LSR Schedule No.
LSR Enquiry No. ZZZ\1288H

M2031

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MOUSE MICRONUCLEUS TEST TO COMPLY WITH
O.E.C.D. GUIDELINE 474 (1983)

29 January 1987

TEST COMPOUND : RC 9927 (FR 45B)

SPONSOR : Pennwalt Corporation
900 First Avenue
P.O. Box C
King of Prussia
Pennsylvania 19406-0018
USA

MONITOR : Dr J Seckar

The ability of this compound to induce chromosome damage resulting in formation of micronuclei in mouse bone marrow erythrocytes will be tested according to Standard Protocol CB60, with the following additions and/or modifications:

4. TEST MATERIAL

Ten millilitre samples of the test material, and all dosing solutions, will be despatched to the Study Sponsor for analysis on completion of the study.

Route of test material administration

The route of administration of RC 9927 will be intraperitoneal, or dermal, as specified below.

5.3 Main study

In addition to the 80 animals which will be dosed intraperitoneally (vehicle controls and RC 9927 treatments) and the 10 animals dosed orally (positive controls), 20 male and 20 female mice will be treated with five daily, dermal applications as follows:

Group		Dose (g/kg)	Number of mice	Animal numbering
6	Vehicle control	2	10M	107-116
			10F	127-136
7	RC 9927	2	10M	117-126
			10F	137-146

6.1 Animal husbandry

All animals treated by the dermal route will be caged individually.

7. TERMINATION, SAMPLE COLLECTION AND SLIDE PREPARATION

Five male and five female mice from Groups 6 and 7 will be killed 18 hours after treatment: the remaining animals will be killed 48 hours after treatment.

MANAGEMENT OF STUDY

Head of Cell Biology Department : James Bootman, B.Sc., M.Sc., M.I.Biol.
Study Director : Galia Hodson-Walker, B.Sc.
Quality Assurance Manager : David J. Ford, B.Sc., Ph.D.

APPROVAL OF PROTOCOL

For LIFE SCIENCE RESEARCH LIMITED

Issued by : D. G. Hodge .....Date : 29.1.87.....

Released by : G. Hodson-Walker .....Date : 29.1.87.....

For PENNWALT CORPORATION

Approved by : Joel A. Sechan .....Date : Feb. 3, 1987.

MOUSE MICRONUCLEUS TEST TO COMPLY WITH O.E.C.D GUIDELINE 474 (1983)

Standard Protocol CB60

of

Life Science Research Limited
Occold, Eye, Suffolk IP23 7PX
England

1. OBJECTIVE AND RATIONALE

1.1 Objective

Assessment of the ability of the test compound to induce chromosomal damage resulting in the formation of micronuclei in bone marrow erythrocytes from the mouse.

1.2 Selection of test system

The micronucleus test has been widely used for investigating the induction of chromosomal damage in vivo. In mitotically dividing cells, such as the bone marrow, chromosome fragments or whole chromosomes which fail to align normally at metaphase or anaphase may be excluded when the daughter nuclei form in telophase. This can result in the formation of small secondary nuclei (micronuclei) in the cells. Micronuclei occur rarely in normal, dividing cells, but are more common in cells taken from animals exposed to known clastogens.

The CD-1 mouse is chosen for this study since it provides a convenient, mammalian model.

2. LOCATION OF STUDY

Life Science Research Limited
Eye, Suffolk, IP23 7PX
England

3. STUDY TIME SCHEDULE

The study will be conducted at a time agreed by both the Sponsor and Life Science Research. The precise dates of animal dosing and termination, together with the date of completion of scoring will be notified in a protocol amendment after acceptance of this protocol.

4. TEST MATERIAL

The test compound will be supplied to Life Science Research by the Study Sponsor; if a declaration of sample purity and stability is required to accompany the final study report, this information should be supplied with the sample.

Unless otherwise directed by the Study Sponsor the compound will be given in aqueous solution if water soluble, or suspended in methyl cellulose if insoluble in water. All solutions or suspensions will be freshly prepared on the day of use.

For compliance with international GLP regulations it may be necessary to analyse a sample of the test material solution or suspension as used in testing, to confirm the homogeneity, concentration and stability of the test material. If requested by the client prior to study commencement, samples of the prepared solutions or suspensions can be retained. These could then be despatched for analysis by the client (or analysed at LSR) at additional cost if required.

The compound will be stored in the dark at 0 - 4°C after receipt unless otherwise specified by the Sponsor.

5. STUDY DESIGN

5.1 Animals

Male and female mice of the CD-1 strain, COBS Swiss Albino, supplied by Charles River Breeding Laboratories (U.K.), Margate, Kent, England, will be used.

Aged 4 - 5 weeks on arrival at Life Science Research, animals will be acclimatised for 4 - 6 days prior to treatment.

5.2 Preliminary toxicity test

The size of the preliminary toxicity test will depend on any information provided by the Sponsor concerning the acute toxicity of the compound. When no information is available, the test will consist of four groups of 2 male and 2 female mice. Four levels of the test compound will be administered on one occasion only. Animals will be dosed orally, by gavage, and the highest level tested will not exceed 2000 mg/kg (unless the route of administration and/or the maximum exposure level are otherwise specified by the Study Sponsor).

Bone marrow smears will be prepared from these animals 72 hours after dosing. Normally a dosage that produces some indication of toxicity, evidenced by weight loss or a depression in bone marrow proliferation, will be used as the highest dosage in the main study. However, if the test compound is non-toxic, the highest practicable dosage will be used.

Animals used in the preliminary toxicity test will be numbered from 1 onwards.

5.3 Main study

Animals will be non-selectively allocated to the following treatment groups:

Group	Treatment	Dose mg/kg	No. of mice	Animal numbering[a]
1	Distilled water or methyl cellulose	-	15 male 15 female	17-31 62-76
2	Test compound	*	5 male 5 female	32-36 77-81
3	Test compound	**	5 male 5 female	37-41 82-86
4	Test compound	***	15 male 15 female	42-56 87-101
5	Chlorambucil	30	5 male 5 female	57-61 102-106

* Lowest level, one fifth of intermediate level.
** Intermediate level, one fifth of highest level.
*** Highest level, to be determined from preliminary toxicity test.
[a] Assuming 16 animals are used in the preliminary toxicity test.

Animals will be treated once only, normally at a volume dosage of 10 ml/kg. The positive control, chlorambucil, will be administered orally in 10% ethanol. All doses will be prepared on the day of administration.

6. ANIMAL CARE

6.1 Animal husbandry

Mice will be housed in single-sex groups of 2 or 5 in high density polypropylene and stainless steel cages (North Kent Plastics Limited). Laboratory animal diet LAD [1/2] (Labsure, Manea, Cambridgeshire, England) will be fed ad libitum throughout the study. This is an expanded autoclaved diet supplied in a discardable outer paper sack and sealed inner polythene bag. It contains no added antibiotic or other chemotherapeutic or prophylactic agent. Normal levels of common contaminants will not influence the outcome of the study. Accordingly, no determination of contaminant levels will be undertaken for diet, water or bedding. The use of vehicle control animals will, in any case, obviate the risk of spurious findings due to contaminants. Drinking water will be supplied to each cage via a polythene bottle and sipper-tube.

Cages housing animals of different treatment groups will be distributed so that the effects of any spatially-variable components are equalised between groups, as far as possible.

6.2 Environmental control

The animals will be housed inside a barriered, limited-access rodent facility designed and operated to minimise the entry of external biological and chemical agents. All personnel entering the facility are required to change into protective clothing.

The animals will be held in a room kept at positive pressure with respect to the outside. The room has its own supply of filtered fresh air, which is passed to atmosphere and not recirculated. Temperature and humidity controls are designed to maintain conditions of 21° ± 2°C and 55% ± 15% RH. There are approximately 15 air changes per hour and a 12-hour light : 12-hour dark cycle operates.

A stand-by power supply is brought into operation should the mains electricity supply fail.

6.3 Animal identification

All animals will be uniquely identified by ear-notch number codes; numbers will be assigned using a set of computer-generated random numbers.

Cage labels, identifying the occupants by experiment, animal numbers, sex and treatment group will be attached to all cages.

6.4 Observations

All animals will be inspected daily during both the acclimatisation period and the period of the study, for evidence of reactions to treatment or ill health. All animals will be weighed on the day of treatment and again immediately before termination. Any deviations from normal and all bodyweights will be recorded. Animals adjudged to be *in extremis* will be killed. Wherever possible, bone marrow smears will be made from moribund animals, or animals found dead.

7. TERMINATION, SAMPLE COLLECTION AND SLIDE PREPARATION

Five male and five female mice per group will be killed 24 hours after treatment, by cervical dislocation following carbon dioxide gassing. Further lots of 5 males and 5 females from Groups 1 and 4 will be killed at 48 and 72 hours after treatment.

Both femurs will be dissected out, cleaned and the marrow cells flushed into clean centrifuge tubes with 2.5 ml foetal calf serum. Each resultant suspension will be centrifuged (1000 rpm, 5 minutes) and the bulk of the supernatant removed. The cell pellet will be resuspended and three smear preparations from each animal made on clean, grease-free microscope slides. After air-drying the slides will be stained, using the Schmid (May-Grunwald and Giemsa) staining technique, and permanently mounted.

8. SLIDE EVALUATION AND DATA ANALYSIS

All slides will be randomly assigned code numbers/letters by a person not subsequently involved in slide evalution, and all other identification markings will be concealed to eliminate bias.

Regions judged to be of suitable technical quality under medium magnification, will be scored at high magnification under the light microscope. A total of at least 2000 mature and polychromatic erythrocytes per animal will be scored for the presence or absence of micronuclei, scoring at least 1000 of each type. Where there is an appreciable shift in the ratio of polychromatic to mature cells, scoring will continue until at least 2000 erythrocytes of the predominant type are examined. The resultant data will be used to calculate the number of micronucleated cells per 1000 polychromatic erythrocytes and per 1000 mature cells for each animal. The counts for mature erythrocytes provide a check on the results of the younger polychromatic cells; an increase in the ratio of mature : polychromatic cells may indicate inhibition of cell division following treatment, and the incidence of micronuclei in the mature cell population of animals killed 24 hours after treatment reflects the pre-treatment situation, since most of these cells were produced before treatment. The frequency of micronuclei in polychromatic cells provides an index of induced genetic change.

9. STATISTICAL ANALYSIS

Comparisons of the number of micronuclei in polychromatic cells will be made between treated and control groups using the Mann Whitney U test. If there is any evidence of a sex-related difference in response, the data from male and female animals will be analysed separately.

10. QUALITY ASSURANCE

The study will be conducted in accordance with current internationally recognised Good Laboratory Practice Regulations and will be subjected to the following quality assurance procedures:

- the protocol is inspected for compliance.
- procedures and data similar to those used and produced on the study are periodically inspected.
- the final report is reviewed to ensure that it accurately describes the methods and relevant Standard Operating Procedures and that the results are in accord with the primary data.

Periodic reports on these activities are made to Management and the Study Director. All raw data pertaining to this study are available for inspection by the Study Monitor (for scientific monitoring). In addition, specified scientists designated by the Sponsor may, upon appointment, examine any set of data from this study.

11. RECORDS TO BE RETAINED

The raw data comprise	:	Operational and observations sheets, covering every aspect of the study. Slide Scoring sheets.

All raw data, slides, test compound information, QA records and reports and other records pertaining to this study are retained in the archives of LSR.

12. REPORTING

The following information and data will be included in the final report:

- Name and address of the facility performing the study and the dates on which the study was initiated and completed.
- Objectives and procedures stated in the approved protocol, including any changes to the original protocol.
- Raw data generated while conducting the study, including calculations performed on the data.
- Statistical methods employed for analysing the data.
- The test substance, identified by name and/or code number.
- Methods used.
- Animals used. The number in the study, sex, bodyweight range, source of supply, species, strain and procedures used for the identification of the animals.
- Dosage, dosage regime, route of administration and duration.
- Any unforeseen circumstances that may have affected the quality or integrity of the study.
- The name of the Study Director.
- A summary of the data, an analysis of the data and a statement of the conclusions drawn from the analysis.
- The location where all raw data and the final report are to be stored.