PROTOCOL TITLE: **Metabolism of T-6292, T-6293, T-6294, and T-6295 by Rat and Human Hepatocytes**

SRI Study No. B011-95

RESEARCH CLIENT

3M Medical Department
Toxicology Services
3M Center
Building 220-2E-02
St. Paul, MN 55133-3220

Study Monitor: Steven C. Gordon, Ph.D., D.A.B.T.

TESTING LABORATORY

SRI International
Toxicology Laboratory
333 Ravenswood Avenue
Menlo Park, CA 94025

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APPROVALS:

Steven C. Gordon — Research Client's Authorized Representative — 6/27/95 Date

Carol E. Green — SRI Study Director — 7/5/95 Date

William Oswald — Quality Assurance — 7/6/95 Date

v. May 3, 1995

I. STUDY OBJECTIVE

The objective of this study is to determine the metabolism of the test articles using isolated hepatocytes prepared from rat and human liver. The data generated by this *in vitro* system will be used to compare the metabolism of the test articles in the two species.

Hepatocytes will be isolated from rat and human liver specimens. The cells will be allowed to attach in monolayer culture and then will be incubated with suitable concentrations of the test chemicals. Aliquots of the culture medium containing cells will be removed at the selected time-points. The protein content and 7-ethoxycoumarin O-deethylase activity, a cytochrome P-450-associated activity, will be determined in cultures from each preparation.

II. STATEMENT OF PURPOSE

The purpose of this study is to provide data that can be used to support applications for research or marketing permits for products regulated by the Food and Drug Administration and submitted pursuant to sections 406, 408, 409, 502, 503, 505, 506, 507, 510, 512-516, 518-520, 706 or 801, or other applicable sections of the Federal Food, Drug and Cosmetic Act or Sections 351 or 354-360F of the Public Health Service Act. This study will be conducted according to SRI Standard Operating Procedures (SOP's) as well as in compliance with the Food and Drug Administration 21 CFR Part 58 Good Laboratory Practice for Nonclinical Laboratory Studies.

III. TEST ARTICLE

A. Test Article Identification

Name: T-6292

Lot No.: To be provided by Research Client

Molecular weight: 571.2

Name: T-6293

Lot No.: To be provided by Research Client

Molecular weight: 685.28

Name: T-6294

Lot No.: To be provided by Research Client

Molecular weight: 527.2

Name: T-6295

Lot No.: To be provided by Research Client

Molecular weight: 538.1

B. Purity and Stability

Documentation of the identity, strength, purity, and stability of the test articles will be the responsibility of the Research Client. Documentation on the identity and purity of the solvent control, DMSO, will be obtained from the supplier of the compound.

C. Handling and Storage

On receipt, the test articles will be placed in a secondary container (lightproof) and stored as recommended by the Research Client. A Material Safety Data Sheet (MSDS) or other comparable document specifying any hazards and identifying first aid and clean-up procedures in case of an accidental spill shall be provided by the Research Client and shall accompany the test articles. The test articles will be logged in and stored in Building L, Room LB286.

D. Disposition of the Test Article

Any remaining unused portion of the test articles will be returned to the Research Client after completion of the study.

IV. TEST SYSTEM

A. Background and Justification for Selection of the Test System

The liver is the major site of metabolism of most organic chemicals, both endogenous and foreign. Species-related differences in the metabolism of xenobiotics are well known and have been documented to be correlated to the toxic effects of chemicals on certain species (Caldwell, 1980; Calabrese, 1983). The recent availability of human tissues for research, particularly high-quality human liver specimens, has contributed greatly to the knowledge base on human xenobiotic metabolism capabilities. In addition, these tissues can be used for *in vitro* investigations on the metabolism of test chemicals early in the development of new products to more reliably predict the metabolic fate of the chemical in humans.

B. Test Species

1. Rat. Adult Sprague-Dawley rats (3 males and 3 females) will be used for the preparation of hepatocytes. They will be purchased from Charles River Laboratories and will weigh approximately 200-250 g and will be at least 2 months old at the time of receipt.

The rats to be used for this study will be housed in Building L, where the cell isolation will be performed. Rats will be taken from the shipping containers, weighed, examined for general health, and placed three per cage in 22 x 12½ x 8-inch suspended polycarbonate cages labeled with their quarantine number assignments. Animals will be quarantined in the same room in which they will be housed. Rats will be fed (*ad libitum*) Purina Certified Rodent Chow (#5002) and the feed supplier will provide SRI with an analytical report identifying the diet to be free of contaminants for each lot of feed received. A copy of each analytical report will be available as part of the study record. Rats will receive purified (deionized and UV-treated) drinking water *ad libitum*. Temperature will be maintained at 72 ± 4°F and relative humidity maintained between 35 and 65%. A light cycle of 12 hours light and 12 hours dark will be maintained, with light starting at 06:00.

The laboratory species will be quarantined for at least 3 days before use in an experiment. The Laboratory Animal Medicine Department at SRI will issue documentation to

the Study Director on the health of animals during the quarantine period. Before isolation of hepatocytes, the rats will be anesthetized with sodium pentobarbital (65 mg/kg).

2. **Human.** Human liver specimens will be acquired through cooperation with organ procurement organizations from brain-dead human organ donors. Liver from two males and two females will be used. Tissues will be processed as for transplant. The organs will be perfused in the operating room with ice-cold organ preservation solution and packed in ice. The tissues will then be shipped to the laboratory for cell isolation by the most expedient method. Hepatocytes will be isolated either by SRI International or they will be obtained from the Human Cell Culture Center (Folkston, GA) .

C. Test System Identification

Each liver specimen will be identified by a letter designating the species (R = rat; H = human), followed by a number indicating the particular specimen.

D. Specimen Characteristics

For the metabolism studies, six rats will be used (three males and three females) and four human specimens (two males and two females). For human specimens, information on age, sex, race, and cause of death will be provided to the Research Client. Other pertinent information that is on drug exposure, smoking, and medical history may be available.

E. Special Considerations Related to Human Tissues

Complete serology testing will be performed on each human donor specimen and only those specimens that test negative to hepatitis B and C viruses, human immunovirus (AIDS), and syphilis will be accepted for use on this project. Many of the donors, however, will have a positive reaction to cytomegalovirus (CMV).

Whatever the results of serology testing, all human tissue will be treated as infectious and technicians will take the necessary precautions in its use for this project, including wearing proper attire while preparing hepatocytes or subcellular fractions and performing incubations. The tissues will be handled in a hood to protect the technician from aerosols that may form during experimental procedures and to keep preparations sterile. Glassware that comes into contact with human tissue will be disinfected with sodium perchlorate, then washed and autoclaved. Disposable materials will be incinerated.

V. EXPERIMENTAL DESIGN

A. Hepatocyte Isolation

Rat hepatocytes will be isolated by either whole liver or biopsy perfusion as convenient. Human hepatocytes will be prepared by the perfusion of biopsy sections (Strom et al., 1981; Green et al., 1986; Allen and Green, 1991). In brief, the whole liver or wedges of the organ will be perfused first with a Ca^{+2}-free buffer, followed by a buffer containing collagenase. Hepatocytes will be combed free from the digested tissue and purified by simple differential centrifugation.

Isolated hepatocytes will be plated onto collagen-coated culture dishes (35 mm diameter) in a modified Waymouth's 752/1 culture medium (CMH1a) that contains 11.2 μg/ml alanine, 12.8 μg/ml serine, 24.0 μg/ml asparagine, 84.0 μg/ml gentamicin sulfate, 0.168 μg/ml aminolevulinic acid, 5.0 μg/ml oleic acid, 5.0 μg/ml linoleic acid, 1.0 μg/ml D,L-tocopherol, 288 ng/ml testosterone, 272 ng/ml estradiol, 393 ng/ml dexamethasone, 7.9 μg/ml thyroxin, 30 ng/ml glucagon, 0.02 U/ml insulin, 0.1% ITS (Collaborative Research, Inc.; final concentrations: 5 μg/ml transferrin, 5 μg/ml insulin, and 5 ng/ml selenium), 0.2 mM L-ascorbic acid 2-phosphate, and 0.2% BSA. The initial plating medium will contain fetal bovine serum. After 2 to 3 hr of incubation at 37°C in an atmosphere of 95% air:5% CO_2, the medium will be aspirated to remove nonviable, unattached cells and replaced with CMH1a containing the test article.

B. Metabolism Experiments

1. Preparation and Administration of Test Article. The test articles will be prepared as stock solutions in DMSO, maintaining the DMSO concentration at 0.1% or less. They will be diluted with culture medium to give a final concentration determined by discussions with the Study Monitor and based on the results of the range-finding cytotoxicity experiment. Stock solutions of the test articles will be prepared immediately prior to the experiment and kept in the refrigerator or on ice until use. Aliquots of these stock solutions will be saved for later analysis by Advanced Bioanalytical Services (Ithaca, NY) to verify the concentrations and homogeneity of the stock solutions.

2. Preparation of Conditioned Medium. Rat hepatocytes will be isolated and cultured on collagen-coated culture dishes for 0 and 6 hr. At the appropriate time point, the hepatocytes will be scraped into the culture medium. The cells and medium will be aspirated and immediately mixed with an equal volume of ice cold methanol. The conditioned medium will then be centrifuged at approximately 1200 X g for 5 minutes and subsequently frozen for

shipment to the Research Client. Approximately 500 ml of conditioned medium at each time point will be prepared.

3. **Incubation of Hepatocytes with Test Article.** Cultures will be incubated in duplicate with the test chemical in CMH1a. Samples will be taken at 2 time-points (0 and 6 hr). The following controls will also be incubated and samples taken at 0 and 6 hr: 1) hepatocytes in media without test article (4 sets of quadruplicate incubations); 2) incubation media containing test articles but no cells. The attached cells will be scraped into the culture medium. Then the cells and medium will be aspirated and immediately added to an equal volume of ice-cold methanol to stop the reaction. The samples will then be centrifuged at high speed in a table top centrifuge for 5 minutes and subsequently frozen for shipment.

4. **Protein Assay.** The protein content of the hepatocyte cultures will be determined by a spectrophotometric procedure, Coomassie blue assay (Bradford, 1976).

5. **Cytochrome P450 Activity.** As a control to indicate the presence of cytochrome P450 associated activity, 7-ethoxycoumarin O-deethylation (ECOD) activity will be determined in each preparation of hepatocytes. The cells will be incubated with 100 μM 7-ethoxycoumarin for 1 hr. The culture medium will be assayed for hydroxycoumarin production using the fluorometric method of Greenlee and Poland (1978). ECOD activity will be calculated as the amount of hydroxycoumarin produced/hr/mg protein.

6. **Analysis of Metabolism.** Samples will be shipped to Dr. Jack Henion at Advanced Bioanalytical Services, Inc. for analysis of metabolism.

7. **Data Collection.** The protein assay and ECOD assay will be performed using a spectrophotometer and fluorometer, respectively. The values will be recorded manually or captured electronically, depending on equipment availability. Data from both assays will be recorded and manipulated on a Microsoft Excel spreadsheet. Information on human liver specimens are kept in a computer data base and are also recorded in a laboratory notebook.

The results of the metabolite analysis (performed by Advanced Bioanalytical Services, Inc.) will be returned to SRI and the data will be calculated as the nmol of metabolite/mg protein. These data will be included in the final report. The results of the analysis of the liver tissue by Mr. James Johnson at 3M Environmental and Pollution Control will also be included in the final report. The raw data generated on the metabolite analysis will be kept and maintained by Advanced Bioanalytical Services. The raw data on levels of the test articles in the human liver samples will be kept and maintained by 3M.

D. Sample Shipping Addresses

The incubation samples, samples of frozen human and rat liver tissue (approximately 1 g), FBS, BSA, L-ascorbic acid 2-phosphate, and media (conditioned and fresh) will be sent to:

Dr. Jack Henion, Ph.D.
Advanced Bioanalytical Services, Inc.
15 Catherwood Road
Ithaca, NY 14850

A sample of each human liver tissue (approximately 1 g) will be sent to:

James D. Johnson
3M Environmental Engineering and Pollution Control
935 Bush Avenue
Building 2-3E-09
PO Box 33331
St. Paul, MN 55133-3331

The data on protein content, ECOD, reports, and excess test article will be sent to:

Dr. Steven C. Gordon
3M Medical Department, Toxicology Services
P. O. Box 33220
St. Paul, MN 55133-3220

VI. CONTROL OF BIAS

Human tissues will be used as they become available.

VII. STATISTICAL EVALUATION OF DATA

Mean and standard deviations will be determined for the replicate incubations for each test article.

VIII. REPORTS

Draft reports will be issued prior to submission of the Final Report. Two copies of the Final Report will be submitted to the Research Client.

IX. RECORDS TO BE MAINTAINED

The laboratory notebooks, all raw data, relevant communications, and the original copy of the Final Report will be transferred to the SRI Records Center, Building B, after completion of the study. These materials will be maintained for 10 years; then the Research Client will be contacted concerning future disposition of the records.

X. GOOD LABORATORY PRACTICES (GLP) AND QUALITY ASSURANCE

This study will be conducted in accordance with Good Laboratory Practice Regulations. The duties of SRI's Quality Assurance Unit (QAU) will include inspecting the calculations, inspecting laboratory work, certifying proper identification and notebook entry of samples, and reviewing the Final Report. A Quality Assurance Statement will accompany the Final Report of the study.

After the study has been initiated, modifications of the protocol—by either the Testing Laboratory or the Sponsor—will be submitted in writing to the other party. All agreed-upon modifications will be in the form of Protocol Amendments, that will state the specific modifications and the reasons for the modifications and will be signed and dated by the Sponsor's Representative and the Testing Laboratory's Study Director. All procedures will be performed in accordance with SRI Standard Operating Procedures.

The Study Director will be made aware of any GLP noncompliance directly. If this area of noncompliance significantly affects the results of the study, the Sponsor will be notified immediately. This will be documented and the agreed upon outcome will be forwarded to the QAU.

Authorized designates of the Sponsor may inspect this study during regular working hours for quality assurance purposes and may copy any study-related original data.

XI. REFERENCES

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DeLean, A., P. J. Munson, and D. Rodbard. 1978. *Am. J. Physiol.* **235**, E97-E102.

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