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DP/ID/SER.A/1472
27 May 1991
ORIGINAL: ENGLISH

MODERN PHARMACEUTICAL FORMULATIONS BASED ON TRADITIONAL THAI PHARMACOPOEIA

DP/THA/87/010

THAILAND

Technical report: Findings and recommendations*

Prepared for the government of Thailand
by the United Nations Industrial Development Organization,
acting as executing agency for the United Nations Development Programme

Based on the work of Ray Waters
Toxicologist

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Vienna

* This document has not been edited.

V.91-24951

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Section 1

Introduction

This visit was first suggested in July 1990 by Mrs. Sasithorn Wasuwat, the now retired director of the Plant and Natural Products Division (PNPD) at the Thailand Institute of Scientific and Technological Research (TISTR). Due to other commitments the mission could not be undertaken until March 1991. It was thus taken when PNPD was under the directorship of Mr. Taweesakdi Rohitasukh.

Objectives

The objectives were i) to educate the staff at PNPD in the genetic toxicology assays legally required for the screening of new pharmaceuticals, and ii) to assist PNPD in the establishment of facilities to undertake the required assays.

Observations

The staff at PNPD were very responsive during lectures and discussions. They were able to grasp the principles of the various methods routinely used in genotoxicity screening. Appendix 1 describes the timetable for my period at TISTR and includes the assays that were covered. These assays are discussed in more detail in Section 2.

Examination of the facilities at PNPD indicated that at present much of the equipment and facilities for genotoxicity testing are unavailable. In fact, only the Ames test and the *in vivo* mouse micronucleus test can currently be undertaken. A list of essential requirements is provided in Appendices 2 and 3. They include approximate costs where known and suggested laboratory modifications.

During my visit the preparation of rat liver S9, the Ames test and the *in vivo* mouse micronucleus assay were the only methods performed in their entirety. It should be noted that with the Ames test only two tester strains, TA98 and TA100 are currently employed at TISTR. It is recommended that additional strains listed in Section 2 and Appendix 4 should also be used. The remaining assays were covered by analysing examples of experimental material brought from the U.K. This included the analysis of slides for chromosome aberrations, slides for micronuclei

in vitro, and slides for *in vivo* rat unscheduled DNA synthesis. These have been left at PNPD as reference material.

The techniques, bar the Ames assay and the *in vivo* micronucleus assay, which are available, generally require a training period of six months before staff become reasonably competent at undertaking them. In light of this I strongly recommend that one staff member attends a laboratory routinely undertaking the mammalian assays for a period of six months. This period of six months is a *minimum requisite* for the individual to be trained to the standard required in genotoxicity testing. A period of less than this would be insufficient as it is essential that TISTR establishes a *competent* unit. A staff member could acquire expertise in mammalian cell culture, the mammalian mutation assay, the mammalian chromosome aberration assay, and the mammalian *in vitro* micronucleus assay whilst at my laboratory in Swansea during a five month period. I could then arrange for them to attend a laboratory that is routinely undertaking unscheduled DNA synthesis in rat hepatocytes during the last month. This should be undertaken only when the facilities at PNPD will be available upon the return of the staff member, hence ensuring the expertise is rapidly established at TISTR (see Appendix 3 for a suggested programme). The *minimum* costs to establish Genetic Toxicology at PNPD are also given in Appendix 3 with recommendations for the eventual staffing of the unit. One research staff member in addition to the toxicologist Mr. Jakkarapong is required. This individual would be responsible for genetic toxicology and undergo training in the UK. Both staff should have one shared technical assistant to aid with work in toxicology and genetic toxicology. Mr. Jakkarapong would have overall responsibility for the unit. He should continue discussions and negotiations with Thai companies and research laboratories concerning TISTR's abilities to screen potential drugs, cosmetics or food additives for toxic or genotoxic activity. This would generate income for TISTR and establish contacts whilst the genetic toxicologist undergoes training.

Expertise in genetic toxicology is essential and will serve TISTR for many years to come and it will complement the pre-existing strengths in toxicology. It will be substantially cheaper to undertake such tests "in house" at TISTR. It should also be noted that the establishment of a toxicology and a genetic toxicology facility will serve not only TISTR's needs. It will also be available for screening potential drugs, cosmetics, pesticides and food additives produced by Thai commercial companies and research institutes. The available tests in toxicology and genetic toxicology should be marketed via a suitable brochure. I additionally suggest that TISTR contacts Pharmaceutical companies, pesticide companies and those producing cosmetics or food additives, based in other S. E. Asian countries. I suspect that many such companies must rely on the above tests being undertaken in European, American or Japanese institutes, all of which would be costly. The establishment of a centre to service S. E. Asia could generate substantial income, the profits from which could be used to subsidise future TISTR projects. This approach is also merited because it is unlikely that the number of potential drugs generated by TISTR will completely occupy a genotoxic toxicology unit all of the time.

I will now describe in some detail the specific assays covered during my visit to TISTR. Precise details of these methods are given in the review articles and books cited most of which have been left at TISTR.No attempt has been made to site original references.

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Section 2

Short term tests for Genotoxicity

Introduction

New drugs or compounds to which humans may be exposed are legally required to undergo screening for toxicological effects. One aspect of this is the examination of genotoxic activity, that is, the examination of whether the compound adversely influences a cell's genetic material.

A relatively large number of different short term tests have been used to screen compounds for genotoxic activity. However, only a proportion of these have been properly validated by examining their responses to a wide array of chemical carcinogens of known activity. It should therefore be realised that at present only a few assays are generally considered as suitable for the routine screening of new drugs. Nevertheless, the field of genetic toxicology is one that is continually being developed. New assays and improvements or modifications of existing procedures are often published. Hence it is imperative to review the literature frequently if one is to keep abreast of the area.

The aim of this portion of the document will be to outline the major assays that are currently routinely used for genotoxic testing and which I recommend for use at TISTR. Details for the procedures described are given in the appendices or in publications cited in the bibliography.

1. Bacterial Mutation Assays

Almost all bacterial mutation tests utilise Salmonella typhimurium strains developed by B. N. Ames. This method is thus termed the "Ames Test". The approach involves examining the ability of a compound to induce reverse mutation in a number of different bacterial strains (1,2,3). Individual strains have been designed to detect the activity of different types of mutagens (mutagens are genotoxins which react directly or indirectly to produce changes in the chemical structure of the genetic material, DNA). Details of some of the strains available and suggested batteries for testing

are given in Appendix 4. The types of mutagens which they react to can be found in references 1,2,3,4,5.

It should be noted that these assays rely on the ability of a compound to revert a pre-existing mutation in a locus which confers a dependence on the amino acid histidine. Hence mutants are detected as prototrophs amongst the auxotrophic population. Many potential mutagens require metabolic activation to DNA-reactive electrophilic species. Unfortunately bacteria do not possess this ability. Hence the Ames test relies on the provision of an exogenous activation system obtained from mammalian liver, and commonly referred to as S9. This is a post-mitochondrial supernatant derived from the homogenised liver of rats (Appendix 5).

The advantages of a bacterial assay are the following:

i) It is rapid to perform (2-3 days)

ii) It is relatively cheap

iii) An enormous data base exists.

However, due to structural differences of the bacterial chromosome the assay will not detect agents that induce the loss or gain of whole mammalian chromosomes by reacting with the spindle apparatus (bacteria have no such system of chromosome segregation at cell division). Neither will the assay detect agents that specifically induce large chromosomal deletions (these would be lethal to a bacterium). Conversely, metabolic mammalian systems may well inactivate some bacteria mutagens.

In light of the above, a positive result should not be construed as signifying a compound will be mutagenic or carcinogenic in mammals or man. A positive result in a bacterial mutation test should merely be construed as an early warning of a potential hazard. The various modifications of the Bacterial Mutation Assays are listed in Appendix 6 along with their strengths and weaknesses. Details of the procedures are given in references 1 to 5.

Further experimentation in higher organisms is now required to clarify any potential hazard.

Mammalian Forward Mutation Assays

A relatively large range of mutant phenotypes can be selected for in cultured mammalian cells. The assays measure forward mutation and are thus able to detect point mutations plus small or large deletions. Only a small fraction of these mutation assays are of use for routine mutagenicity testing. The cell lines generally employed for this purpose are given in Appendix 7. The appendix also lists the loci studied and how the mutants are detected. Basic details concerning mammalian cell culture are given in references 6 to 8.

Chinese hamster cell lines (V79 and CHO) are used to monitor mutation in the gene coding for the enzyme hypoxanthine-guanine phosphoribosyl transferase (HPRT). This gene is located on the X chromosome in mammals and is thus functionally haploid in both male and female cells. HPRT is one of the 'salvage' enzymes for purines whose function is to salvage the degradation products of nucleic acid synthesis. As such it is not essential for cell survival as bases can be synthesised de novo. Mutants of the HPRT locus with non-functional or zero levels for the HPRT enzyme can be detected because they do not incorporate the toxic purine analogue 6-thioguanine (6TG). Hence in the presence of 6TG normal cells will die but the mutant cells survive to produce colonies.

Mutation can also be monitored at the thymidine kinase locus (TK). This locus has an autosomal location, and therefore exists as two copies. Furthermore a TK mutation is recessive. Hence a cell line that is heterozygous for the TK mutation must be used; for example, the murine transformed cell ling L5178Y TK^{+}/TK^{-}. Again TK is a non-essential salvage enzyme and TK^{-}/TK^{-} mutants can be selected for by virtue of growth in trifluorothymidine (TFT) which is a toxic analogue of thymidine.

In both of the above cases the concentration of the toxic selective agent (ie. 6TG or TFT) is critical. It must be high enough to completely kill all non-mutant cells.

Mutants cannot be selected for immediately after treatment with a mutagen. They must first be cultured in non-selective medium for a number of days so as i) the mutagen induced DNA damage is fixed as a mutation and ii) the level of the non-mutant enzyme is reduced to a negligible amount by the routine "turnover" of the enzyme in question. The expression time varies with the cell line and the selective system. For mutants at the HPRT locus in V79 or CHO cells it is 6-7 days, yet in the L5178Y TK^{+}/TK^{-} cell line it is only 2-3 days. Thus in the case of L5178Y cells they must be grown for 2-3 days in non-selective medium and then transferred to a selective medium for the selection of mutants. These expression times must be adhered to because the number of mutants can diminish if culturing in non-selective medium occurs for periods longer than those recommended. References 6 to 8 contain procedural details of these assays for the detection of mammalian mutation. Comments on the maintenance of the cell lines are given in Appendix 8. The strengths and weaknesses of the systems are listed in Appendix 9.

Assays for measuring chromosome aberrations in mammalian cells.

The microscopic examination of mammalian chromosomes for structural aberrations and numerical alterations is an established means of examining the potential genotoxic effects of an agent. Experimentation can be undertaken using cultured Chinese hamster cells (V79 or CHO) or human lymphocytes stimulated to divide by the addition of phytohaemagglutinin (PHA). These two approaches rely on DNA damage present during DNA synthesis (S Phase) being converted into visible structural changes in chromatids and chromosomes. These are breaks or exchanges between chromosomal material. This damage is observed in metaphase chromosomes. Cells in this stage of the cell cycle can accumulate after the incubation of cultures in colcemid for a few hours. These are then treated hypotonically, fixed and the chromosomes stained for analysis.

Cell cultures should be routinely maintained as for the mammalian mutation assay. Human lymphocytes are obtained from donors who have not been exposed to X-rays or suffered with viral disease within two months. Details of the practical procedures are given in references 9 to 11. The strengths and weaknesses of this assay are listed in Appendix 10.

The Mammalian Micronucleus Assay

This assay measures the appearance of chromosome fragments or whole chromosomes that undergo non-disjunction. They are detected as micronuclei totally separate from the main nucleus in interphase cells. As stated above the assay can detect the non-disjunction of whole chromosomes. This may arise after exposure to agents that react with a cell's spindle apparatus but not DNA. Hence the assay provides a valuable means of detecting the activity of a class of genotoxins not detectable by many of the assays previously discussed. It can be performed in vitro with cultured cells or in vivo by examining bone barrow. The in vitro approach has seen a number of technical advances which improve the interpretations of data. First, Fenech et al (12) developed the use of cytochalasin B. This agent blocks cytokenesis. Therefore cells that have undergone cell division have two nuclei i.e. they are binucleate. The scoring of micronuclei in these cells indicates how many micronuclei have occurred due to the replication of damaged DNA or damage to the spindle apparatus. The percent binucleate cells amongst the total population provides information on the mitotic index. The processing of slides can involve the use of a kinetochore antibody which is detected by fluorescence. This is used to identify chromosomes (or fragments) containing a centromere. An agent that induces micronuclei only which possess centromeres would be classed as an aneugen (an aneuploidy inducing agent) but not a DNA interacting mutagen. The ability of this assay to detect mutagens and aneugens, plus the simplicity of scoring the slides when compared to analysing chromosome aberrations, makes the micronucleus assay an attractive method for

genotoxicity screening. Furthermore automated slide analysis should be available in the near future. Details of this procedure are given in reference 12.

The mouse micronucleus assay is now being routinely used as an indicator of genotoxicity *in vivo*. Micronuclei are estimated in bone marrow cells. Here it is important to note that the genotoxin must reach the bone marrow to elicit an effect. It must be borne in mind that some agents may not reach this tissue although they might be genotoxic to other tissues (e.g. stomach, liver). A negative with this assay should thus never be construed as indicative of the agent being totally non-genotoxic *in vivo*. The advantages and disadvantages of the micronucleus assay are listed in Appendix 11.

Unscheduled DNA synthesis in mammalian cells

This method detects the synthesis of new DNA following the excision repair of DNA damage. This is done by virtue of measuring the incorporation of ^{3}H Thymidine into DNA. The amount of repair detected depends on:

i) the types of DNA damage induced

ii) the number of lesions repaired during the period studied, and

iii) the amount of synthesis associated with the repair of each lesi .

Unscheduled DNA synthesis (UDS) has been detected in cultured cells from a number of mammalian species, in various cell types and after exposure to different DNA damaging agents. Human fibroblasts of finite life span have been used but have limitations for routine screening. Cultures of transformed cells of infinite life span are more usually utilised. A disadvantage of these cultures is that they do not possess the ability to activate proximate carcinogens and the addition of S9 is essential when screening agents. Furthermore, because these cell cultures are actively growing, semi-conservative replication would normally interfere with the assay unless it is suppressed. To circumvent both of these drawbacks UDS is more commonly undertaken *in vitro* with primary rat hepatocyte cultures or *in vivo* in rat liver. In the former case freshly perfused hepatocytes are exposed to the agent *in vitro*. These cells are non-dividing and retain the

liver's metabolic capability for activating proximate carcinogens. In the second case the animals are treated with the agent *in vivo* and the hepatocytes examined after sacrifice. UDS may be detected by either measuring the amount of ^{3}H-thymidine incorporated into total culture or liver DNA by scintillation counting or by determining the number of nuclear grains seen in the nuclei of cells using autoradiography. The latter approach has many advantages over the former method and it is this which predominates in mutagenicity screening. Thus this section will restrict itself to considering the measurement of UDS by autoradiography. Precise details of the above approaches are listed in references 15 to 18. The strengths and weaknesses of the *in vivo* and *in vitro* rat hepatocyte assays are given in Appendix 12. The *in vivo* approach may be considered more relevant, however it is also more costly in terms of animals, radioisotope and test agent.

The analysis of slides and what actually constitutes a positive result has been the subject of much debate (see references 15 to 18). It is recommended to determine by counting at least 50 cells per slide:

i) the grains per $100\mu M^{2}$ of nuclear area

ii) the grains per $100\mu M^{2}$ of cytoplasmic area.

Cells with more than 100 grains/nucleus are generally considered to be part of the small number of hepatocytes in "S" phase and are thus not considered. Ideally an automated grain counter should be used if UDS is to be routinely undertaken. This computerised analysis system could be purchased so as to also screen slides for micronuclei (the software for which will shortly be available).

Finally the detection of "S" phase cells is simple. The estimation of the percentage cells in this stage can be used to identify agents which may not induce UDS but which can stimulate cell division in the liver.

The Analysis of Genetic Damage to Germ Cells

Cytogenetic tests with male or female germ cells are not used in general screening procedures. However, investigators may wish to include such an assay in specific circumstances where they suspect solely germ cell effects may be

occurring. Methods exist for investigating chromosome aberrations in spermatogonia, spermatocytes and oocytes. As these are not routine methods and require specialist training which is available in only a small number of laboratories they will not be discussed further. Details can be found in references 13 and 14.

The dominant lethal test can be undertaken to identify agents that damage germ cells (19). However this is an expensive and lengthy assay of limited sensitivity. Very few of the many genotoxic agents could be considered as germ cell specific mutagens. Hence this assay is not recommended for routine screening.

Concluding Remarks

All of the assays described have different strengths and weaknesses. Hence a battery of tests should be employed to screen for potential genotoxins. The precise nature of the battery employed can vary. A typical group would be:

1) Ames Test
2) Mammalian mutation or chromosome aberrations
3) Micronucleus assay *in vitro* and *in vivo*
4) Rat hepatocyte UDS assay *in vitro*

However if an agent (eg. a hormone) is suspected of specifically targeting germ cells then assays for genetic damage in germ cells should be undertaken. Obviously one should refer to the results obtained with the standard histological analysis of tissues from a routine toxicology study when considering what tissues might be targeted.

This report is intended as an introduction to routine assays in genetic toxicology. The references cited, many of which are in reading material left at TISTR (Appendix 13) should be read. With the advent of modern molecular biology it is likely that some assays will be modified and newer, more sensitive/rapid approaches developed in the coming years. It is imperative to keep abreast of such developments and introduce new validated approaches as necessry.

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Appendix 1

Programme for Training Provided at TISTR

Date	Topic
Fri. 8th March	Bacterial Mutation Assays: (Lecture and discussions)
Mon. 11th March	Mammalian Mutation Assays: (lecture and discussions)
Tues. 12th March	Chromosome Aberration Assays: (lecture, discussions, analysis of prepared slides brought from the U.K.)
Wed. 13th March	Preparation of S9 from mammalian liver: laboratory experiment. (Supervision of TISTR personnel continuing analysis of slides from Tues.)
Thurs. 14th March	The mammalian micronucleus assay (lecture, discussions and analysis of prepared slides brought from the U.K.). Mouse micronucleus assay - laboratory experiment.
Fri. 15th March	Continued, supervised analysis of slides from Thurs. by TISTR personnel.
Mon. 18th March	The Ames test: practical undertaking of the assay using strains TA98 and TA100 with 4 nitroquinoline 1-oxide and Benzo(a)pyrene with and without S9 activation.
Tues. 19th March	The unscheduled DNA synthesis assay (lecture, discussion and demonstrations using prepared slides brought from the U.K.)
Wed. 20th March	Scoring of the Ames plates, data analyses and interpretations. Scoring of *in vivo* micronucleus slides.
Thurs. 21st March	Unscheduled DNA synthesis (continued analysis of prepared slides brought from the U.K.).
Fri. 22nd March	Continued, supervised scoring of UDS slides by TISTR personnel.
Mon. 25th March	The Basics of Molecular Biology (lectures).

Tues. 26th March	The uses of molecular biology in genetic toxicology (lectures).
Wed. 27th March	New Developments in Genetic Toxicology-TISTR seminar
Thurs. 28th March	Discussions with TISTR staff concerning all of the approaches covered.
Fri. 29th March	As Thursday 28th.

Days Monday 1st April to Friday 5th April were used to:

a) Compile recommendations to TISTR concerning the purchasing of equipment for genetic toxicology (Appendix 2 and 3).

b) Produce a suggested format for future training of personnel (Appendix 3).

c) Formulate a short draft of the final report to be used in debriefing in Vienna.

Appendix 2

Equipment and Facility Recommendations

Equipment

1. Class II. Pharmaceutical Safety Cabinet:

   This is essential for any institute developing drugs. It affords protection to operaters against unwitting exposure to drugs or "positive" control chemicals in genotoxicity testing. Furthermore it can be used to manipulate mammalian cells used in the assays, many of which are tumour cell lines.

2. CO_2/air mammalian cell culture incubator and CO_2 change over unit:

   An incubator for culturing mammalian cells is an essential component of a genotoxicity testing laboratory developing pharmaceuticals as the majority of the tests use cultured mammalian cells.

3. Balance:

   A sensitive balance should be used for weighing out pharmaceuticals or positive control chemicals which are carcinogens. This balance should, for safety reasons, be used solely for this purpose.

4. Inverted Microscope:

   An inverted microscope is essential for monitoring cultured mammalian cells during growth. Conventional microscopes are not suitable due to the depth of culture vessels.

5. A fluorescence attachment for the pre-existing Nikon microscope:

   Many techniques in modern toxicology/genetic toxicology can use fluorescence to monitor events. The acquisition of this capability will enhance the spectrum of end points measurable in some assays, especially the micronucleus assay. The system can also be used to routinely monitor cultures for mycoplasma contamination.

6. A refrigerated bench top centrifuge:

   This will be used in the cell culture laboratory solely for spinning down cultured cells.

7. A -20 °C freezer to store sera, trypsin and other media components - located in cell culture laboratory.

8. A water purification system e.g. Milli Q (marketed by Millipore):

   This will provide tissue culture grade water routinely (it will also be of use for all routine biochemistry requisites).

9. UV lights on a timer and a positive pressure air system in the cell culture laboratory. This will dramatically reduce the risk of contamination in cultures.

10. Media Filtration Unit:

    Media may be prepared at lower cost if purchased as a powder, hydrated in the laboratory, and filtered to sterilise. This is not an essential item, but if a great deal of cell culturing is undertaken it can substantially reduce the costs.

11. An automated slide analysis system:

    This will enable the analysis of UDS and micronucleus slides to be undertaken rapidly. Although not absolutely essential all genetic toxicology laboratories involved in routine screening possess this item. It substantially speeds up analysis.

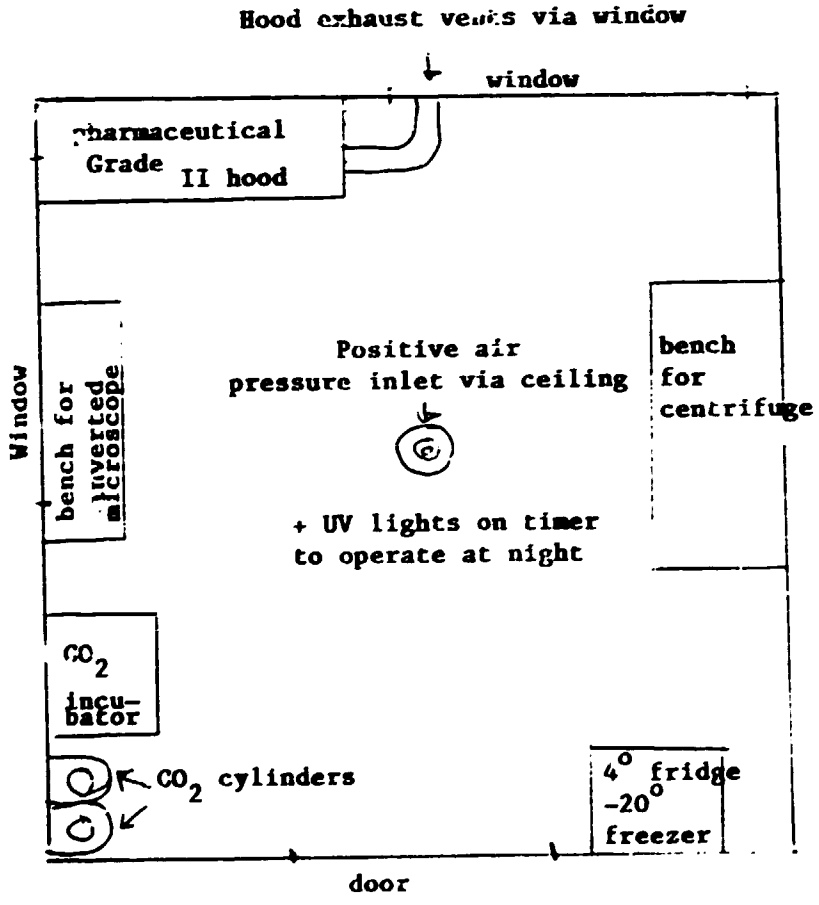
Approximate Cost of Items based on U.K. prices*

1. £5,000
2. £2,000
3. £1,500
4. £1,500 - £2,000
5. £3,000? - unsure as to current costs
6. £2,000
7. £3,000
8. £4,000? - insure as to cost)
9. Cost unknown
10. Cost unknown

11. Cost unknown

Facilities

A laboratory dedicated to mammalian cell culture is essential for the manipulation of mammalian cells. The present media/food store room on the second floor of TISTR is ideal for this purpose. It is large enough to accommodate the class II pharmaceutical grade hood, tissue culture incubator with CO_2 cylinders, bench centrifuge, a bench for the inverted microscope and balance, -20° freezer and a refrigerator. Ideally this room should be under a positive pressure of clean air due to its proximity to the animal facility on the same floor. This will dramatically decrease contamination problems. The room is already equipped with UV lighting. The lights should be attached to an automatic timer which switches on the UV lights for a selected period at night, thus routinely sterilising work surfaces and floors. A suggested plan is shown overleaf.



Appendix 3

Suggested Training Scheme for a TISTR Staff Member

1. Basic mammalian cell culture.
2. Micronucleus assay *in vitro*.
3. Chromosome Aberrations *in vitro*.
4. Mammalian Mutation *in vitro*.
5. Unscheduled DNA synthesis: rat hepatocyte *in vitro*.

Items 1-4 would be undertaken at Swansea during a period of 5 months, whereas item 5 would be undertaken at ICI or Glaxo during month 6. A period of 6 months would therefore be suitable to acquire these methods. The candidate should be trained only when TISTR has the equipment to undertake these assays. This means that he/she could establish the assays at TISTR immediately upon return, without a lag during which some of the skills may be lost.

Minimum Costs to Establish Genetic Toxicology at TISTR

1. Equipment[2]	Cost (£)
Class II pharmaceutical hood	5,000
CO_2/air tissue culture incubator	2,000
Inverted microscope	1,500
[3]Refrigerated bench top centrifuge	2,000
-20° Freezer for cell culture laboratory	400
Balance for weighing toxic chemicals	1,500
Total	£12,400
2. Six month training of a staff member in the UK[4]:	
Living expense (based on UK post graduate grants which are not taxed)	3,000
Return air fare Thailand/UK	800
Travel within UK	500
Cost of training (bench fees)	1,000
Total	£5,300

Grand total = £17,700 or

£15,700[5]

1. Not inclusive of local tax.
2. Based on U.K. costs
3. Possibly available at TISTR
4. Based on costs at Swansea. Has not used an UNIDO per diem that might apply
5. If item in 3 is available

Suggested Staffing of Toxicology and Genetic Toxicology at PNPD

At present the sole permanent staff member in the above disciplines is Mr. Jakkrapong Limpanussorn, a toxicologist. It is totally unrealistic to expect a single person to undertake all of the tests required in above two areas. I therefore recommend that a second staff member be appointed to work with Mr. Jakkrapong. This individual should receive training in genetic toxicology. The two staff would require one technician to assist them. The technician's time would be divided between the two disciplines of toxicology and genetic toxicology.

Appendix 4

Strains of Salmonella typhimurium most commonly used in Bacterial Mutation Assays

Strain	Histidine mutation	Type of mutation	Target	Other mutations	Plasmid
TA97[ac]	his D6610	Frameshift	GC	rfa[1] uvrB[2]	pkM101[3]
TA98[ab]	his D3052	Frameshift	GC	rfa[1] uvrB[2]	pkM101[3]
TA100[ab]	his G46	base pair substitution	GC	rfa[1] uvrB[2]	pkM101[3]
TA102[abc]	his G428	base pair substitution	AT	rfa[1]	pAQ1[4]
TA1535[b]	his G46	base pair substitution	GC	rfa[1] uvrB[2]	No
TA1537[b]	his C3076	Frameshift	GC	rfa[1] uvrB[2]	No

[1]The presence of the rfa mutation increases the cell wall permeability.

[2]The uvrB mutation confers a defect in excision repair, thus making the cells more sensitive to the lethal and mutagenic effects of an agent.

[3] pkM101 is a plasmid carrying the Muc$^+$ gene. This gene's product participates in SOS repair. Its presence increases a cell's resistance to the lethal effects of an agent at the expense of increased mutability.

[4]pAQ1 is a multicopy plasmid which carries the his G428 mutation. Hence its presence substantially increases the targets for reversion of the his$^-$ phenotype.

a) Strains recommended by Levin and Ames (1986) for routine screening (ref. 5).

b) Strains recommended by Gatehouse et al (1990) for routine screening (ref. 3).

c) These strains have been difficult to maintain in a number of laboratories.

All strains should be checked from time to time to confirm their genotype and spontaneous mutation rates (2,3). "Diagnostic" mutagens may also be employed to check them (4,5).

Appendix 5

An outline[1] for preparing a liver microsomal fraction for metabolising proximate carcinogens

1. Take 6-8 week old male inbred rats[2] weighing about 200g.
2. Wearing gloves, inject interpitoneally 500mg/kg body weight of Aroclor 1245.
3. After five days the rats are killed and their livers removed.
4. Rinse the livers with ice cold saline until washings are no longer coloured. Blot the livers dry.
5. Weigh the livers in a preweighed beaker.
6. Use 3 times the weight in volume of ice-cold 0.15M KCl.
7. Chop the livers into small pieces with a scissors.
8. Add two thirds the KCl and homogenise the livers (either mechanically or by hand). Transfer to a conical flask on ice.
9. Use the remaining KCl to rinse the homogeniser and add to 8. Shake gently to achieve a uniform suspension.
10. Spin the homogenate in a prechilled centrifuge (2 °C) at 9,000g for 20 minutes.
11. Remove the supernatant and keep it on ice whilst dispensing aliquots of a chosen volume into ampoules for immediate storage at -70 °C.

[1] Details can be found in reference 2.

[2] The rats should only be handled by qualified personnel.

Appendix 6

Variations of Bacterial Mutation Assays routinely used in Genotoxicity Tests

1. Plate incorporation assays

Ths involves mixing the agent in study with or without S9 in molten agar at 45 ℃ and overlaying plates containing bottom agar. Revertants are counted as distinct colonies.

A liquid preincubation step is more efficient for detecting certain classes of mutagens (2). Volatile materials require forming pour plates and exposing the plates with top and bottom agar to a vapour in a dessicator at 37 ℃ (2).

2. The Fluctuation Test

This test was originally devised to distinguish between adaptation and mutation. It has been modified to form a simple, sensitive mutation assay (2,3). Here the method relies on measuring the growth of mutated cultures in liquid. The assay is performed in microtitre plates, so as many replicates can be undertaken at a given dose. Growth can be measured because it reduces the pH of the medium. The degree of change in pH is estimated by the colour change of an indicator dye in the medium.

The advantages of the fluctuation test over the pour plate assay are:

i) It is more sensitive.

ii) In the pour plate assay the test agent can diffuse into the bottom agar. This can alter the actual concentrations of the agent in the top agar.

iii) Soluble S9 components can diffuse into the bottom agar.

iv) Mutation can be measured in absolute terms with the fluctuation test (mutations/cell/division)

v) The fluctuation test can also detect cell growth caused by nutrients (eg amino acids) which may be in the test material.

vi) Finally the fluctuation test gives an estimation of cell killing.

Appendix 7

Cultures routinely used in Mammalian Mutation Assays

Cell Line	Locus Studied	Selective Agents
Chinese hamster ovary (CHO)	hypoxanthine-guanine phosphoribosyl transferase	6 Thioguanine (6TG) or 8-azaguanine (8AG)
Chinese hamster lung (V79)	hypoxanthine-guanine phosphoribosyl transferase	6 Thioguanine (6TG) or 8-azaguanine (8AG)
Mouse lymphoma (L5178YTK^{+}/TK^{-})	Thymidine kinase (TK)	Trifluorothymidine (TFT) or 5-bromodeoxyuridine (5BrdUrd)

If the cultures exhibit a high spontaneous mutation frequency they can be purged of spontaneous mutants by growing the cells for twenty four hours in medium with 5 x 10^{-5}M hypoxanthine, 4 x 10^{-7}M methotrexate and 5 x 10^{-6}M thymidine. This is replaced with medium containing only hypoxanthine and thymidine. Subsequent cultures are given medium without any of these additions (6,7,8).

Appendix 8

Maintenance of Cultures for Mutagenicity Screening

1. New cultures must always be screened for mycoplasma contamination. Contaminated cultures should be discarded. Twice yearly screening of established cultures is recommended.
2. Stocks of cultures should be kept over liquid nitrogen.
3. The plating efficiency of cultures should be determined prior to use, and should be within the norms for that cell line.
4. The spontaneous mutation rate of the cell line should be measured. If higher than normal cells should be reselected for using selective medium.
5. The cell line should definitely not be routinely maintained in medium selective for normal cells (as is recommended by some authors!). This can give rise to abnormal cells in the culture. The occasional reselection as in 4 is sufficient.

Detail of the above are available in references 6 to 8.

Appendix 9

Comments on Mammalian Mutation Assays

1. The assays are sensitive.
2. They are forward mutation assays and thus detect base pair changes, frameshift mutations plus large or small deletions.
3. Care must be taken to supply sufficient selective agent to eliminate normal cells.
4. The assays involve substantial cell culturing. Hence they are more prone to contamination in the hands of inexperienced operators.

Appendix 10

The Assay for Chromosome Aberrations

Advantages

1. Overall ease of assay.
2. Relatively short time to complete the assay.
3. Ability to use human cells for testing.
4. Sensitivity.
5. Ease of scoring.

Disadvantages

1. Long time required to score aberrations.
2. The need to have an experienced observer.
3. The absence of intrinsic metabolism*.
4. Subjectiveness of scoring.
5. Large number of cells needed to detect weak effects.
6. Difficult to correlate in vitro effects to whole body effects.

* Chinese hamster cell lines expressing cloned P450 genes are now available.

Appendix 11

The Mammalian Micronucleus Assay

Advantages

1. The assay is relatively rapid.
2. Scoring is easy and can be automated.
3. It detects genotoxins that react with DNA.
4. It detects genotoxins that react with the spindle.
5. It is sensitive.

Disadvantages

1. Extrapolation from the *in vitro* assay to the whole body is difficult.
2. The *in vivo* assay only detects genotoxins that reach the bone marrow.

Appendix 12

Rat Hepatocyte Unscheduled DNA Synthesis Assay

Advantages

1. The in vivo assay reflects events in the animal.
2. The assay is relatively sensitive.
3. Scoring can be automated.
4. Both the in vitro and in vivo assays possess intrinsic metabolism.
5. Agents that stimulate cell division but do not damage DNA to induce UDS can be detected.

Disadvantages

1. The in vivo assay only reflects agents that can damage the liver DNA. Tissue specific agents for other organs would not be detected.
2. It is not quantitative for the amount of damage.
3. It does not measure the consequences of damage.
4. Results with the in vitro assay are not easily correlated to in vivo whole body effects.

Appendix 13

Materials donated to TISTR

Books

Mutagenicity Testing: a practical approach. eds. S. Vennitt and J. M. Parry. IRL Press Oxford (1984).

Radioisotopes in Biology: a practical approach. ed. R. J. Slater. IRL Press Oxford (1990).

HPLC of small molecules: a practical approach. ed. C. K. Lim. IRL Press Oxford (1986).

United Kingdom Environmental Mutagen Society Guidelines for Mutagenicity Testing Part 1. pubs. UKEMS (1983) and Part II pub. UKEMS (1984).

OECD Guidelines for Testing of Chemicals (1981).

Reviews

1. E. R. Nestmann (ed). Recommended protocols based on a survey of current practice in genotoxicity testing laboratories. Mutation Res. 246, No. 2 (Special Issue) 227-330. (Reviews on a) Mammalian Mutation, b) Chromosome aberrations, and c) Rat hepatocyte UDS in vitro).

2. A. D. Mitchell and J. C. Mirsalis. Unscheduled DNA synthesis as an Indicator of Genotoxic Exposure. Topics in Chemical Mutagenesis 2, 165-216 (1984).