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Protocol for *in vitro* unscheduled
DNA synthesis assays with various
coded samples

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1. STUDY ORGANISATION**1.1. BIBRA Project No:**

1363 1/0
June 1993

1.2. Title:

Protocol for *in vitro* unscheduled DNA synthesis assays with various coded samples

1.3. Testing Facility:

BIBRA Toxicology International
Woodmansterne Road
Carshalton
Surrey SM5 4DS
UK

1.4. Sponsor:

B.A.T. (UK & Export) Ltd
Research & Development Centre
Regent's Park Road
Southampton SO9 1PE
UK

1.5. Critical dates:

A detailed timetable will be issued as a protocol addendum following receipt of instructions from the sponsor to proceed.

1.6. Responsible Staff:

Study Director : S.D. Blowers. PhD.

1.7. Good Laboratory Practice

The study described in this protocol will be carried out to the highest scientific standards and in accordance with written Standard Operating Procedures.

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1.8. Sponsor representative

The sponsors will appoint a representative to assure them of the adequate conduct of the study and to act as the contact between the sponsor and BIBRA.

1.8.1. Definition of representative

The sponsor representative will be:

Dr E.D. Massey
B.A.T. (UK & Export) Ltd
Research & Development Centre
Regent's Park Road
Southampton, SO9 1PE

1.8.2. Access of representative to the study

The sponsor representative or any nominated individual will have access at all times within normal working hours (Monday to Friday 09.00 to 17.00 hr) to all stages of the study, all data generated as part of the study and to the scientific staff responsible for its conduct.

It is expected that such access would normally be by agreement with the Study Director.

Access to various parts of the BIBRA facility will depend upon compliance with codes of practice relating to use and safety.

1.8.3. Contact

The representative will be informed of the progress of the study by:

- receiving copies of any documents passed from BIBRA to the sponsor relating to the conduct of the study
- any visits made (1.7.2.). If such visits are made by a nominated individual, it will be the responsibility of that individual to report to the sponsor representative
- contact by the most rapid means available should unexpected events occur. All contacts will be confirmed in writing.

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1.8.4. Integrity of the study

In the event of unexpected occurrences requiring action, BIBRA will contact the sponsor representative. Should the representative not be available or not respond in reasonable time, BIBRA will take the action considered to be in the best interests of the study. Details of the reason and the action will be documented to the representative and will form part of the study record.

2. SCIENTIFIC OBJECTIVE

To assess various coded samples for their ability to induce DNA repair in cultured rat hepatocytes using the unscheduled DNA synthesis (UDS) assay.

3. TEST AND CONTROL ARTICLES

3.1. Test article

3.1.1. Definition

The test articles will be coded samples provided by the sponsor with any relevant data relating to their stability and storage conditions. Unless otherwise indicated samples will be stored in the containers provided at room temperature.

3.2. Positive control article

2-Acetyl aminofluorene (2AAF) will be used as the diagnostic mutagen in the UDS assay and will be provided by BIBRA.

3.3. Negative control article

This will be the solvent used for the test article, and will be provided by BIBRA.

4. TEST SYSTEM

4.1. Isolation of hepatocytes

The hepatocytes will be derived from 180-220 g, male, Fischer 344 rats and will be isolated using a two-step collagenase perfusion technique (Gray *et al.*, 1983, Toxicol. Appl. Pharmacol. 32, 355-367). The viability of the cells will be assessed and only cultures with greater than 75% viable hepatocytes will be used.

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4.2. Preparation of hepatocyte cultures

The hepatocytes will be seeded at 5×10^5 cells per plate layered over a 20 mm circular glass coverslip. The suspension medium will be RPMI with 5% Foetal Calf serum and the plates will be incubated at 37°C in an air/carbon dioxide atmosphere (95:5).

5. EXPERIMENTAL DESIGN

For each test article, 8 dose levels will be selected in discussion with the sponsor. Groups of 5 cultures will be exposed to each of these doses, a positive control treatment and a solvent control treatment (6.1.). After autoradiography (6.2.) and staining (6.3.), scheduled and unscheduled DNA synthesis will be assessed (6.4.). A total of 150 cells (from at least 3 slides) will be evaluated for both negative and positive control groups and for 3 test article concentrations up to the limit of toxicity.

6. PROCEDURES

6.1. Treatment of hepatocytes

Two hours after seeding, cultures will be washed and refed with 1 ml of serum free medium (RPMI 1640) containing tritiated thymidine ($5 \mu\text{Ci/ml}$) and the test or control article at the appropriate concentrations. After twenty hours of incubation at 37°C, the cells will be examined for signs of toxicity, washed free of medium and cultured with 1 ml of serum free medium containing 'cold' thymidine at 0.25 M for 2 hours. The cells will then be fixed, with 3:1 ethanol:acetic acid, washed with water and air dried. When dry the coverslips will be mounted on microscope slides and left to set in a desiccated container.

6.2. Autoradiography

The prepared slides will be coated (in the dark) with Ilford K-5 photographic emulsion. The coated slides will be exposed in the dark for five days at 4°C before being developed.

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6.3. Staining

The developed slides will be stained using haematoxylin and eosin to highlight the nucleus and cytoplasm.

6.4. Measurement of UDS

A quantitative assessment of the silver grains in the nucleus and cytoplasm will be carried out using a Seescan Image analysis system. Grain counts will be made for the whole nucleus area and from three equal sized areas in the cytoplasm for each cell scored. The mean of the cytoplasmic counts will be subtracted from the nuclear count giving a net grain (NG) count. Fifty cells per slide will be assessed, initially from two slides per treatment. The slides will be coded and examined blind. For each slide the population average of NG and the standard deviation (SD) will be calculated. Also the percentage of cells undergoing repair (those with greater than 5NG increase) and those in S phase (scheduled synthesis) will be recorded.

6.5. Statistical assessment

The data derived will be presented as the mean NG for each dose group. In addition the percentage of cells undergoing repair in each dose group will be given.

A hierarchical analysis of variance will be used to compare variability between dose levels, between slides within a dose and between cells within a slide. Comparisons between individual dose levels and the negative controls will be carried out using the least significant difference (LSD) criteria. If appropriate other statistical methods may be used to help in the interpretation of the experimental results.

7. REPORTING

7.1. Final report

A report will be prepared which:-

- defines the responsible staff
- defines the test article
- defines the test system

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- defines the procedures used
- summarizes the findings
- evaluates the findings
- lists the data generated

8. **PROTOCOL ACCEPTANCE**

I hereby declare that the study described in this protocol will be conducted under my supervision to the highest scientific standards and in accordance with written Standard Operating Procedures.

Study Director:
S. Blowers, PhD.

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Signature..... *BJ Phillip* Date. *8th June 1993*

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