

- I. Study Type: Gene Mutation in Mammalian Cells in vitro:
CHO/HGPRT Assay
(Guideline §84-2)

Study Title: CHO/HGPRT Mutation Assay in the Presence and Absence
of Exogenous Metabolic Activation
Test Article
SENCOR Technical (Metribuzin)

EPA Identification Numbers: EPA ID No. 3125-305
EPA Accession No. 262227
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Shaughnessy Code: 101101-4
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Document No.

Sponsor: Mobay Chemical Corporation

Testing Laboratory: Microbiological Associates, Inc.
5221 River Road
Bethesda, Maryland 20816

Study Number: MA Study No. T4485.332
Mobay Toxicology Report No. 733
Mobay Ag Chem Report No. 91760

Study Date: March 26, 1986

Study Author: Li Lillian Yang, Ph.D.

Test Material: SENCOR®, Technical (also known as Metribuzin)
Lot: 77-297-50
Purity = 92.6%

Dosages: Metribuzin was added to cells at levels of 1000, 900, 800, 700 or 600 ug/ml in the non-activated study and at levels of 200, 175, 150, 100 or 50 ug/kg in the presence of a S-9 activation system. Ethyl methanesulfonate (EMS, Aldrich, lot 0422 BM) was used as positive control in the non-activated study at a concentration of 0.2 ul/ml. Benzo(a)pyrene (BaP, Sigma, lot 13F-9006) was used as positive control in the activated study at a concentration of 4 ug/ml. Solvent control was acetone (Fisher, lot 851079) at the "same concentration as test article groups".

Test Cultures: CHO-K₁-BH₄ cells (Dr. Abraham Hsie, Oak Ridge National Laboratories, Oak Ridge, TN).

Metabolic Activator: S-9, 9000 x g supernatant of an Arochlor-1254 induced Fischer 344 rat liver homogenate.

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This study was designed to assess the mutagenic potential of Metribuzin based on its ability to induce forward mutation at the HGPRT locus of CHO cells.

III. Materials and Methods: A copy of the Material and Methods section from the investigators report is appended. The following comments and highlights are noted:

The S-9 metabolic activation system was prepared from livers of adult male Fischer rats weighing 200-250 gm who received a single IP injection of Arochlor-1254 at a dosage of 500 mg/kg body weight. The procedure is described on attached materials and methods. According to the investigators "Each bulk preparation of S-9 is assayed for its ability to metabolize 2-aminoanthracene and 7,12 dimethyl-benz(a)anthracene to forms mutagenic to Salmonella typhimurium TA100".

The investigators performed a range finding study based on colony forming efficiency. They exposed CHO cells to either solvent alone (control) or to 9 concentrations of Metribuzin ranging from 0.1 ug/ml to 1000 ug/ml for 5 hours $37 \pm 1^\circ\text{C}$ in the presence and absence of metabolic activation.

The mutation assay used published methodologies of Mechanoff, R., O'Neill, J.P., and Hsie, A.W. (Quantitative analysis of cytotoxicity and mutagenicity of benzo(a)pyrene in mammalian cells (CHO/HGPRT), Chem. Biol. Interactions 34:1-10, 1981) and O'Neill, J.P., Brimer, P.A., Machanoff, R., Hirsch, G.P., and Hsie, A.W. (A quantitative assay of mutation induction at the hypoxanthine-guanine phosphoribosyl transferase locus in Chinese hamster ovary cells (CHO/HGPRT system): Development and definition of the system, Mutation Research 45:91-101, 1977).

Exponentially growing CHO-K₁-BH₄ cells were plated in specially prepared Ham's F-12 medium (see attached materials and methods). The time the cells were first exposed to Metribuzin was designated as day 0. The plated cells were exposed in duplicate to 4 concentrations of Metribuzin for 5 hours at $37 \pm 1^\circ\text{C}$. This was done for both the metabolically activated and non-activated study. After treatment "the cells were subcultured to assess cytotoxicity and to initiate the phenotypic expression period".

For cytotoxicity evaluation, the replicates from the primary mutation study for each treatment group were pooled and subcultured in triplicate, and after a further 7 to 10 day incubation the cells were then fixed, stained and counted.

For mutant phenotype expression, the replicates from the primary mutation study for each treatment group were pooled and subcultured in duplicate and further subcultured at 2-3 day intervals for the 7-9 day expression period. The cells were then selected for the TG-resistant phenotype by pooling and replating (in quintuplicate) from each treatment group. Also, for cloning efficiency determinations, cells were plated in triplicate. After a 7-10 day incubation period, the colonies were fixed, stained and counted for cloning efficiency and mutant selection.

The cytotoxicity was expressed relative to solvent control, as the relative cloning efficiency.

The mutation frequency (MF) was calculated by dividing the total number of mutant colonies counted by the number of plates selected, corrected for the cloning efficiency of the cells. This cloning efficiency is determined prior to mutant selection. The MF is expressed as TG-resistant mutants per 10^6 number of clonable cells. If no mutant colonies are observed, mutation frequencies will be expressed as less than the frequency obtained with one mutant colony. If doses give $\leq 10\%$ relative survival they were not considered as valid data points for mutation frequencies.

The investigators feel that calculation of mutagenic response based only on a "fold increase" in mutation frequency is not a reliable measure for some loci with very low spontaneous mutation frequencies. They decided that "For assays characterized by a wide degree of variation in the frequency of spontaneous mutants found in the negative or solvent controls, a confidence interval can be calculated by the application of a one-sided Student's t test ($p < .05$) from the historic background mutation frequency" (Gupta, R.S. and Sing, B., 1982, Mutagenic responses of five independent genetic loci in CHO cells to a variety of mutagens: development and characteristics of a mutagen screening system based on selection for multiple drug resistant markers, Mutation Research 94:449-466). They decided that the mutagenic response after treatment will only be considered significant if the treatment mutation frequency is increased above the negative controls by at least 8.7 mutants/ 10^6 clonable cells and at least twice that of the solvent and untreated controls.

According to the investigators, "The assay will be considered positive in the event a dose-dependent increase in mutation frequency is observed with one or more of the five concentrations tested inducing a mutation frequency which is at least twice that of the solvent control, and also is increased above that of the solvent control and the untreated control by at least 8.7 mutants/ 10^6 clonable cells. The study will be considered suspect if there is no dose response but one or more dose induce a mutation frequency which is considered significant. The assay will be considered negative if none of the doses tested induce a mutation frequency which is considered significant."

For a valid test the investigators stated that: "The cloning efficiency of the solvent and untreated controls must be no less than 50%. The spontaneous mutation frequency in the solvent and untreated controls must fall within the range of 0-20 mutants per 10^6 clonable cells. And further: "The positive control must induce a mutation frequency at least three times that of the solvent control."

A Quality Assurance Statement was included in this report relating to inspection dates, phases of the study inspected and report dates of the QA inspections.

IV: Results

The range finding study conducted to determine doses to be used in the CHO/HGPRT assay determined the cloning efficiency after exposure to either the solvent alone or to one of nine concentrations of Metribuzin ranging from 0.1 ug/ml to 1000 ug/ml both in the presence and absence of S-9 activation. The results are reported in attached Table 1. It was noted that Metribuzin without metabolic activation produced relative cloning efficiency comparable to solvent control at all dose levels tested. Metribuzin in the presence of S-9 activation produced relative cloning efficiency of 96, 100, 95, 91, 82, 76, 42, 0 and 1 % for the 0.1, 0.3, 1, 3, 10, 30, 100, 300 and 1000 ug/ml respectively. The dose levels chosen for the primary study were 600, 700, 800, 900 and 1000 ug/ml without metabolic activation and 50, 100, 150, 175 and 200 ug/ml in the presence of S-9 activation.

The cytotoxicity results from the 5 hour treatment of CHO cells (in the presence and absence of S-9 activation) in primary study are reported in Table 2. For the non-activated study the doses of 600, 700, 800, 900 and 1000 ug/ml of Metribuzin produced relative cloning efficiency of 118, 80, 68, 55 and 61% respectively. For the metabolically activated study the doses of 50, 100, 150, 175 and 200 ug/ml produced relative cloning efficiencies of 123, 63, 40, 0.4 and <0.4% respectively. The positive control produced relative cloning efficiencies of 66 and 34 for the nonactivated and metabolically activated studies respectively. Similar effects on survival were seen in the concurrent cytotoxicity studies with the mutation assay (reported in attached Tables 3 and 4).

The CHO/HGPRT mutation assay in the absence of metabolic activation is reported in Table 3. The solvent control produced 8 thioguanine-resistant mutant colonies which calculates to a mutation frequency of 10.13 mutants per 10^6 clonable cells. The doses of Metribuzin of 600, 700, 800, 900 and 1000 ug/ml showed a total number of mutant colonies of 3, 4, 7, 0 and 1 (giving mutation frequencies of 4.55, 4.55, 9.21, <1.28 and 1.30 mutants per 10^6 clonable cells). Therefore, no significant effect was noted. The positive control showed a total of 171 mutants with a mutation frequency of 259.09 mutants per 10^6 clonable cells.

The CHO/HGPRT mutation assay in the presence of S-9 activation is reported in Table 4. The solvent control produced 4 mutant colonies which gives a mutation frequency of 4.60 mutants per 10^6 clonable cells. The high dose (200 ug/ml) was too toxic to carry out "mutant expression and mutant selection". The doses of Metribuzin of 50, 100, 150 and 175 ug/ml showed a total number of thioguanine-resistant mutant colonies of 9, 1, 6 and 4 (giving mutation frequencies of 9.28, 1.09, 6.32, and 4.82 mutants per 10^6 clonable cells). None of the dosage levels tested showed levels significantly above that of the solvent control. The positive control showed a total of 343 mutants with a mutation frequency of 581.36 mutants per 10^6 clonable cells.

V: Conclusions

Under conditions of this study, Metribuzin is negative in the CHO/HGPRT mutation assay.

Core Classification: Acceptable.