Mouse Lymphoma Assay

1. Purpose

Mammalian cell culture systems can be used to detect mutations induced by chemical substances. One of the most commonly used mammalian cell mutagenesis system; the L5178Y$^{TK+/-}$ mouse lymphoma-TK assay detects the mutations at the thymidine kinase locus caused by base pair changes, frameshift and small deletions. Mutant cells, deficient in TK due to the forward mutation in the TK locus (from TK^+ to TK^-), are resistant to the cytotoxic effect of pyrimidine analogues such as trifluorothymidine (TFT). The mutagenicity of the test agents is indicated by the increase in the number of mutants after treatment.

2. Background

Thymidine monophosphate (TMP) occupies a unique position in DNA replication. Of the four principle desoxyribonucleotide monophosphates, TMP alone does not undergo significant conversion to other nucleotides. This conservation makes the TMP pool size quite small and constant under normal growth condition, which serves as a regulator for DNA synthesis. If the TME is replaced by other lethal TMP analogues, the cell will be killed. The phosphorylation of these analogues is mediated by the "salvage" enzyme thymidine kinase (TK), which normally phosphorylates thymidine to TMP in most mammalian cells. TK-deficient cells lack this enzyme activity and therefore are resistant to the cytotoxic effect of the lethal analogues. In the mouse lymphoma cell forward mutation assay, the TK-conpetent L5178Y (TK+/+ or TK+/-) cells are treated with the test agents. After certain period of expression, the cells are shifted to a selective medium containing the lethal analogues such as trifluorothymidine (TFT).

Only the mutant cells (TK-/-) can survive under the selection condition, and the mutagenicity of the test compound is evident by the increase in the number of mutants.

3. Test Methods

1. Cells and culture maintenance:

The L5178Y $TK^{+/-}$ mouse lymphoma cells are used throughout the study. The cells used in the mutagenesis assay should have a high cloning efficiency and low spontaneous mutation frequency. The cells are maintained as suspension culture in RPMI 1640 media in culture flasks and incubated at 37°C and 5% CO_2.

The cells have a doubling time as 10-11 hours. Each week the cells will be grown in the RPMI 1640 media containing THMG (thymidine, hypoxanthine, methotrexate and glycine) to select against newly arising $TK^{-/-}$ mutants, and then placed in the RPMI 1640 media containing THG (thymidine, hypoxanthine, and glycine) for 1-3 days prior to use in mutagenesis study.

2. Metabolic activation system:

Cells will be exposed to the test agent both in the presence and absence of an appropriate metabolic activation system. Cofactor- supplemented liver S9 from Aroclor-induced rats will be used in each assay.

3. Test agent:

The test agent will be freshly dissolved with distilled water prior to each use. A preliminary range finding experiment will be conducted using 10 doses over a 3-4 log range with 5000 µg/mL as the top concentration. The procedures for range

finding are identical to that used for mutagenesis except that the cultures are terminated after 24-48 hours without further cloning. The toxicity is indicated by the decrease of cell number in the suspension culture compared with that in untreated control. Four to five concentrations will be selected based on the result and used in the mutagenesis assay. The highest dose should produce a low level of survival (approximately 10-15%), and the survival in the lowest dose should be the same as the negative control.

4. Controls:

Negative control without treatment and positive control with known mutagens should be included in each assay. Methylmethanesulphonate (MMS, without S9 mixture) and Cyclophosmphamide or Benzo(a)pyrene (with S9 mixture) will be used as the positive controls. Both mutagens are dissolved in DMSO, and corresponding solvent control will also be included.

5. Mutagenesis assay:

a. Exposure:

Cells (6 x 10^6 cells in 10 ml medium for each culture) are treated with test agents with and without S9 mixture, and incubated at 37°C for 3-4 hours. Chemicals are removed and cells are washed twice by centrifugation then resuspended in non-selective medium at a density of 3 x 10^5 cells/ml, and maintained in the incubator for 2 days at 37°C.

b. Expression:

The 2 day maintenance after exposure is the expression period for mutation. During this period, cell density is checked daily and adjusted to 3 x 10^5 cells/ml.

c. Cloning:

On the second day of expression, 1.6 cells are seeded on 96well plates to determine the survival and the mutation frequency. For each dose group, two 96well plates containing 1.6 cells/well in non-selective medium are set up for viability measurement, another two 96well plates with 2000 cells/well in selective medium containing TFT (4 µg/ml) are used for mutant counting. Plates are incubated at 37°C in an atmosphere of 5% CO_2 .95% air.

d. Colony counting and sizing:

Colonies are counted and sized 11-14 days after cloning using. The mutant frequency is calculated and adjusted based on the survival percentage.

4. Evaluation/Analysis

The toxicity of the test agent will be indicated by a decrease in colony forming efficiency (CFE: number of colonies/number of cells plated [%]).
The mutagenicity of the test agent will be evident by the increase in mutation frequency based on the number of mutants and adjusted by the survival fraction of cells:

5. Interpretation of results

A test agent will be considered to be positive in the mouse lymphoma cell mutagenesis assay if it induces a statistically significant dose-related increase in the mutant frequency, or generates a reproducible and statistically significant increase in the mutant frequency for at least one concentration.

A positive result in mouse lymphoma cell mutagenesis assay indicates that under the experimental conditions, the test compound induces gene mutation in the cells used.
A high amount of small colonies is an indication of a compound's clastogenicity.
A test agent which does not produce either a statistically significant dose-related increase or a reproducible and statistically significant increase of the mutant frequency in any one of the concentrations tested will be considered nonmutagenic in this system.
A negative result indicates that under the experimental conditions, the test compound does not produce gene mutation in the cells used.

6. References

- Clive D and Spector J: Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Clive D and Spector J: Laboratory procedure for assessing specific locus mutations at the TK locus in cultured L5178Y mouse lymphoma cells; Mutation Research 31: 17-29 (1975)
- Oberly T, Yount D, Garriott M: A comparison of the soft agar and microtitre methodologies for the L5178Y TK+/- mouse lymphoma assay; Mutation Research 388: 59-66 (1997)