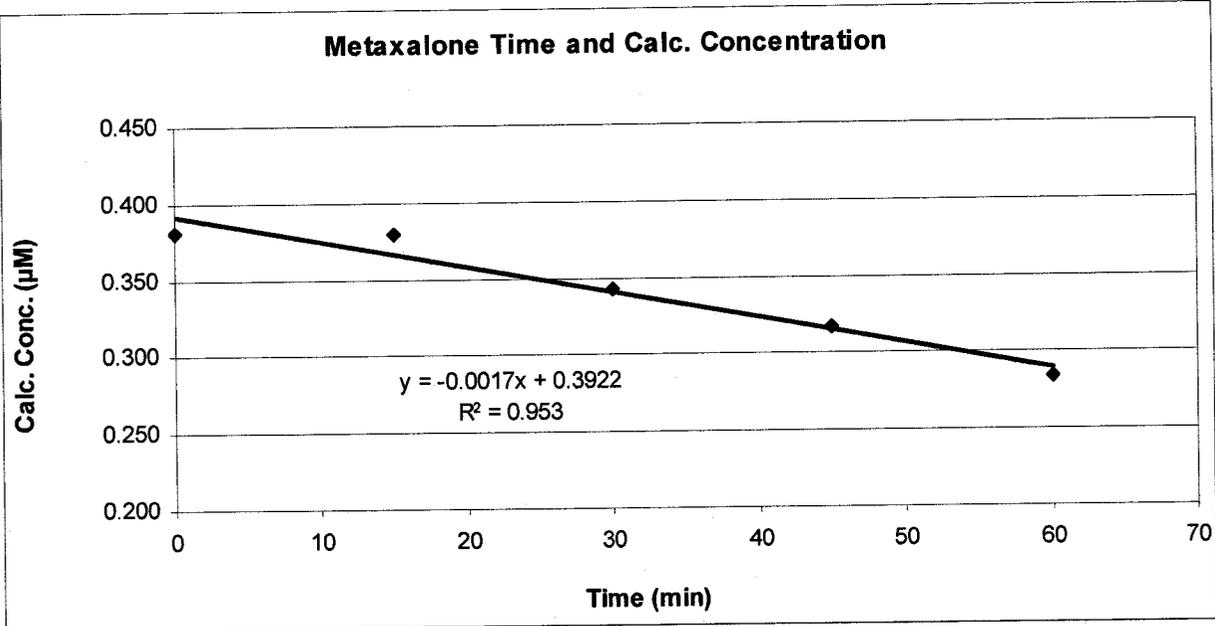


Figure 2: Protein Dependence on Metaxalone Metabolic Turnover

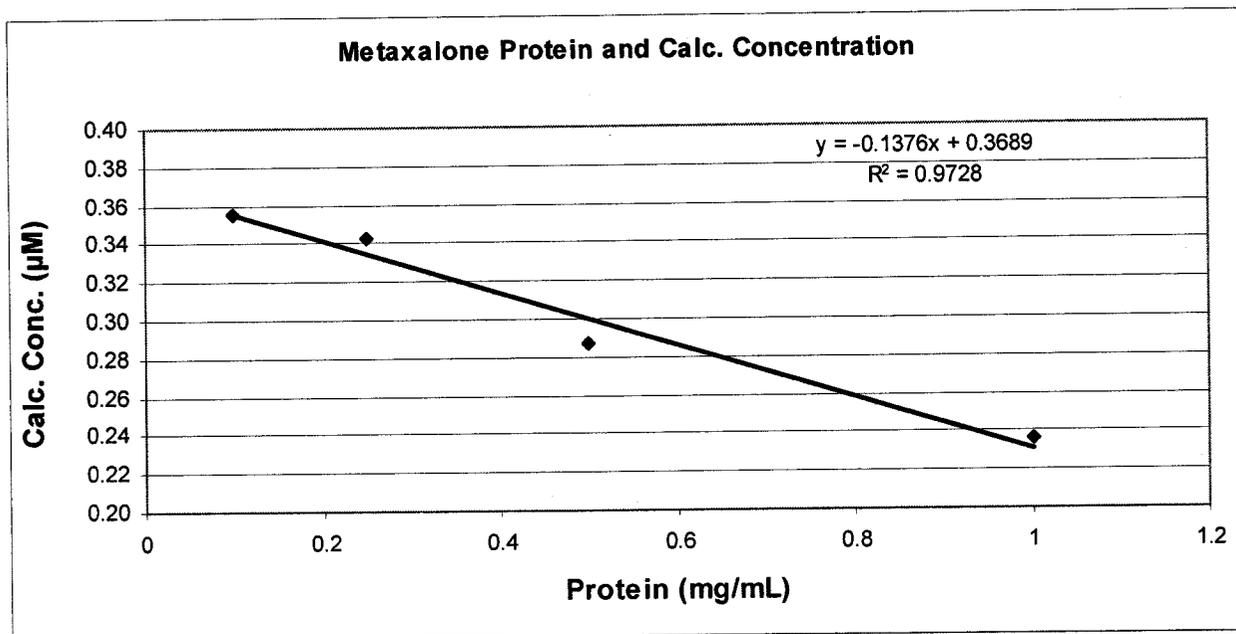


Figure 3: Metaxalone (0.75 and 7.5 μ M) Phenotyping in Human Liver Microsomes with Selective CYP450 Inhibitors

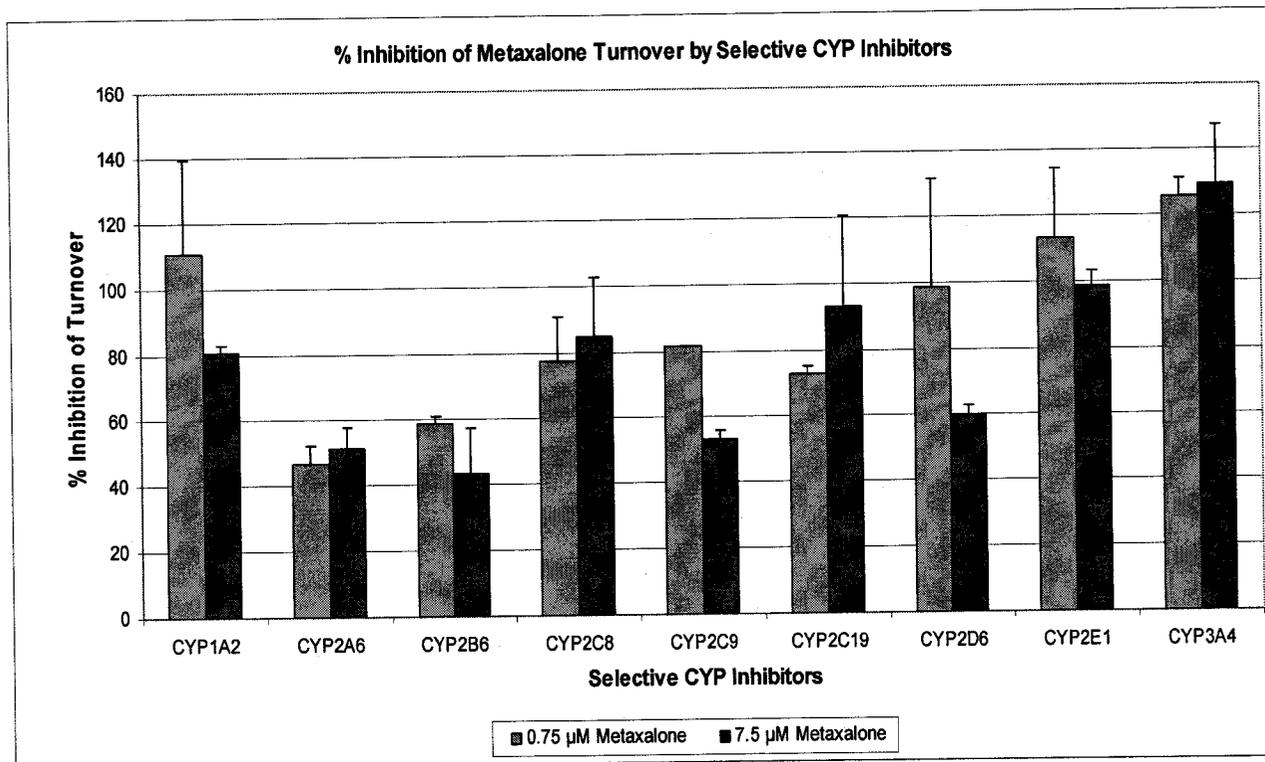


Figure 4: Metaxalone (0.75 μ M) Phenotyping Using Recombinant CYP450s

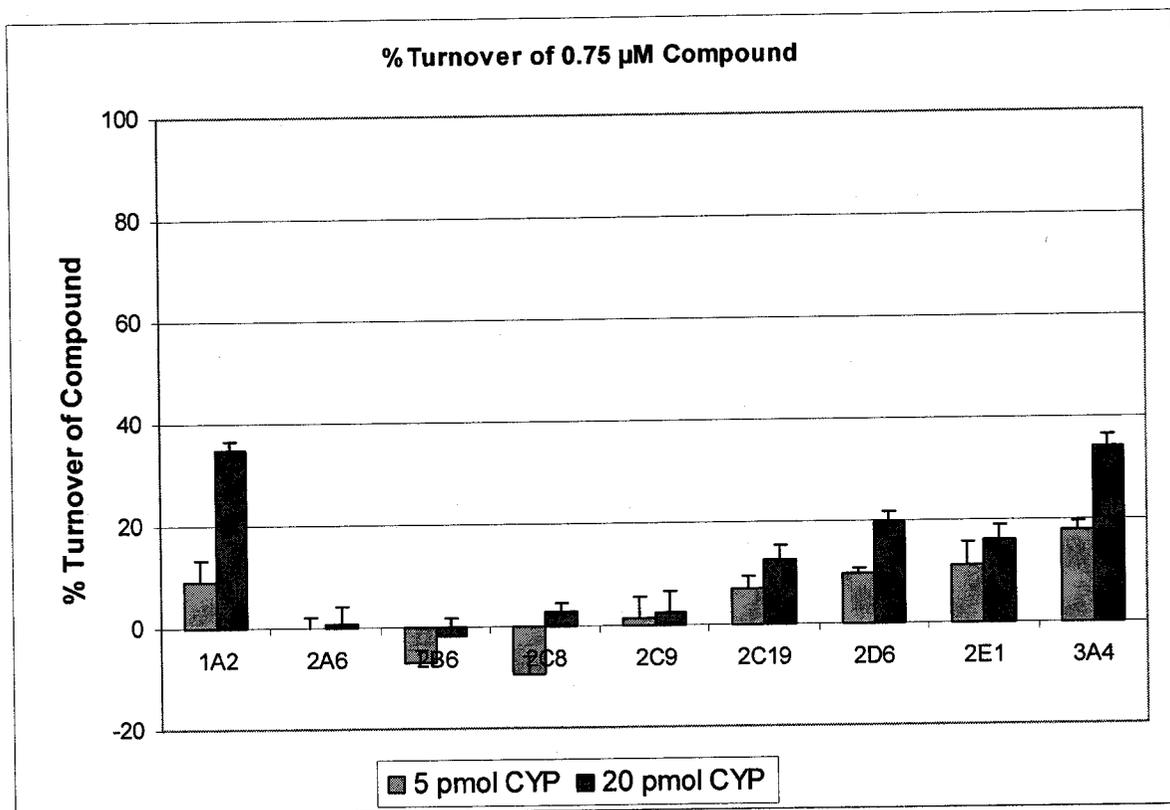
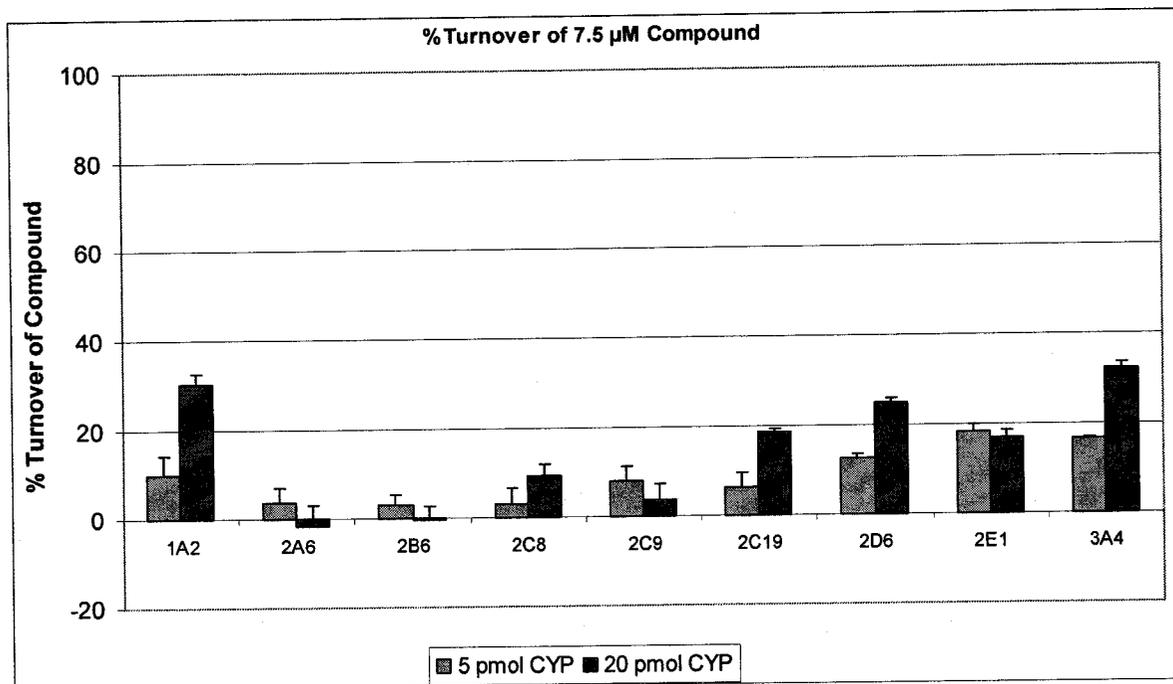


Figure 5: Metaxalone (7.5 μ M) Phenotyping Using Recombinant CYP450s



8.0 DATA TABLES

Table 2: Time and Protein Dependence on Metaxalone Metabolic Turnover

Sample	ID	0.5 μ M Metaxalone Concentration (μ M)
Time 0min - Protein 0.5mg/mL	0.5uM-T0-P0.5-1	0.380
Time 0min - Protein 0.5mg/mL	0.5uM-T0-P0.5-2	0.383
Time 15min - Protein 0.5mg/mL	0.5uM-T15-P0.5-1	0.374
Time 15min - Protein 0.5mg/mL	0.5uM-T15-P0.5-2	0.385
Time 30min - Protein 0.5mg/mL	0.5uM-T30-P0.5-1	0.355
Time 30min - Protein 0.5mg/mL	0.5uM-T30-P0.5-2	0.331
Time 45min - Protein 0.5mg/mL	0.5uM-T45-P0.5-1	0.319
Time 45min - Protein 0.5mg/mL	0.5uM-T45-P0.5-2	0.316
Time 60min - Protein 0.5mg/mL	0.5uM-T60-P0.5-1	N12
Time 60min - Protein 0.5mg/mL	0.5uM-T60-P0.5-2	0.285
Time 60min - Protein 0.1mg/mL	0.5uM-T60-P0.1-1	0.356
Time 60min - Protein 0.1mg/mL	0.5uM-T60-P0.1-2	0.355
Time 60min - Protein 0.25mg/mL	0.5uM-T60-P0.25-1	0.336
Time 60min - Protein 0.25mg/mL	0.5uM-T60-P0.25-2	0.349
Time 60min - Protein 0.5mg/mL	0.5uM-T60-P0.5-1	0.275
Time 60min - Protein 0.5mg/mL	0.5uM-T60-P0.5-2	0.300
Time 60min - Protein 1.0mg/mL	0.5uM-T60-P1.0-1	0.248
Time 60min - Protein 1.0mg/mL	0.5uM-T60-P1.0-2	0.224
Heat Treated	HT-0.5uM-T60-P1.0-1	0.383
Heat Treated	HT-0.5uM-T60-P1.0-2	0.414
No-NADPH	No-0.5uM-T60-P1.0-1	0.426
No-NADPH	No-0.5uM-T60-P1.0-2	0.422

HT - Heat Treated

No - No NADPH

N12 - NOT INCLUDED IN CALCULATIONS; VALUE BELOW THE LLOQ.

Report: Reaction Phenotyping for Human CYP450 by Metaxalone

Table 3: Chemical Inhibitor Data in Human Liver Microsomes with 0.75 µM Metaxalone

0.75 µM Metaxalone

Inhibitor	CYP	ID	Concentration		% Turnover	Mean	Standard Error	% Inh of turnover	Mean % inh of turnover	Standard Error
			(µM)	Mean (µM)						
	0 min.	0.75µM-T0-P1.0-1	0.730							
		0.75µM-T0-P1.0-2	0.815							
		0.75µM-T0-P1.0-3	0.770	0.772						
	60 min.	0.75µM-T60-P1.0-1	0.536		30.5					
		0.75µM-T60-P1.0-2	0.561		27.3					
		0.75µM-T60-P1.0-3	0.529	0.542	31.5	29.8	1.3			
Furafylline	CYP1A2	0.75µM-T60-P1.0-F-1	0.824		-19.7			166		
		0.75µM-T60-P1.0-F-2	0.767		0.60			99.0		
		0.75µM-T60-P1.0-F-3	0.701	0.797	9.2	-3.29	6.57	69.1	111	29
Pilocarpine	CYP2A6	0.75µM-T60-P1.0-P-1	0.664		13.9			53.2		
		0.75µM-T60-P1.0-P-2	0.658		14.7			50.6		
		0.75µM-T60-P1.0-P-3	0.622	0.648	19.4	16.0	1.7	34.9	46.2	5.7
Thio-TEPA	CYP2B6	0.75µM-T60-P1.0-TT-1	0.678		12.1			59.3		
		0.75µM-T60-P1.0-TT-2	0.684		11.3			62.0		
		0.75µM-T60-P1.0-TT-3	0.665	0.676	13.8	12.4	0.7	53.7	58.3	2.4
Quercetin	CYP2C8	0.75µM-T60-P1.0-Qr-1	0.661		14.3			51.9		
		0.75µM-T60-P1.0-Qr-2	0.769		0.39			98.7		
		0.75µM-T60-P1.0-Qr-3	0.728	0.719	5.8	6.78	4.06	81.1	77.2	13.6
Sulfaphenazole	CYP2C9	0.75µM-T60-P1.0-S-1	0.730		5.4			81.9		
		0.75µM-T60-P1.0-S-2	0.728		5.6			81.1		
		0.75µM-T60-P1.0-S-3	0.729	0.729	5.8	5.53	0.08	81.3	81.4	0.3
Ticlopidine	CYP2C19	0.75µM-T60-P1.0-Ti-1	0.698		9.6			67.9		
		0.75µM-T60-P1.0-Ti-2	0.714		7.5			74.7		
		0.75µM-T60-P1.0-Ti-3	0.715	0.709	7.4	8.16	0.70	75.2	72.6	2.3
Quinidine	CYP2D6	0.75µM-T60-P1.0-Qi-1	0.694		10.0			66.4		
		0.75µM-T60-P1.0-Qi-2	0.920		-19.2			165		
		0.75µM-T60-P1.0-Qi-3	0.692	0.769	10.3	0.376	9.796	65.4	99.7	32.9
Clomethiazole	CYP2E1	0.75µM-T60-P1.0-Clo-1	0.780		-1.1			104		
		0.75µM-T60-P1.0-Clo-2	0.896		-16.1			154		
		0.75µM-T60-P1.0-Clo-3	0.729	0.802	5.6	-3.87	6.39	81.4	113	21
Ketocozazole	CYP3A4	0.75µM-T60-P1.0-K-1	0.644		-9.4			132		
		0.75µM-T60-P1.0-K-2	0.807		-4.8			116		
		0.75µM-T60-P1.0-K-3	0.842	0.831	-9.1	-7.71	1.54	130	126	5

Report: Reaction Phenotyping for Human CYP450 by Metaxalone

Table 4: Chemical Inhibitor Data in Human Liver Microsomes with 7.5 μ M Metaxalone

7.5 μ M Metaxalone

Inhibitor	CYP	ID	Concentration		% Turnover	Mean	Standard Error	% inh of turnover	Mean % inh of turnover	Standard Error
			(μ M)	Mean (μ M)						
0 min.		7.5uM-T0-P1.0-1	8.34							
		7.5uM-T0-P1.0-2	8.55							
		7.5uM-T0-P1.0-3	8.65	8.51						
60 min.		7.5uM-T60-P1.0-1	6.40		24.8					
		7.5uM-T60-P1.0-2	6.48		23.9					
		7.5uM-T60-P1.0-3	6.38	6.42	25.1	24.6	0.3			
Furafylline	CYP1A2	7.5uM-T60-P1.0-F-1	8.07		5.2			78.8		
		7.5uM-T60-P1.0-F-2	8.21		3.6			85.4		
		7.5uM-T60-P1.0-F-3	8.05	8.11	5.5	4.77	0.59	77.7	80.6	2.4
Pilocarpine	CYP2A6	7.5uM-T60-P1.0-P-1	7.76		8.8			64.2		
		7.5uM-T60-P1.0-P-2	7.28		14.5			41.2		
		7.5uM-T60-P1.0-P-3	7.41	7.48	13.0	12.1	1.7	47.1	50.8	6.9
Thio-TEPA	CYP2B6	7.5uM-T60-P1.0-TT-1	7.86		7.7			88.7		
		7.5uM-T60-P1.0-TT-2	7.26		14.8			40.0		
		7.5uM-T60-P1.0-TT-3	6.86	7.33	19.4	14.0	3.4	21.1	43.3	13.8
Quercetin	CYP2C8	7.5uM-T60-P1.0-Qr-1	8.67		-1.8			107		
		7.5uM-T60-P1.0-Qr-2	8.48		0.45			98.2		
		7.5uM-T60-P1.0-Qr-3	7.43	8.19	12.7	3.77	4.50	48.5	84.7	18.3
Sulfaphenazole	CYP2C9	7.5uM-T60-P1.0-S-1	7.49		12.1			51.0		
		7.5uM-T60-P1.0-S-2	7.65		10.2			58.5		
		7.5uM-T60-P1.0-S-3	7.44	7.52	12.6	11.8	0.7	48.8	52.8	2.0
Ticlopidine	CYP2C19	7.5uM-T60-P1.0-Ti-1	8.02		5.8			76.5		
		7.5uM-T60-P1.0-Ti-2	7.58		11.0			55.5		
		7.5uM-T60-P1.0-Ti-3	9.50	8.37	-11.6	1.71	6.83	147	93.1	27.7
Quinidine	CYP2D6	7.5uM-T60-P1.0-Qi-1	7.62		10.5			57.5		
		7.5uM-T60-P1.0-Qi-2	7.80		8.4			65.9		
		7.5uM-T60-P1.0-Qi-3	7.57	7.66	11.1	10.0	0.8	55.1	59.5	3.3
Clomethiazole	CYP2E1	7.5uM-T60-P1.0-Clo-1	8.29		2.7			89.2		
		7.5uM-T60-P1.0-Clo-2	8.54		-0.25			101		
		7.5uM-T60-P1.0-Clo-3	8.63	8.48	-1.3	0.356	1.197	105	98.6	4.9
Ketocoazole	CYP3A4	7.5uM-T60-P1.0-K-1	8.49		0.28			98.8		
		7.5uM-T60-P1.0-K-2	9.78		-14.9			161		
		7.5uM-T60-P1.0-K-3	9.12	9.13	-7.1	-7.26	4.39	129	130	19

Table 5: Metaxalone (0.75 μ M) Phenotyping 5 pmol CYP450

CYP	Sample ID	Concentration (μ M)	Mean (μ M)	% Turnover	Mean % Turnover	Standard Error
Control	IC-20uM-T0-1	0.786	0.791			
	IC-20uM-T0-2	0.802				
	IC-20uM-T0-3	0.785				
1A2	1A2-0.75uM-T60-5pmol-1	0.653	0.719	17.5	9.20	4.20
	1A2-0.75uM-T60-5pmol-2	0.742		6.2		
	1A2-0.75uM-T60-5pmol-3	0.761		3.9		
2A6	2A6-0.75uM-T60-5pmol-1	0.777	0.790	1.8	0.143	2.158
	2A6-0.75uM-T60-5pmol-2	0.824		-4.1		
	2A6-0.75uM-T60-5pmol-3	0.769		2.8		
2B6	2B6-0.75uM-T60-5pmol-1	0.878	0.845	-11.0	-6.77	2.38
	2B6-0.75uM-T60-5pmol-2	0.813		-2.8		
	2B6-0.75uM-T60-5pmol-3	0.843		-6.6		
2C8	2C8-0.75uM-T60-5pmol-1	0.812	0.865	-2.6	-9.25	3.48
	2C8-0.75uM-T60-5pmol-2	0.877		-10.9		
	2C8-0.75uM-T60-5pmol-3	0.905		-14.3		
2C9	2C9-0.75uM-T60-5pmol-1	0.743	0.779	6.1	1.57	4.20
	2C9-0.75uM-T60-5pmol-2	0.749		5.4		
	2C9-0.75uM-T60-5pmol-3	0.845		-6.8		
Control	IC-0.75uM-T0-1	0.738	0.712			
	IC-0.75uM-T0-2	0.697				
	IC-0.75uM-T0-3	0.701				
2C19	2C19-0.75uM-T60-5pmol-1	0.696	0.662	2.2	6.9	2.5
	2C19-0.75uM-T60-5pmol-2	0.658		7.6		
	2C19-0.75uM-T60-5pmol-3	0.634		11.0		
2D6	2D6-0.75uM-T60-5pmol-1	0.649	0.642	8.8	9.9	0.9
	2D6-0.75uM-T60-5pmol-2	0.629		11.6		
	2D6-0.75uM-T60-5pmol-3	0.647		9.1		
2E1	2E1-0.75uM-T60-5pmol-1	0.693	0.631	2.7	11.3	4.4
	2E1-0.75uM-T60-5pmol-2	0.604		15.1		
	2E1-0.75uM-T60-5pmol-3	0.596		16.3		
3A4	3A4-0.75uM-T60-5pmol-1	0.567	0.585	20.3	17.9	1.6
	3A4-0.75uM-T60-5pmol-2	0.582		18.3		
	3A4-0.75uM-T60-5pmol-3	0.605		15.0		

Note: Calculations performed with machine precision of Microsoft Excel.

Table 6: Metaxalone (0.75 μ M) Phenotyping 20 pmol CYP450

CYP	Sample ID	Concentration (μ M)	Mean (μ M)	% Turnover	Mean % Turnover	Standard Error
Control	IC-20 μ M-T0-1	0.786	0.791			
	IC-20 μ M-T0-2	0.802				
	IC-20 μ M-T0-3	0.785				
1A2	1A2-0.75 μ M-T60-20pmol-1	0.540	0.516	31.7	34.8	1.6
	1A2-0.75 μ M-T60-20pmol-2	0.511		35.5		
	1A2-0.75 μ M-T60-20pmol-3	0.496		37.3		
2A6	2A6-0.75 μ M-T60-20pmol-1	0.837	0.784	-5.7	0.910	3.327
	2A6-0.75 μ M-T60-20pmol-2	0.762		3.7		
	2A6-0.75 μ M-T60-20pmol-3	0.753		4.8		
2B6	2B6-0.75 μ M-T60-20pmol-1	0.748	0.805	5.5	-1.77	3.70
	2B6-0.75 μ M-T60-20pmol-2	0.845		-6.7		
	2B6-0.75 μ M-T60-20pmol-3	0.823		-4.0		
2C8	2C8-0.75 μ M-T60-20pmol-1	0.790	0.768	0.19	2.95	1.73
	2C8-0.75 μ M-T60-20pmol-2	0.743		6.1		
	2C8-0.75 μ M-T60-20pmol-3	0.771		2.5		
2C9	2C9-0.75 μ M-T60-20pmol-1	0.722	0.772	8.7	2.42	4.15
	2C9-0.75 μ M-T60-20pmol-2	0.760		4.0		
	2C9-0.75 μ M-T60-20pmol-3	0.834		-5.4		
Control	IC-0.75 μ M-T0-1	0.738	0.712			
	IC-0.75 μ M-T0-2	0.697				
	IC-0.75 μ M-T0-3	0.701				
2C19	2C19-0.75 μ M-T60-20pmol-1	0.659	0.623	7.4	12.5	2.8
	2C19-0.75 μ M-T60-20pmol-2	0.618		13.3		
	2C19-0.75 μ M-T60-20pmol-3	0.591		16.9		
2D6	2D6-0.75 μ M-T60-20pmol-1	0.546	0.573	23.3	19.5	2.0
	2D6-0.75 μ M-T60-20pmol-2	0.593		16.7		
	2D6-0.75 μ M-T60-20pmol-3	0.581		18.5		
2E1	2E1-0.75 μ M-T60-20pmol-1	0.608	0.597	14.6	16.2	2.6
	2E1-0.75 μ M-T60-20pmol-2	0.622		12.6		
	2E1-0.75 μ M-T60-20pmol-3	0.560		21.3		
3A4	3A4-0.75 μ M-T60-20pmol-1	0.486	0.470	31.7	34.0	2.5
	3A4-0.75 μ M-T60-20pmol-2	0.434		39.0		
	3A4-0.75 μ M-T60-20pmol-3	0.488		31.4		

Note: Calculations performed with machine precision of Microsoft Excel.

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Table 7: Metaxalone (7.5 µM) Phenotyping 5 pmol CYP450

CYP	Sample ID	Concentration (µM)	Mean (µM)	% Turnover	Mean % Turnover	Standard Error
Control	IC-200uM-T0-1	8.52	8.45	14.3		
	IC-200uM-T0-2	8.41				
	IC-200uM-T0-3	8.40				
1A2	1A2-7.5uM-T60-5pmol-1	7.24	7.60	NA	10.0	4.3
	1A2-7.5uM-T60-5pmol-2	N14		NA		
	1A2-7.5uM-T60-5pmol-3	7.97		5.7		
2A6	2A6-7.5uM-T60-5pmol-1	7.69	8.14	9.0	3.66	3.27
	2A6-7.5uM-T60-5pmol-2	8.08		4.3		
	2A6-7.5uM-T60-5pmol-3	8.64		-2.3		
2B6	2B6-7.5uM-T60-5pmol-1	8.56	8.20	-1.3	2.95	2.46
	2B6-7.5uM-T60-5pmol-2	8.19		3.0		
	2B6-7.5uM-T60-5pmol-3	7.84		7.2		
2C8	2C8-7.5uM-T60-5pmol-1	7.58	8.20	10.3	2.96	3.69
	2C8-7.5uM-T60-5pmol-2	8.43		0.21		
	2C8-7.5uM-T60-5pmol-3	8.58		-1.6		
2C9	2C9-7.5uM-T60-5pmol-1	7.24	7.78	14.3	7.86	3.35
	2C9-7.5uM-T60-5pmol-2	8.20		2.9		
	2C9-7.5uM-T60-5pmol-3	7.90		6.4		
Control	IC-7.5uM-T0-1	7.36	7.71			
	IC-7.5uM-T0-2	7.85				
	IC-7.5uM-T0-3	7.93				
2C19	2C19-7.5uM-T60-5pmol-1	7.69	7.23	0.25	6.3	3.3
	2C19-7.5uM-T60-5pmol-2	7.17		7.1		
	2C19-7.5uM-T60-5pmol-3	6.82		11.6		
2D6	2D6-7.5uM-T60-5pmol-1	6.89	6.74	10.7	12.6	1.1
	2D6-7.5uM-T60-5pmol-2	6.75		12.5		
	2D6-7.5uM-T60-5pmol-3	6.60		14.5		
2E1	2E1-7.5uM-T60-5pmol-1	6.41	6.29	16.9	18.4	1.6
	2E1-7.5uM-T60-5pmol-2	6.42		16.7		
	2E1-7.5uM-T60-5pmol-3	6.04		21.7		
3A4	3A4-7.5uM-T60-5pmol-1	6.36	6.43	17.5	16.6	0.5
	3A4-7.5uM-T60-5pmol-2	6.43		16.7		
	3A4-7.5uM-T60-5pmol-3	6.50		15.7		

N14 - NOT INCLUDED IN CALCULATIONS; NO PEAK DETECTED.

Note: Calculations performed with machine precision of Microsoft Excel.

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Table 8: Metaxalone (7.5 µM) Phenotyping 20 pmol CYP450

CYP	Sample ID	Concentration (µM)	Mean (µM)	% Turnover	Mean % Turnover	Standard Error
Control	IC-200uM-T0-1	8.52	8.45			
	IC-200uM-T0-2	8.41				
	IC-200uM-T0-3	8.40				
1A2	1A2-7.5uM-T60-20pmol-1	5.63	5.88	33.3	30.4	2.4
	1A2-7.5uM-T60-20pmol-2	5.72		32.3		
	1A2-7.5uM-T60-20pmol-3	6.29		25.5		
2A6	2A6-7.5uM-T60-20pmol-1	8.58	8.59	-1.6	-1.76	4.59
	2A6-7.5uM-T60-20pmol-2	7.93		6.1		
	2A6-7.5uM-T60-20pmol-3	9.27		-9.8		
2B6	2B6-7.5uM-T60-20pmol-1	8.95	8.46	-6.0	-0.197	2.935
	2B6-7.5uM-T60-20pmol-2	8.29		1.8		
	2B6-7.5uM-T60-20pmol-3	8.14		3.6		
2C8	2C8-7.5uM-T60-20pmol-1	8.09	7.66	4.3	9.28	2.68
	2C8-7.5uM-T60-20pmol-2	7.59		10.2		
	2C8-7.5uM-T60-20pmol-3	7.31		13.4		
2C9	2C9-7.5uM-T60-20pmol-1	8.54	8.13	-1.1	3.73	3.58
	2C9-7.5uM-T60-20pmol-2	8.31		1.6		
	2C9-7.5uM-T60-20pmol-3	7.54		10.7		
Control	IC-7.5uM-T0-1	7.36	7.71			
	IC-7.5uM-T0-2	7.85				
	IC-7.5uM-T0-3	7.93				
2C19	2C19-7.5uM-T60-20pmol-1	6.16	6.27	20.2	18.7	0.8
	2C19-7.5uM-T60-20pmol-2	6.32		18.0		
	2C19-7.5uM-T60-20pmol-3	6.34		17.8		
2D6	2D6-7.5uM-T60-20pmol-1	5.87	5.79	23.9	24.9	1.2
	2D6-7.5uM-T60-20pmol-2	5.90		23.5		
	2D6-7.5uM-T60-20pmol-3	5.61		27.3		
2E1	2E1-7.5uM-T60-20pmol-1	6.33	6.39	18.0	17.2	1.4
	2E1-7.5uM-T60-20pmol-2	6.25		19.0		
	2E1-7.5uM-T60-20pmol-3	6.60		14.5		
3A4	3A4-7.5uM-T60-20pmol-1	5.07	5.22	34.3	32.4	1.2
	3A4-7.5uM-T60-20pmol-2	5.18		32.8		
	3A4-7.5uM-T60-20pmol-3	5.40		30.0		

APPENDICES

Appendix 1: Study Protocol



Proposal # SP046306
Reaction Phenotyping

***In Vitro* Assessment of Reaction Phenotyping (Enzyme Identification)
for Human Cytochrome P450 Enzymes by Metaxalone**

Proposal Number: SP046306 / MPC-003-06-0002

Date: November 17, 2006

Sponsor: Salamandra, LLC.

Sponsor Contact: J. Michael Morgan, Ph.D., DABT
Senior Consultant
4800 Hampden Lane
Bethesda, MD 20814
Tel: 301-652-6110 ext. 131
Fax: 301-652-6739
Email: mmorgan@salamandra.net

CellzDirect: CellzDirect, Inc.
480 Hillsboro Street
Suite 130
Pittsboro, NC 27312
Tel: 919-545-9959
Fax: 919-545-9890

CellzDirect Contact: Jeff Hergenrader
CellzDirect, Inc.
Tel: 919-842-5436
Email: jeffh@cellzdirect.com

Approved By: Chris Black, Ph.D.
Vice President Scientific Operations
CellzDirect, Inc.

Confidentiality: This protocol contains confidential or proprietary information concerning experimental design that should not be shared with a third party without the expressed consent of CellzDirect, Inc.

Purpose of Study: The aim of these studies is to identify the cytochrome P450 enzymes involved in the *in vitro* biotransformation of Metaxalone in support of drug development efforts at URL Mutual, in care of Salamandra, LLC. The metabolism of the test compound, provided by the Sponsor, will be examined in human liver microsomes, utilizing specific inhibitors. Based on the results from these studies, the major hepatic cytochrome P450 enzymes involved in the metabolism of the test article will be determined.

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Reaction Phenotyping

INTRODUCTION

Biotransformation is the major route of elimination for a majority of drugs and metabolism by Phase I oxidative cytochrome P450 enzymes (P450) is the most common metabolic pathway. To characterize the metabolic clearance of a drug candidate, the relative contribution of P450 enzymes to the overall elimination process is required along with identification of the enzymes responsible for the oxidative reactions. Reaction phenotyping (enzyme identification) studies are conducted to identify the specific enzymes responsible for the metabolism of a drug. Identification of cytochrome P450 enzymes *in vitro* can prove useful in predicting the potential for drug interactions, polymorphic impact on drug disposition and formation of toxic or active metabolites, and is warranted if P450 enzymes contribute to greater than 25% of a drug's total clearance (1-3). Alternatively, evidence that certain metabolic pathways are not important via *in vitro* studies may preclude the need for further clinical investigations, or help focus the design of clinical drug-drug interaction studies (4-6). While this proposal is limited to P450 enzymes, other Phase I (flavin-containing monooxygenase, monoamine oxidase, epoxide hydrolase) and Phase II (UDP-glucuronyltransferase, glutathione S-transferase, sulfotransferase, methyltransferase, and N-acetyltransferase) drug metabolizing enzymes may also contribute to metabolism and should be considered.

The goal in reaction phenotyping is to determine the metabolic profile of a drug and the relative contribution of specific enzymes to overall metabolic clearance, followed by systematic characterization of the specific enzymes involved in formation of each major metabolite. To do this successfully, more than one approach should be used. Microsomal preparations containing individual recombinant human P450 isoform are a valuable tool for evaluating the intrinsic ability of each individual isoform to metabolize a drug candidate (7). If the test compound is metabolized by multiple enzymes, further studies with highly selective chemical inhibitors and/or antibodies can be conducted in human hepatic microsomes to address the relative contribution (1). An appropriate approach for an inhibition study is to examine the concentration-dependent effect of a particular chemical/antibody on the metabolism of the test compound to enable evaluation of the relative contribution of a P450 isoform. Chemical inhibitors for which there is prior clinical data should be used to aid in valid *in vitro* *in vivo* correlations. The disadvantage of this system is that few highly specific chemical inhibitors are available. Potent inhibitory monoclonal antibodies against various human P450 isoforms are more specific inhibitors. A fourth approach is more time intensive and involves mechanistic determinations (K_m and V_m) of the test compound with each active recombinant P450 enzyme, to enable calculations of intrinsic clearance (V_m/K_m). Combined with knowledge of the relative abundance of each P450 isoform in the pool of human liver microsomes being used, the relative contribution of different P450 enzymes in the metabolism of the compound of interest in human liver microsomes can be evaluated (7, 8).

CellzDirect recommends at least two experimental approaches to determine the liver-derived enzymes that are involved in the metabolism of the investigational drug. For this study, only the use of CYP450-specific inhibitors will be used to discern which hepatic CYP450(s) may be involved in the metabolism of metaxalone.

Proposal # SP046306
Reaction Phenotyping

EXPERIMENTAL DESIGN

The primary objective of this study is to determine which human liver CYP450 isoforms may be involved in the *in vitro* biotransformation of the test compound. The scope of work to be performed for this is outlined in the following tasks: Task 1) Set-up of Analytical Methodology, Preliminary Incubations, and Validation; Task 2) CYP450 Selective Inhibition Studies; and Task 3) Report.

Task 1: Set-up of Analytical Methodology and Preliminary Incubations

The objective of Task 1 is to set-up an analytical method that will allow for chromatographic resolution and quantitation of the parent test article contained within an incubation matrix. The Sponsor will provide CellzDirect with adequate parent material and internal standard (if available) as well as structural information. CellzDirect ideally requests approximately 50 mg of parent material. Set-up of the analytical method will involve determining the parent and daughter ions for the compound by direct infusion. Secondly, the compound will be injected in mobile phase onto an isocratic LC-MS/MS chromatographic system to assess peak shape, retention times, and signal to noise ratio of a known amount on column. CellzDirect will then make an assessment based on the signal to noise ratio and estimate the lower limit of quantitation. A standard curve, comprised of at least 8 calibration standards, will be prepared and assessed.

Preliminary Incubations (Time and Protein Dependence): CellzDirect will conduct pilot incubations using parent test article to establish the appropriate incubation time points. Specifically, this will entail analysis of the time and protein dependence on test article depletion. In brief, both incubation time and protein concentration linearity will be established to determine if reaction phenotyping can be conducted under initial rate conditions. Next, a standard curve range will be selected to ensure that test article depletion can be quantified. Negative controls (no NADPH and heat-treated microsomes) will be included to account for any non-enzymatic or non-NADPH dependent reactions. An organic solvent will be used to terminate the enzymatic incubations. The incubations will be analyzed using the LC-MS/MS system.

Validation: A one day validation will be performed to establish the analytical method using pooled human liver microsomes. To this end, a triplicate standard curve and six QCs (at three different concentration levels) will be analyzed. Standard curves will be run at the beginning and end of each run. The validation will aim to address the following:

- Intra-Day Accuracy
- Intra-Day Precision (ruggedness)
- Linearity (Correlation Coefficient)
- Specificity (blank matrix)

CellzDirect will consider the validation acceptable if standard and QC samples meet criteria set forth in CellzDirect SOPs. Specifically, if the back-calculated values for each calibration standard are within $\pm 20\%$ ($\pm 25\%$ at the LLOQ) of the theoretical value and 50% of the back-calculated QC samples at each concentration level and 2/3rd of the back-calculated QC samples overall are within 20% of their theoretical values.

CellzDirect will perform Tasks 2-3 at two different concentrations of the test article (typically these concentrations are approximately C_{max} and $0.1 \times C_{max}$, if the C_{max} value is available). Based on estimated

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Proposal # SP046306
 Reaction Phenotyping

clinical plasma concentrations of Metaxalone, concentrations will be determined in conjunction with the Sponsor.

Task 2: Studies with Specific Inhibitors

The objective of these experiments is to identify CYP450 isoform(s) involved in the potential metabolism of the parent test article in a microsomal system that approximates the *in vivo* distribution of hepatic enzymes. This is accomplished through the use of pooled human liver microsomes from at least 15 donors, selective CYP450 substrate probes, and known CYP450-specific inhibitors. Using the assay validated in Task 1, evaluation of substrate depletion in the presence and absence of selective chemical inhibitors and antibodies will be performed. The following CYP450 isoforms and inhibitors will be investigated: CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. The specific chemical inhibitors to be used are detailed in Table 1 below. The incubation time and substrate concentrations will be chosen based on the results from Task 1 and in conjunction with the sponsor. Incubations will be conducted in triplicate in the presence of inhibitors. Samples will be analyzed by LC-MS/MS and the rates of test article depletion will be compared with control (no inhibitor, in triplicate).

Table 1. Protocol for *in vitro* Assessment of Chemical Inhibition in Human Liver Microsomes.

P450 ISOFORM	SPECIFIC CHEMICAL INHIBITOR	CONCENTRATION
CYP1A2	Furafylline	50 μ M
CYP2A6	Pilocarpine	100 μ M
CYP2B6	Thio-TEPA	75 μ M
CYP2C8	Quercetin	10 μ M
CYP2C9	Sulfaphenazole	20 μ M
CYP2C19	Ticlopidine	1 μ M
CYP2D6	Quinidine	10 μ M
CYP2E1	Clomethazole	100 μ M
CYP3A4	Ketoconazole	1 μ M

Task 3: Data Analysis and Report

CellzDirect will prepare a report on the findings of the study). Specifically, CellzDirect will detail the method developed, validation results and incubation specifics in the experimental section. Results and discussion will include which isoforms appear to be responsible for the metabolism of the test compound. The data will be presented in tables and figures.

CellzDirect, Inc. will provide the Sponsor with a draft report for comment prior to finalization. The final report will be provided using CellzDirect's standard format, unless specified otherwise by the Sponsor. The report shall contain, but is not limited to, a title page, table of contents, summary of results and conclusions, introduction, materials and methods, results, discussion, references, and appendices containing all figures and raw data. Any deviation from the protocol will be noted and the significance of the deviation discussed. The Study Director and a reviewer will sign the report.



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Reaction Phenotyping

ETHICS AND COMPLIANCE STATEMENT

This study will not be conducted in strict accordance with the FDA Good Laboratory Practice (GLP) Regulations, 21 CFR Part 58, but will be conducted to the high standards of record keeping, and in accordance with the applicable Standard Operating Procedures of CellzDirect, Inc. All study procedures, observations, comments, results, and data will be clearly documented.

QUALITY CONTROL PLAN

All study procedures, observations, comments, results, and data will be clearly documented. All personnel participating in these studies are trained and certified. Analytical runs will be evaluated on the basis of the acceptance criteria set forth in the study protocol and applicable CellzDirect Standard Operating Procedures (SOPs). CellzDirect's Quality Control Unit will subject the data and all study documentation to review and verification. Associated documentation includes, but is not limited to, equipment records, training records, observations and comments noted during the study, and receipt of compounds of interest.

TIMELINES

DRAFT REPORT	January 2, 2007
FINAL REPORT	3 days after receiving client comments



Proposal # SP046306
Reaction Phenotyping

APPROVAL SIGNATURES

Signatures from CellzDirect, Inc.

Signature:  Date: 30 Nov 2006
Jeff Hergenrader
Regional Sales Manager
CellzDirect, Inc.

Signature:  Date: 11/30/2006
Chris Black, Ph.D.
Vice President Scientific Operations
CellzDirect, Inc.

Signatures from Sponsor

Signature:  Date: 21-Nov-2006
J. Michael Morgan, Ph.D., DABT
Senior Consultant
Salamandra, LLC

Signature:  Date: 29-NOV-2006
Matthew Davis, MD, RPh.
Vice President, Branded Products & Medical Affairs
URL Mutual

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Reaction Phenotyping

REFERENCES

- (1) Lu, A., Wang, R. and Lin, J. (2003) Cytochrome P450 in vitro reaction phenotyping: a re-evaluation of approaches used for P450 isoform identification. *Drug Metab Dispos* 31, 345-350.
- (2) Williams, J., Bauman, J. N., Cai, H., Conlon, K., Hensel, S., Hurst, S., Sadagopan, N., Tugnait, M., Zhang, L. and Sahi, J. (2005) in vitro ADME phenotyping in drug discovery: current challenges and future solutions. *Current Opinion Drug Discovery Development* 8, 78-88.
- (3) Williams, J., Hurst, S., Bauman, J., Jones, B., Hyland, R., Gibbs, J., Obach, R. and Ball, S. (2003) Reaction phenotyping in drug discovery: moving forward with confidence? *Curr Drug Metab* 4, 527-534.
- (4) Venkatakrisnan, K., Von Moltke, L. and Greenblatt, D. (2001) Human drug metabolism and the cytochromes P450: application and relevance of in vitro models. *J Clin Pharmacol* 41, 1149-1179.
- (5) Streetman, D., Bleakley, J., Kim, J., Nafziger, A., Leeder, J., Gaedigk, A., Gotschall, R., Kearns, G. and Jr, B. J. (2000) Combined phenotypic assessment of CYP1A2, CYP2C19, CYP2D6, CYP3A, N-acetyltransferase-2, and xanthine oxidase with the "Cooperstown cocktail". *Clin Pharmacol Ther* 68, 375-383.
- (6) Williams, J., Hyland, R., Jones, B., Smith, D. A., Hurst, S., Goosen, T., Peterkin, V., Koup, J. and Ball, S. (2004) Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUC_i/AUC) ratios. *DMD* 32, 1201-1208.
- (7) Crespi, C. L. and Miller, V. P. (1999) The use of heterologously expressed drug metabolizing enzymes--state of the art and prospects for the future. *Pharmacol Ther* 84, 121-131.
- (8) Rodrigues, A. D. (1999) Integrated cytochrome P450 reaction phenotyping: attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. *Biochem Pharmacol* 57, 465-480.
- (9) White NJ, Loacareesuwan S, Warrell DA, Warrell MJ, Bunnag D, Harinasuta T. (1982) Quinine pharmacokinetics and toxicity in cerebral and uncomplicated *Falciparum* malaria. *Am J Med*. 73, 564-72.

Appendix 2: Certificate of Analysis



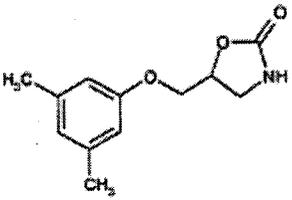
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Certificate No.: 3279

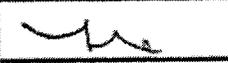
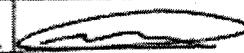
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Retest date:

Feb. 2007

Compound Name: Metaxalone		Structure: 
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Molecular Formula:	C ₁₂ H ₁₃ NO ₃	
Source:	SynFine Research	
Source Lot No.:	P-1055-116A1	
Storage conditions:	Store at room temperature in a well closed container	

Test Description	Specifications	Results
Visual Description	White to off white solid	Conforms
Identification MS ¹ H NMR	Conforms to structure Conforms to structure	Conforms Conforms
Chromatographic Purity by HPLC	Not less than 98.0%	99.8%
Recommendation:	Released	

Name	Department	Signature	Date
Reviewed and approved by: Juan Xu	Analytical Services		Feb. 09, 2006
Approved by: Tina Shahed	Quality Assurance		Feb 15, 2006

SynFine Research, Inc., P.O. Box 433, Station A, Richmond Hill, Ontario, Canada L4C 4Y8
 Tel: (905) 737-2702, Fax: (905) 737-6299, e-mail: research@synfine.com
www.synfine.com

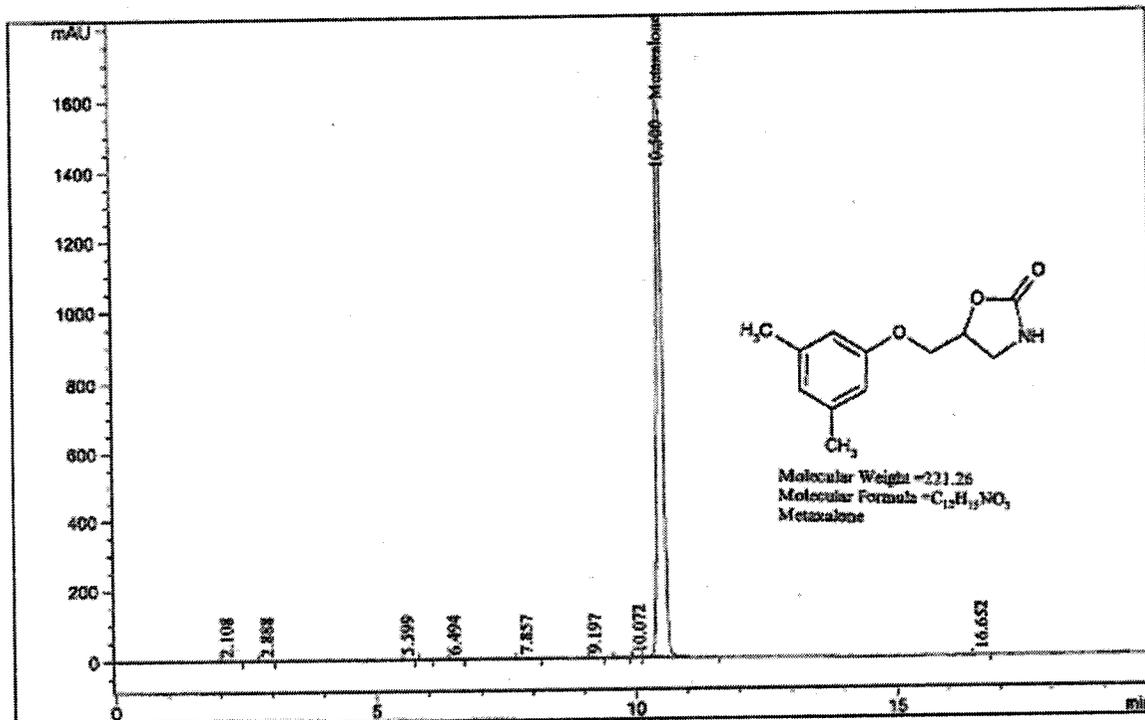


REVIEWED AND APPROVED
Wm Feb. 08, 2006

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Lot No.: P-1055-116A1

Data Acquired: 2/6/2006 8:22:32 PM



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2	2.888	MM	0.43	0.00	
3	5.599	BB	0.83	0.01	
4	5.921	BB	2.06	0.01	
5	6.494	BB	1.44	0.01	
6	7.657	BB	4.42	0.03	
7	9.197	BB	6.17	0.04	
8	9.704	BB	10.30	0.07	
9	10.072	MF	1.21	0.01	
10	10.500	FM	13741.55	99.80	Metaxalone
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MultiView 1.3

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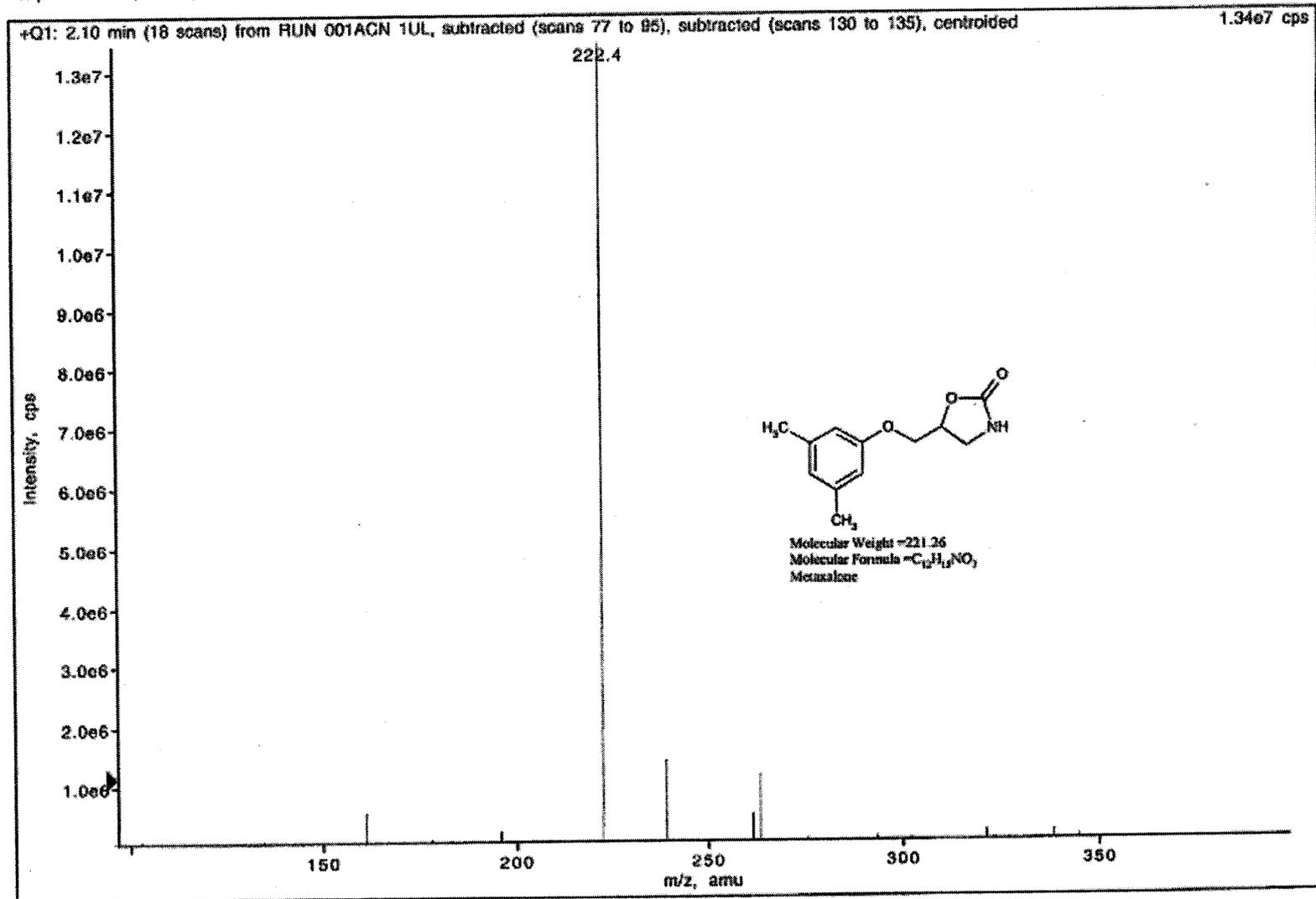
REVIEWED AND
APPROVED
Feb. 08. 2006

page 1 of 1

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37

Report: Reaction Phenotyping for Human CYP450 by Metaxalone

P-1055-116A1, 1H NMR in CDCl3

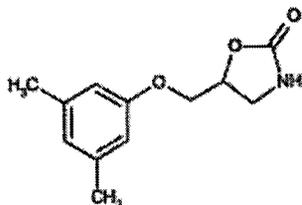
REVIEWED AND APPROVED
Mr Feb 7, 2006

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PROCNO: 1

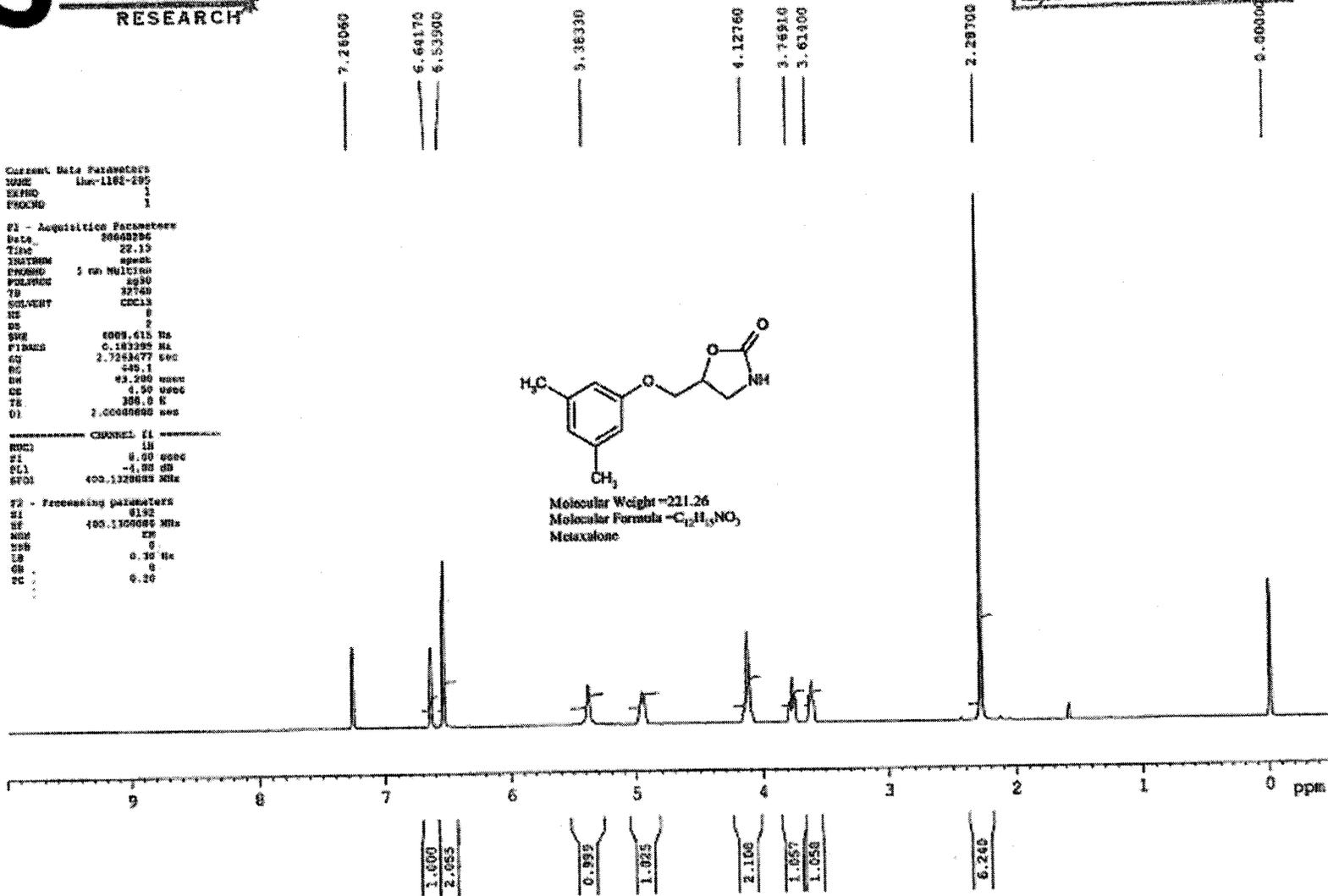
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NS: 8
DS: 2
SWH: 6009.615 Hz
FIDRES: 0.183295 Hz
AQ: 2.7243477 sec
RG: 648.1
RM: 0.1200000000
CC: 4.50 usec
TE: 300.2 K
D1: 2.0000000000 sec

----- CHANNEL f1 -----
NUC1: 1H
P1: 0.00 usec
PL1: -1.00 dB
SFO1: 400.1326000 MHz

F2 - Processing parameters
SI: 8192
SF: 400.1326000 MHz
WDW: EM
SSB: 0
LB: 0.10 Hz
GB: 0
PC: 0.20



Molecular Weight ~221.26
Molecular Formula -C₁₇H₁₉NO₃
Metaxalone



Appendix 3: Microsomal Characterization

HUMAN LIVER MICROSOMES

Characterization Data for Pool HMMC-PL020

OVERVIEW

The following highlights the characterization data for our GLP-validated 15-donor human microsome pool (HMMC-PL020). CellzDirect's human microsomes are available from individual donors or in a pooled format. Custom pools are available upon request, and can be specifically tailored to meet the customers' specification. If you need more information, please contact us at 866.952.3559.

DONOR MEDICAL HISTORY

Lot No.	Sex	Age	Race	Alcohol Use	Medications/Drug Use	Smoker	Cause of Death
HMMC-SD115	M	54	C	Yes	Alcohol and drug abuse (cocaine and marijuana)	UNK	Unknown
HMMC-SD118	M	67	C	Yes	None	No	Head trauma
HMMC-SD119	M	40	AA	No	None	No	Subarachnoid hemorrhage
HMMC-SD120	F	53	C	UNK	None	UNK	Intracranial hemorrhage
HMMC-SD121	M	48	C	No	Hypertension medications	No	Head trauma
HMMC-SD122	M	57	C	UNK	None	UNK	Subarachnoid hemorrhage
HMMC-SD123	F	62	C	Yes	None	Yes	Intracranial hemorrhage
HMMC-SD124	M	62	C	UNK	None	No	Intracranial hemorrhage
HMMC-SD128	F	80	C	Yes	None	No	Intracranial hemorrhage
HMMC-SD129	M	28	C	Yes	Drug abuse (cocaine, marijuana and methamphetamines)	Yes	Anoxia
HMMC-SD132	M	64	H	Yes	Hypertension medications	No	Intracranial hemorrhage
HMMC-SD134	F	74	C	UNK	None	UNK	Anoxia
HMMC-SD135	M	44	C	Yes	Hypertension medications	Yes	Intracranial hemorrhage
HMMC-SD136	M	54	C	Yes	None	Yes	Anoxia/cardiovascular
HMMC-SD003	F	24	C	UNK	None	UNK	Anoxia

Key: AA = African American, C = Caucasian, H = Hispanic, UNK = Unknown,

GENOTYPING RESULTS

For Individual Donors Included in Pool

Lot No.	CYP2C9	CYP2C19	CYP2D6
HMMC-SD115	*2/wt	wt/wt	*4/wt
HMMC-SD118	wt/wt	wt/wt	*4/wt
HMMC-SD119	wt/wt	*2/wt	wt/wt
HMMC-SD120	*3/wt	wt/wt	wt/wt
HMMC-SD121	wt/wt	*2/wt	*4/wt
HMMC-SD122	wt/wt	*2/wt	*4/wt
HMMC-SD123	wt/wt	*2/wt	wt/wt
HMMC-SD124	*2/wt	wt/wt	wt/wt
HMMC-SD128	*2/wt	*2/wt	wt/wt
HMMC-SD129	wt/wt	*2/wt	*4/wt
HMMC-SD132	wt/wt	*2/wt	wt/wt
HMMC-SD134	*2/wt	wt/wt	*3/wt
HMMC-SD135	wt/wt	wt/wt	*4/wt
HMMC-SD136	wt/wt	wt/wt	wt/wt
HMMC-SD003	wt/wt	wt/wt	wt/wt

KINETIC PARAMETERS

For Pool HMMC-PL020

Isoform	K_m (μM)	V_{max} (nmol/min/mg)
CYP1A2	41	0.81
CYP2A6	1.0	1.2
CYP2B6	75	0.48
CYP2C8	6.7	0.27
CYP2C9	130	0.31
CYP2C19	54	0.054
CYP2D6	4.6	0.22
CYP2E1	84	2.0
CYP3A4 6BT	56	6.0
CYP3A4 MDZ	2.2	0.98

Key: 6BT = 6 β -Testosterone Hydroxylase, MDZ = Midazolam 1'-Hydroxylase

Additional Information

Cytochrome b ₅ (nmol/mg)	0.35
Cytochrome c reductase (nmol/min/mg)	120
Cytochrome P450 content (nmol/mg)	0.26

These data were generated by and are the property of CellzDirect. These data are not to be reproduced, published, or distributed without the expressed written consent of CellzDirect.

HUMAN LIVER MICROSOMAL ENZYMATIC ACTIVITIES
 Graphical Representation of Enzymatic Activities for Single Donor Lots and Pool HMMC-PL020

