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ATTACHMENT B.1
INHIBITION STUDY: PROTOCOL

In Vitro Technologies, Inc.
Protocol No. 1179
Version: Final (27 September 2005)

**Determination of the Inhibitory Potential of Metaxalone on the
Activities of CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9,
CYP2C19, CYP2D6, CYP2E1, and CYP3A4 in Human Liver
Microsomes**

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INC. AND MUTUAL PHARMACEUTICAL COMPANY.

Objective

The objective of this study is to determine the potential of metaxalone to inhibit the activities of cytochrome P450 (CYP) isoforms CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 in human liver microsomes.

Test Article Information

The test article will be identified in this study as follows:

- Metaxalone (molecular weight = 221 g/mol)

Mutual Pharmaceutical Company will provide metaxalone and will be responsible for the derivation, characterization, retention, and stability testing of metaxalone. Additionally, Mutual Pharmaceutical will be responsible for providing In Vitro Technologies with detailed information regarding handling and storage requirements, diluents or cosolubilizers, and safety hazards and precautions (Material Safety Data Sheet or other documentation) for metaxalone, before or upon initiation of this study.

Test System Identification

The test system that will be used in this study is human liver microsomes.

Test System Justification

The liver represents the major organ for drug metabolism and contains the CYP enzymes, the major enzyme system for xenobiotic metabolism (1). Microsomes prepared from the liver constitute a physiologically relevant experimental model for the evaluation of potential drug-drug interactions related to the inhibition of CYP enzyme activities.

Differences in drug-metabolizing enzymes among species, especially in CYP isoforms, often account for the inability to predict human clinical responses based on data obtained from laboratory animal studies. Human liver microsomes can be used as an experimental model to reduce concerns about species differences (2). Liver microsomes provide a readily available and well-characterized biological model for use in CYP enzyme inhibition studies.

Description of Study

Microsomes will be incubated in the presence of metaxalone and a selective substrate for each CYP isoform. The formation of the selective metabolite from its substrate will be measured by

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high-performance liquid chromatography (HPLC) or liquid chromatography/mass spectrometry (LC/MS).

Experimental Methods

Test Article Preparation

Metaxalone stock solutions will be prepared in methanol at 100 times (100X) the final concentration. The stock solutions will be added to incubation mixtures to obtain the final concentrations of 0.3, 1, 3, 30, and 100 μM ¹, each containing 1% methanol. Modifications in test article preparation, which pertain to changes in solvent used or changes in dosing concentrations, may be made with the approval of the Study Director. These modifications and their rationale will be communicated to Mutual Pharmaceutical and will be described in the study report.

Positive Control Preparation

Ketoconazole will be prepared in methanol as a 100X stock solution.

Substrates for the CYP isoforms

The activity of each of the CYP isoforms will be measured in the presence of the following isoform-selective substrates. Each substrate will be prepared as 100X stock solutions in the solvents and final concentrations listed below:

CYP isoform	Isoform-selective substrate	Substrate concentration	Solvent
CYP1A2	Phenacetin	50 μM	ACN
CYP2A6	Coumarin	8 μM	ACN
CYP2B6	S-Mephenytoin	1 mM	ACN
CYP2C8	Paclitaxel	5 μM	ACN
CYP2C9	Tolbutamide	150 μM	ACN
CYP2C19	S-Mephenytoin	50 μM	ACN
CYP2D6	Dextromethorphan	5 μM	Water
CYP2E1	Chlorzoxazone	50 μM	ACN
CYP3A4	Testosterone	100 μM	ACN

Microsome Preparation

Microsomes were prepared by differential centrifugation of liver homogenates (3) pooled from at least ten human donors. For this study, human liver microsomes will be obtained from the microsome bank maintained at In Vitro Technologies.

¹ The concentrations in ng/mL will be: 66.3, 221, 663, 6,630, and 22,100 ng/mL.

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Test Article Incubations

All incubations will be conducted at 37 ± 1 °C in a shaking water bath. The sample size will be N = 3 replicates for experimental groups.

Incubation mixtures will be prepared in 0.1 M Tris buffer and will contain microsomes (0.25 mg protein/mL for CYP2C9, CYP2D6, CYP2E1, and CYP3A4; 0.5 mg protein/mL for CYP1A2, CYP2A6, CYP2B6, CYP2C8, and CYP2C19), metaxalone (at each concentration), and a CYP isoform-selective substrate. After a 5-minute preincubation, NADPH regenerating system (NRS) will be added to initiate the reaction. CYP2A6 and CYP3A4 incubations will be continued for 10 minutes. All other incubations will be continued for 30 minutes.

Incubations for CYP2C8 will be terminated by adding 1.0 mL of ACN. All other incubations will be terminated by adding 1.0 mL of methanol. Samples will be transferred to cryovials. If analysis does not occur immediately after incubation, samples will be stored at -70 °C \pm 10 °C.

Control Incubations

All incubations will be conducted at 37 ± 1 °C in a shaking water bath. The sample size will be N = 4 replicates for the vehicle and positive control groups, and N = 2 for the test article interference control group.

Positive Control

Ketoconazole, a selective inhibitor of CYP3A4, will be added to microsome incubations to verify that the test system is responsive to inhibitors.

Incubation mixtures will be prepared in 0.1 M Tris buffer and will contain microsomes (0.25 mg protein/mL), 1 μ M ketoconazole, and 100 μ M testosterone. After a 5-minute preincubation, NRS will be added to initiate the reactions. Incubations will be continued for 10 minutes.

The test system will be considered responsive to inhibitors if the mean specific activity of CYP3A4 in the positive control samples treated with ketoconazole is \leq 50% of the mean specific activity in the corresponding vehicle control samples. If this criterion is not met, the study will be repeated.

Vehicle Control

Vehicle control samples will be included to establish a baseline value for enzyme activity. Incubation mixtures will be prepared in 0.1 M Tris buffer and will contain microsomes (0.25 mg protein/mL for CYP2C9, CYP2D6, CYP2E1, and CYP3A4; 0.5 mg protein/mL for CYP1A2, CYP2A6, CYP2B6, CYP2C8, and CYP2C19), 1% methanol, and a CYP isoform-selective substrate. After a 5-minute preincubation, NRS will be added to initiate the reactions. CYP2A6 and CYP3A4 incubations will be continued for 10 minutes. All other incubations will be continued for 30 minutes.

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Test Article Interference Control

Test article interference control samples will be included to investigate the possibility of interference by metaxalone or its metabolites. Incubation mixtures will be prepared in 0.1 M Tris buffer and will contain microsomes (0.25 mg protein/mL for CYP2C9, CYP2D6, CYP2E1, and CYP3A4; 0.5 mg protein/mL for CYP1A2, CYP2A6, CYP2B6, CYP2C8, and CYP2C19), 100 μ M metaxalone, and 1% substrate solvent. After a 5-minute preincubation, NRS will be added to initiate the reactions. CYP2A6 and CYP3A4 incubations will be continued for 10 minutes. All other incubations will be continued for 30 minutes. If interference is observed in these samples, Mutual Pharmaceutical will be notified to discuss a course of action.

Termination of Control Incubations

CYP2C8 incubations will be terminated by adding 1.0 mL of ACN. All other incubations will be terminated by adding 1.0 mL methanol. Samples will be transferred to cryovials. If analysis does not occur immediately after incubation, samples will be stored at $-70\text{ }^{\circ}\text{C} \pm 10\text{ }^{\circ}\text{C}$.

Analyses

In Vitro Technologies will measure the formation of metabolites from CYP isoform-selective substrates using the following analytical procedures:

Phenacetin O-Deethylase (CYP1A2)

The activity of CYP1A2 in microsomes will be determined by measuring the formation of acetaminophen. Samples will be analyzed using an LC/MS method.

Coumarin 7-Hydroxylase (CYP2A6)

The activity of CYP2A6 in microsomes will be determined by measuring the formation of 7-hydroxycoumarin. Samples will be analyzed using an HPLC method.

S-Mephenytoin N-Demethylase (CYP2B6)

The activity of CYP2B6 in microsomes will be determined by measuring the formation of nirvanol. Samples will be analyzed using an LC/MS method.

Paclitaxel 6-Hydroxylase (CYP2C8)

The activity of CYP2C8 in microsomes will be determined by measuring the formation of 6-hydroxypaclitaxel. Samples will be analyzed using an LC/MS method.

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Tolbutamide 4'-Methyl Hydroxylase (CYP2C9)

The activity of CYP2C9 in microsomes will be determined by measuring the formation of 4'-methylhydroxytolbutamide. Samples will be analyzed using an LC/MS method.

S-Mephenytoin 4'-Hydroxylase (CYP2C19)

The activity of CYP2C19 in microsomes will be determined by measuring the formation of 4'-hydroxymephenytoin. Samples will be analyzed using an LC/MS method.

Dextromethorphan O-Demethylase (CYP2D6)

The activity of CYP2D6 in microsomes will be determined by measuring the formation of dextrophan. Samples will be analyzed using an LC/MS method.

Chlorzoxazone 6-Hydroxylase (CYP2E1)

The activity of CYP2E1 in microsomes will be determined by measuring the formation of its metabolite, 6-hydroxychlorzoxazone. Samples will be analyzed using an LC/MS method.

Testosterone 6 β -Hydroxylase (CYP3A4)

The activity of CYP3A4 in microsomes will be determined by measuring the formation of 6 β -hydroxytestosterone. Samples will be analyzed using an HPLC-UV method.

Description of Data Calculations

The concentration of metabolites will be reported. Enzyme activity for each CYP isoform will be reported as specific activity (pmol/minute/mg protein) in the presence (SA_T) and absence (SA_C) of metaxalone. The data will be expressed as mean \pm standard deviation. SA_T relative to SA_C for each CYP isoform will be expressed as a percent using the following equation:

$$\% \text{ of vehicle control} = \frac{SA_T}{SA_C} \times 100$$

The IC_{50} value will be determined where it can be calculated.

Criteria for Data Acceptance

Bioanalytical data will be accepted in accordance with the In Vitro Technologies standard operating procedure(s) on bioanalytical data acceptance.

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Study Report

A copy of the final study report will be issued to Mutual Pharmaceutical and will include, but not be limited to, the following information:

- Participating Personnel
- Study Dates and Data Retention
- Statement of Compliance
- Quality Assurance Statement
- Summary
- Introduction
- Experimental Methods
- Results
- Conclusions
- Description of Data Calculations
- Copy of study protocol

Data Retention

In Vitro Technologies will retain all supporting documentation, including raw data and written records, for a period of up to five years following issuance of the final report. At the end of this period, Mutual Pharmaceutical will be notified to determine whether the data (excluding proprietary information) will be transferred, retained, or destroyed.

Protocol Approval

Sponsor Approval

This protocol has been reviewed and approved by the following:

Jie Du, Ph.D.

Sponsor Representative
Mutual Pharmaceutical Company

Signature

Date

9/27/05

Study Director Review

This study will be conducted using good documentation practices and using equipment that is properly maintained and calibrated in accordance with In Vitro Technologies standard operating procedures. The study will be conducted under my scientific guidance and management. I have reviewed the procedures outlined in this protocol.

Genfu Chen, Ph.D.

Study Director
In Vitro Technologies

Signature

Date

29 Sept 2005

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References

1. Spatzenegger, M.; Jaeger, W. Clinical importance of hepatic cytochrome P450 in drug metabolism. *Drug Metab. Rev.* **1995**, *27*, 397-417.
2. Chauret, N.; Gauthier, A.; Martin, J.; and Nicoll-Griffith, D.A. In vitro comparison of cytochrome P450-mediated metabolic activities in human, dog, cat, and horse. *Drug Metab. Dispos.* **1997**, *25(10)*, 1130-1136.
3. Guengerich, F. P. Analysis and characterization of enzymes. In *Principles and Methods of Toxicology* (A.W. Hayes, Ed.). Raven Press, New York, 1989, pp. 777-813.

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