



**ATTACHMENT C.1**

**INDUCTION STUDY: PROTOCOL**

**In Vitro Technologies, Inc.**  
**Protocol No. 1188**  
**Version: Final (20 October 2005)**

**Evaluation of the Induction Potential of Metaxalone on the  
Activities of CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9,  
CYP2C19, CYP2D6, CYP2E1, and CYP3A4 in Human Hepatocytes**

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## Objective

The objective of this study is to evaluate the potential of metaxalone to induce the activities of cytochrome P450 (CYP) isoforms CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 in human hepatocytes following *in vitro* exposure.

## 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 cryopreserved human hepatocytes.

## 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). Hepatocytes isolated from the liver constitute a physiologically relevant experimental model for the evaluation of potential drug-drug interactions related to the inhibition or induction 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 hepatocytes can be used as an experimental model to reduce concerns about species differences (2). Cryopreserved hepatocytes provide a readily available and well-characterized biological model for use in CYP enzyme induction studies.

## Description of Study

Hepatocytes will be incubated in the presence of metaxalone for  $48 \pm 3$  hours, after which a selective substrate for each CYP isoform will be added. The formation of a specific metabolite

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from its substrate will be measured by high-performance liquid chromatography with UV detection (HPLC-UV) or liquid chromatography/mass spectrometry (LC/MS).

## Experimental Methods

### Media

The following media, as prepared at In Vitro Technologies, will be used in this study.

- DMEM Stock: Dulbecco's modified Eagle's medium (DMEM) supplemented with bovine serum albumin, fructose, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonate) (HEPES), and sodium bicarbonate
- Hepatocyte Plating Medium: DMEM stock supplemented with antibiotics, bovine serum, hydrocortisone, insulin, and minimum essential medium (MEM) non-essential amino acids
- Sandwich Medium: Hepatocyte plating medium supplemented with Vitrogen™
- Incubation Medium: DMEM stock supplemented with antibiotics, hydrocortisone, insulin, and MEM non-essential amino acids
- Supplemented KHB: Krebs-Henseleit buffer (KHB) supplemented with antibiotics, calcium chloride, heptanoic acid, HEPES, and sodium bicarbonate

### Test Article Preparation

Metaxalone stock solutions will be prepared in methanol at 100 times (100X) the final concentration. The stock solutions will be diluted with incubation medium to produce incubation solutions with final concentrations of 0.4, 4, and 40 µM, each containing 1% methanol. Stock solutions will be prepared fresh prior to use. Modifications in test article preparation, which pertain to changes in solvent used or changes in incubation 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 Article Preparation

Omeprazole and rifampin will be prepared as 100X stock solutions in methanol. Positive control article stock solutions will be diluted with incubation medium to the final concentrations listed below.

CYP isoform	Positive Control Article	Concentration
CYP1A2	Omeprazole	50 µM
CYP3A4	Rifampin	25 µM

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## Reference Control Article Preparation

Phenobarbital will be prepared as a 100X stock solution in deionized water. All other reference control articles will be prepared as 100X stock solutions in methanol. Reference control article stock solutions will be diluted with incubation medium to the final concentrations listed below.

<b>CYP isoform</b>	<b>Reference Control Article</b>	<b>Concentration</b>
CYP2B6	Phenobarbital	1 mM*
CYP2C8	Rifampin	25 $\mu$ M
CYP2C9	Rifampin	25 $\mu$ M
CYP2C19	Rifampin	25 $\mu$ M

\* Methanol will be added to the dosing solution to achieve a final methanol concentration of 1%.

## CYP Isoform Substrate Preparation

The activity of each of the CYP isoforms will be measured in the presence of the following isoform-selective substrates. Isoform-selective substrates will be prepared as 100X stock solutions in acetonitrile and diluted with supplemented KHB to the final concentrations listed below.

<b>CYP isoform</b>	<b>Isoform-selective substrate</b>	<b>Concentration</b>
CYP1A2	Phenacetin	100 $\mu$ M
CYP2A6	Coumarin	100 $\mu$ M
CYP2B6	S-Mephenytoin	1 mM
CYP2C8	Paclitaxel	50 $\mu$ M
CYP2C9	Tolbutamide	50 $\mu$ M
CYP2C19	S-Mephenytoin	100 $\mu$ M
CYP2D6	Dextromethorphan	16 $\mu$ M
CYP2E1	Chlorzoxazone	300 $\mu$ M
CYP3A4	Testosterone	125 $\mu$ M

## Hepatocyte Preparation

Hepatocytes were isolated and cryopreserved based on published methods (3-5). For this study, hepatocytes from three human donors will be obtained from the cryopreserved hepatocyte bank maintained at In Vitro Technologies. Donor demographics and medical histories will be provided in the study report. Cryopreserved hepatocytes will be thawed and counted to determine yield, viability will be measured, and cell seeding density will be adjusted accordingly. Hepatocytes will be transferred to collagen-coated 48-well plates for attachment. After hepatocytes attach to the collagen matrix, plating medium will be replaced with sandwich medium and the hepatocytes will be incubated until use.

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## Test Article Incubations

All incubations will be conducted at  $37 \pm 1$  °C, 95% air/5% CO<sub>2</sub>, and saturating humidity. The sample size will be N = 3 replicates for experimental groups.

After the cultures are established, sandwich medium will be removed and the hepatocytes will be treated with an incubation solution containing metaxalone for  $24 \pm 1.5$  hours. The incubation solution will be aspirated and replaced with incubation solution containing the same concentration of metaxalone as was used in the initial dosing and incubated for an additional  $24 \pm 1.5$  hours. The total treatment period will be  $48 \pm 3$  hours.

After the treatment period of  $48 \pm 3$  hours, the incubation solution will be replaced with 150 µL of supplemented KHB. The hepatocytes will be incubated for 10 minutes to remove residual metaxalone. The supplemented KHB will be replaced with 150 µL of supplemented KHB containing an isoform-selective substrate. The hepatocytes will be incubated for 4 hours.

CYP2C8 incubations will be terminated by adding 150 µL of acetonitrile. All other incubations will be terminated by adding 150 µL of ice-cold 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  $37 \pm 1$  °C, 95% air/5% CO<sub>2</sub>, and saturating humidity. The sample size will be N = 4 replicates for the vehicle control, positive control, and reference control groups; and N = 2 replicates for the test article interference control groups.

### Vehicle Control

Vehicle control samples will be included to establish a baseline value for analysis.

After the cultures are established, sandwich medium will be removed and the hepatocytes will be treated with incubation medium containing 1% methanol for  $24 \pm 1.5$  hours. The incubation medium containing 1% methanol will be aspirated and replaced with incubation medium containing 1% methanol and incubated for an additional  $24 \pm 1.5$  hours. The total treatment period will be  $48 \pm 3$  hours.

After the treatment period of  $48 \pm 3$  hours, the incubation medium containing 1% methanol will be replaced with 150 µL of supplemented KHB and will be incubated for 10 minutes. The supplemented KHB will be replaced with 150 µL of supplemented KHB containing an isoform-selective substrate. The hepatocytes will be incubated for 4 hours.

### Positive Control

Positive controls samples will be included to verify that the test system is responsive to known inducers.

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Omeprazole is a selective inducer of CYP1A2 and will be used to verify that the test system is responsive to CYP1A2 inducers. After the hepatocytes are established, sandwich medium will be replaced with incubation medium containing 50  $\mu$ M omeprazole for  $24 \pm 1.5$  hours. The incubation medium containing 50  $\mu$ M omeprazole will be aspirated and replaced with incubation medium containing 50  $\mu$ M omeprazole and incubated for an additional  $24 \pm 1.5$  hours. The total treatment period will be  $48 \pm 3$  hours.

After the treatment period of  $48 \pm 3$  hours, the incubation solution will be replaced with 150  $\mu$ L of supplemented KHB and will be incubated for 10 minutes to remove residual positive control article. The supplemented KHB will be replaced with 150  $\mu$ L of supplemented KHB containing 100  $\mu$ M phenacetin. The hepatocytes will be incubated for 4 hours.

Rifampin is a selective inducer of CYP3A4 and will be used to verify that the test system is responsive to CYP3A4 inducers. After the hepatocytes are established, sandwich medium will be replaced with incubation medium containing 25  $\mu$ M rifampin. The incubation medium containing 25  $\mu$ M rifampin will be aspirated and replaced with incubation medium containing 25  $\mu$ M rifampin and incubated for an additional  $24 \pm 1.5$  hours. The total treatment period will be  $48 \pm 3$  hours.

After the treatment period of  $48 \pm 3$  hours, the incubation solution will be replaced with 150  $\mu$ L of supplemented KHB and will be incubated for 10 minutes to remove residual positive control article. The supplemented KHB will be replaced with 150  $\mu$ L of supplemented KHB containing 125  $\mu$ M testosterone. The hepatocytes will be incubated for 4 hours.

The test system will be considered inducible if the mean specific activities of both CYP1A2 and CYP3A4 in the positive control samples treated with omeprazole and rifampin, respectively, are  $\geq 200\%$  of the mean specific activities in the corresponding vehicle control samples. If these criteria are not met, the study will be repeated.

#### Reference Control

Reference control samples will be included to evaluate the inducibility of CYP2B6, CYP2C8, CYP2C9, and CYP2C19 in the test system.

After the hepatocytes are established, sandwich medium will be replaced with incubation medium containing reference control article for  $24 \pm 1.5$  hours. The incubation medium containing reference control article will be aspirated and replaced with incubation medium containing the same concentration of reference control article as was used in the initial dosing and incubated for an additional  $24 \pm 1.5$  hours. The total treatment period will be  $48 \pm 3$  hours.

After the treatment period of  $48 \pm 3$  hours, the incubation solution will be replaced with 150  $\mu$ L of supplemented KHB and will be incubated for 10 minutes to remove residual positive control article. The supplemented KHB will be replaced with 150  $\mu$ L of supplemented KHB containing an isoform-selective substrate. The hepatocytes will be incubated for 4 hours.

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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 their metabolites.

After the hepatocytes are established, sandwich medium will be removed and the hepatocytes will be treated with an incubation solution containing 40  $\mu$ M metaxalone for  $24 \pm 1.5$  hours. The incubation solution will be aspirated and replaced with incubation solution containing 40  $\mu$ M metaxalone and incubated for an additional  $24 \pm 1.5$  hours. The total treatment period will be  $48 \pm 3$  hours.

After the treatment period of  $48 \pm 3$  hours, the incubation solution will be replaced with 150  $\mu$ L of supplemented KHB and will be incubated for 10 minutes to remove residual metaxalone. The supplemented KHB will be replaced with 150  $\mu$ L of supplemented KHB containing 1% acetonitrile. The hepatocytes will be incubated for 4 hours. If interference is observed in these samples, then Mutual Pharmaceuticals will be notified to determine a course of action.

### **Termination of Control Incubations**

CYP2C8 incubations will be terminated by adding 150  $\mu$ L of acetonitrile. All other incubations will be terminated by adding 150  $\mu$ L of ice-cold methanol. Samples will be transferred to cryovials. If analysis does not occur immediately after incubation, samples will be stored at  $-70^{\circ}\text{C} \pm 10^{\circ}\text{C}$ .

### **Analyses**

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

#### **Phenacetin O-Deethylase (CYP1A2)**

The activity of CYP1A2 in hepatocytes 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 hepatocytes will be determined by measuring the formation of 7-hydroxycoumarin and its conjugated derivatives, 7-hydroxycoumarin glucuronide and 7-hydroxycoumarin sulfate. Samples will be analyzed using an HPLC-UV method.

#### **S-Mephenytoin N-demethylase (CYP2B6)**

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

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**Paclitaxel 6-Hydroxylase (CYP2C8)**

The activity of CYP2C8 in hepatocytes will be determined by measuring the formation of 6-hydroxypaclitaxel. Samples will be analyzed using an HPLC-UV method.

**Tolbutamide 4'-Methyl Hydroxylase (CYP2C9)**

The activity of CYP2C9 in hepatocytes 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 hepatocytes 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 hepatocytes 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 hepatocytes will be determined by measuring the formation of 6-hydroxychlorzoxazone. Samples will be analyzed using an LC/MS method.

**Testosterone 6 $\beta$ -Hydroxylase (CYP3A4)**

The activity of CYP3A4 in hepatocytes will be determined by measuring the formation of 6 $\beta$ -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/million cells) 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:

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

Except for test article interference samples, samples with back-calculated concentrations below the lower limit of quantitation (LLOQ) will be assigned the LLOQ value for calculation.

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## Criteria for Data Acceptance

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

The test article may be cytotoxic at one or more of the concentrations tested. This is an acceptable outcome of induction studies.

## Study Report

A copy of the final study report will be issued to Mutual Pharmaceuticals 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 Pharmaceuticals will be notified to determine whether the data (excluding proprietary information) will be transferred, retained, or destroyed.

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## Protocol Approval

### Sponsor Approval

This protocol has been reviewed and approved by the following:

Jie Du, Ph.D.

Sponsor Representative  
Mutual Pharmaceuticals Company

Signature

Date

10/20/05

### 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

21 Oct 05 RE BC 21 Oct 05  
20 Oct 05

## References

1. Li, A. P. Primary hepatocyte cultures as an in vitro experimental model for the evaluation of pharmacokinetic drug-drug interactions. *Adv. Pharmacol. Series* 1997, 43, 103-130.
2. Li, A. P.; Lu, C.; Brent, J. A.; Pham, C.; Fackett, A.; Ruegg, C. E.; Silber, P. M. Cryopreserved human hepatocytes: characterization of drug-metabolizing enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. *Chem. Biol. Interact.* 1999, 121, 17-35.
3. Li, A. P.; Roque, M. A.; Beck, D. J.; Kaminski, D. L. Isolation and culturing of hepatocytes from human liver. *J. Tiss. Culture Methods* 1992, 14, 139-146.
4. Loretz, L. J.; Li, A. P.; Flye, M. W.; Wilson, A. G. Optimization of cryopreservation procedures for rat and human hepatocytes. *Xenobiotica* 1989, 19, 489-498.
5. Ruegg, C. E.; Silber, P. M.; Mughal, R. A.; Ismail, J.; Lu, C.; Bode, D. C.; Li, A. P. Cytochrome-P450 induction and conjugated metabolism in primary human hepatocytes after cryopreservation. *In Vitro Toxicol.* 1997, 10, 217-222.

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