UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

JAN 15 1982

MEMORANDUM

OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

DATE:

SUBJECT: ORYZALIN, EPA Reg. No. 1471-100, 6(a)(2) data Submission - Mutagenicity Studies. CASWELL #623A

FROM: R. Bruce Jaeger, Section Head
Review Section #1
Toxicology Branch/HED (TS-769)

TO: Mr. Robert Taylor, PM#25
Registration Division (TS-767)

Subject mutagenicity studies, submitted as 6(a)(2) data, have been reviewed by Dr. I. Mauer, report 1/5/82 attached.

Dr. Mauer's findings of subject reports are self-explanatory. His review of the Rat Dominant Lethal assay, previously reviewed by TB, support our conclusion that the study protocol is inadequate to generate meaningful data and therefore, unacceptable.

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DATE:

SUBJECT: ORYZALIN EL-119, Lilly Compound 67019: Review and Evaluation of Genetic Toxicology Studies for Registration
CASWELL 623A

FROM: Irving Mauer, Ph.D.
Review Section #3
Toxicology Branch/HED (TS-769)

TO: Robert Jaeger, Section Head
Review Section #1
Toxicology Branch/HED (TS-769)

Oryzalin [3,5-dinitro-N^4, N^4 di (n-propyl) sulfanilamide] was tested for potential genotoxicity in a battery of in vitro and in vivo assays. Evaluation of these studies results in the following summary assessments (for detailed DER's see below):

Study I. The Effect of Lilly Compound 67019, Oryzalin, upon Bacterial Systems Known to Detect Mutagenic Events (Lilly Research Laboratories, December 4, 1979).

The reporting of this study is judged inadequate to support the negative conclusion ascribed to the assay.

Study II. The Effect of Oryzalin (Lilly Compound 67019) on the Induction of DNA Repair Synthesis in Primary Cultures of Adult Rat Hepatocytes. Study 810217-337-UDS. (Lilly Research Laboratories, June 17, 1981).

The study is judged adequate, and the conclusion (negative response for DNA repair) acceptable.

Study III. A Dominant Lethal Study with Compound 67019 (Oryzalin) in the Rat. Study R-149. (Lilly Research Laboratories, November 26, 1979).

The study conditions are inadequate to generate valid data, and the result (no dominant lethals induced by treatment) is unacceptable.

Study IV. The Effect of Oryzalin (Compound 67019) on the in vivo Induction of Sister Chromatid Exchange in Bone Marrow of Chinese Hamster. Studies 810601 SCE, 810707 SCE, 810720 SCE. (Lilly Research Laboratories, December 14, 1981).

The study is adequate, and the results (positive response by the intraperitoneal route, negative orally) acceptable.

DER: Study I

Procedure:

Agar plates containing concentration gradients of the test compound dissolved in DMSO were prepared, approximating four dose ranges: 100 - 1000 mcg/ml, 10-100 mcg/ml, 1-10 mcg/ml and 0.1-1.0 mcg/ml. Eight tester strains of Salmonella typhimurium LT-2 (the histidine auxotrophs G46, TA 1535, TA 100, C3076, TA 1537, C3052, TA 1538, and TA 98) and two tryptophane auxotrophic strains of Escherichia coli (WP 2, and WP 2 uvrA-) were streaked across these plates which were incubated at 37°C for 48 hours. If the compound were mutagenic, the frequency of colony appearances (revertents to prototrophy) would increase along the increasing gradient to a concentration at which maximal mutation occurs or, conversely, would decrease along the decreasing gradient to a concentration below which only background lawn is observable. Thus the concentration range over which the compound is mutagenic under the test conditions is reported. The minimum inhibitory concentration (MIC) at which cytotoxicity was observed was also recorded. Gradient plate assays were performed both in the absence as well as the presence of a mammalian metabolic activation system, consisting of liver microsomes from Arochlor 1254-treated male adult Fischer rats, prepared according to standardized procedures (1 ml of 9,000 x g supernate from liver homogenate equivalent to about 250 mg wet liver), combined with the generating co-factors, TPN^+, G-6-PO_4, $MgCl_2$, KCl, and Na_2 PO_4 buffer. In addition to plates containing test compound, two sets of positive controls were prepared, one with streptozoticin, a mutagen active without metabolic activation, the other with 2-acetoaminofluorene, which requires liver microsomes for mutagenic activity in these bacterial strains.

Results and Conclusion:

It is stated that reversion to prototrophy did not occur in either non-activated or activated plates, and thus the test compound was not mutagenic at the levels tested, 0.1 through 1000 mcg/ml. The two positive controls, on the other hand, apparently gave the expected positive results.

Evaluation:

The gradient method employed for these studies is an expeditious attempt to rapidly screen a test substance with a brod range of concentrations. This design procedure, however, qualifies only as a modified spot test, i.e., a qualitative assay, lacking the quantitation attainable in plate incorporation assays employing single concentrations. In addition, there are a number of reporting deficiencies which cast doubt that an adequate assay has been performed:

1. No raw data representing exact counts of revertent colonies induced are presented, nor any assurance that the assay was conducted with strains exhibiting acceptable spontaneous mutation rates.

2. Only a single data summary sheet is included in the report.

3. Apparently only one plate per treatment was employed.

4. There is no cytotoxicity screen. Although MIC's are reported, these are not related to concentration levels at which auxotrophs were inhibited .

5. It is not certain an effective range of concentrations was tested (customarily, from 10% to 90% survival), nor whether the 1000 mcg/ml is the maximal concentration which could be tested.

6. It is not stated whether among the "negative control" run was a solvent control (DMSO).

7. Purity of the test compound is not stated. Herbicides of this type are known to contain nitrosamines, either as a carry-over from their manufacture, or as breakdown products of storage.

Oryzalin reported to contain [redacted] has been reported to give positive results in a dose dependent fashion in the Ames (Salmonella) test at concentrations as low as 100 mcg/plate, both with and without metabolic activation, provided by a mouse liver microsome preparation (CBIB Virology Unit Report: "Microbial Bioassay of (the) Herbicide Oryzalin", EPA, September 30, 1979).

DER: Study II

Procedure:

Freshly prepared hepatocytes (HPC's) form a 175 g adult male Fischer 344 rat were exposed in vitro at 37°C for 20 hours in culture medium containing 10 uCi/ml tritiated thymidine (^{3}H-TdR), and concentrations of 0.5, 1.0, 5, 10, 50, 100, 500, or 1000 nM/ml oryzalin (97%, by HPLC/colorimetry).

After staining, microscope slide preparations were treated for detection of unscheduled DNA synthesis (UDS) by standardized autoradiographic (AR) procedures (i.e., dipping in NTB-2 liquid photographic emulsion, refrigeration in light-tight dessicator containers at 4°C for 7 days, developing in D-19, and fixing.

Developed silver grains were counted by oil immersion microscopy over 20 morphologically unaltered cell nuclei per treatment, using a semi-automated colony counter. Net nuclear grain count was determined after substracting mean cytoplasmic grain counts. A positive response for UDS is judged when two successive compound concentrations produce nuclear grain counts exceeding those of the control by three standard deviations of the control value.

One negative and two positive controls were run with each of the eight concentrations of the test compound: The solvent, DMSO (at 0.0005% through 1%); the direct-acting mutagen/carcinogen, N-methyl-N^1-nitro-N-nitrosoguanidine (MNNG), and the pro-carcinogen, 2-acetylaminofluorene (2-AAF) requiring metabolic activation (both the positive controls at equivalent concentrations, 0.5 through 1000 n M/ml).

Results:

Cytotoxicity incompatible with evaluating UDS was reported at 500 and 1000 n M/ml oryzalin, as well as at the same concentrations of MNNG and 2-AAF. At all doses below these cytotoxic levels, grain counts for oryzalin treatment were no different from those of the solvent control, whereas positive, concentration-dependent responses were recorded for both positive controls. "Some cytotoxicity" was also recorded in HPC's treated with 10, 50 and 100 n M/ml 2-AAF, coinciden with these positive responses; silver grain density, however, was reported to be too excessive for automated counting.

Conclusions:

Compared to positive responses of MNNG and 2-AAF in inducing UDS repair in cultured rat HPC's as determined by grain counts in AR preparations, oryzalin was inactive (negative response) at exposures up to cytotoxic concentrations.

Evaluation:

Under the conditions reported, this assay appears to have been conducted in a manner adequate to generate valid results, and the negative response, as judged by the single summary table of AR grain counts, is acceptable.

Caution, however, is advised in an overly hasty acceptance of the negative results in such assays employing rat hepatocytes. Firstly, from a survey of over 200 chemicals published recently by Probst and colleagues of the Lilly Research Labs. (presented as REF. 3 of the report here reviewed), it is apparent that the HPC-DNA repair test is not responsive to chemicals with nitro-aromatic groups, represented by oryzalin, inter alia. Since this class of chemicals produce bacterial mutation in the absence of a mammalian metabolic activation system, and since bacteria contain endogenous aerobic nitroreductase activity, the lack of a UDS response in hepatocytes may be attributed to the apparent inhibition of hepatic nitroreductase activity under aerobic conditions.

Further, identification of DNA repair events (and thereby AR counts) in HPC's depends upon incorporation of tritiated thymidine in quantities sufficient to be detectable by AR, a relatively insensitive method for quantifying beta-radiation. Therefore, low levels of DNA repair may not be detected by AR. Although liquid scintillation counting has been suggested as a more sensitive method for measuring UDS (repair), AR counting has proved to be a more expedient method of choice for most investigators.

The following specific omissions in the reporting of this study were noted:

1. No indication of the levels of cytotoxicity (% survival at each concentration) were given. The footnoted statement in Table I: "c Cytotoxic, cells unevaluable for UDS" for the oryzalin 500 & 1000 n M concentrations is insufficiently quantitative, especially when contrasted with (same location)": b Cytotoxic surviving cells positive for UDS".

2. Since no statements are made to the contrary, apparently only one test was performed, and only one slide per treatment sampled. Documentation is lacking for selection of only 20 cells for grain counting.

3. It is not stated whether, and/or by what means, selective S-phase (scheduled DNA synthesis) was prevented from confounding grain counts of the repair assay.

4. Although mentioned in the protocol sheet (Appendix B) there is no statistical analysis of the data (ANOVA, and comparison of means by Dunnett's method).

DER: Study III

Procedures:

Ten Adult male Wistar-derived rats (428 + 6.7g) were given a single oral (gavage) dose of 5.0 g/kg oryzalin (99% purity, with [redacted] in a 10% (w/v) aqueous acacia solution, (dose volume, 20 ml/kg), then mated weekly to different untreated Wistar females (190.7 + 0.9 g) for 8 consecutive weeks. Selection of the 5 g/kg dose was considered maximal, since the volume necessary to achieve a higher level would be excessive. Previously it had been determined that 10 g/kg of this preparation is an LD0 in rats. An equivalent volume of the solvent was given to 10 control males. (A positive control was not included.)

Females with copulatory plugs were killed on day 18, 19 or 20 of gestation; those without such physical evidence of mating were killed 2 weeks after separation from males. Ovaries were examined for number of corpora lutea, and uteri for the number and location of fetuses and resorptions. Viability of fetuses was recorded, and resorptions classified as early or late to indicate relative time of mortality.

For each set of 10 females, indices for fetal viability, resorptions and implantations were recorded. Statistical analyses (one-way ANOVA) on pooled bi-weekly data were performed after arcsin square root transformation. The error rate (probability) was set at 0.05. Tabulated individual data, mean values and reproduction indices are presented. The male is used as the experimental unit.

Results:

There were no deaths, and no signs of toxicity reported in treated males. Fertility in treated and control groups was sufficient for a meaningful evaluation of the dominant lethal effect. There were no reported effects of treatment on litter size, resorption or implantation rate, or in reproduction indices during the 8 weeks. The total of 1583 live fetuses all appeared grossly normal. No statistical differences in implantation occurrence or resorption incidence in any treatment week were found, indicating no effect of treatment on pre-implantation losses or post-implantation deaths.

Conclusions:

Oryzalin did not induce dominant lethals when administered to male rats in a single oral dose of 5.0 g/kg prior to mating.

Evaluation:

This appears to be an inadequate study, not conducted according to accepted procedures for dominant lethal assays, and hence the results and their interpretation are considered invalid for the protocol conditions as stated.

1. Among the crucial deficiencies causing this study to be unacceptable is the selection of only one large oral dose (5 g/kg) for the test. The dominant lethal is a relatively insensitive test, in that negative results have followed the administration of single high doses of such potent mutagens as N-methyl-N^1-nitro-N-nitrosoguanidine (MNNG), nitrogen mustard (HN_2), and dimethylnitrosamine (DMN). There is also some question whether any, or only an inconsequential portion, of the dose administered was absorbed. The report states that males treated with the test compound had orange-colored feces the day following dosing (oryzalin has an orange color). No quantitative recovery data are presented for this excretion. Other dosage schedules have been found preferable for optimal absorption

of test substances, and hence assure a more adequate test for dominant lethality. These include multiple doses over a 5 to 7 day period, or chronic dosage over a period covering the spermatogenic cycle of the rat (usually 10 weeks).

Other inadequacies in performing this study are:

2. Lack of a positive control (such as TEM), the inclusion of which is desirable to verify the responsiveness of the experimental organism.

3. The three-day delay between dosing and mating.

4. Mating for 8 instead of 10 weeks, unless it can be shown that the spermatogenic cycle in the strain of rat used is completed in the lesser period of time.

5. The mating of males to one female per week, rather than the customary 1:2 weekly cycle.

DER: Study IV

Procedure:

Inbred female Chinese hamsters (28-36 g) were implanted subcutaneously with bromodeoxyuridine tablets (63-65 mg BUdR), and 5 hours later, either injected intraperitoneally or dosed orally with single doses of 500, 400, 300 or 200 mg/kg oryzalin (96%, solubilized in DMSO and diluted in either corn oil for i.p. use, or 10% acacia for oral dosing, to give a final DMSO concentration of 1.9%). Cyclophosphamide 25 mg/kg (dissolved in 0.9% saline for parenteral use) or 50 mg/kg (in 10% acacia for oral administration), served as concurrent positive control, while the solvent-diluent for each route of administration (corn oil-DMSO, i.p.; 10% acacia, p.o.) was used in each phase of the study. Each animal received a 10 ml/kg volume of material.

Nineteen hours after treatment each animal was given a single 1 mg/kg i.p. injection of the stathmokinetic agent, Velban, and sacrificed two hours later. Bone marrow cells were prepared on microscope slides by standard procedures for metaphase analysis, kept from light for 24 hours, then stained by a modification of the FPG (fluorescence-plus-Giemsa) technique, consisting of "pre-staining" in the water soluble dye, Hoechst 33258 (1 mg/ml), exposure to u v light (wavelength, 266 nm) for one hour (3 cm), followed by incubation in 10x SSC (sodium chloride-sodium citrate) for 20 minutes at 60°C, and staining in 3% aqueous Giemsa.

Twenty-five metaphase cells demonstrating the typical second-division sister chromatid differentiation were selected for each animal, and scored for sister chromatid exchanges (SCE). A positive response was recorded when dose-related increases in SCE frequency were found for at least two doses statistically different from controls according to Dunnett's t-test.

Two studies were performed by the intraperitoneal route (Studies 810601 SCE, 810707 SCE), and one oral test (Study 810720 SCE). In each study, 3 animals per group received oryzalin, 2/group the respective solvent-diluent, and one per group served as positive control. Data for each study were tabulated for individual animals as well as summarized.

Results:

Cytotoxicity was reported from the 500 and 400 mg/kg oryzalin treatments for both routes of administration. This effect was recorded qualitatively as an "index," graded according to the following scheme:

0 = no toxicity

1 = number of second-division cells reduced as compared to controls (with a corresponding accumulation of first-division cells).

2 = moderate inhibition of mitotic index, second division cells much reduced as compared to controls (with accumulation of first division cells)

3 = severe depression of mitotic index, second division cells unscorable.

4 = complete inhibition of mitotic index - cells unevaluable.

Cyclophosphamide produced slight (grade-1) cytotoxicity in one of the two i.p. tests (one animal), but not orally. No cytotoxicity was noted among animals in either the 300 or 200 mg/kg oryzalin groups.

The positive control (cyclophosphamide) induced significantly increased SCE's in both types of study (7 times solvent control values). Significant dose-related positive responses (1.5-2.0x) were also recorded in all i.p. oryzalin dosage groups, but not in cells from animals treated orally. It was suggested in the report that this differential response reflects contrasting metabolic pathways for oryzalin, the i.p. route favoring the formation of a genotoxic metabolite, whereas the oral route involves detoxification primarily, or a formation of non-genotoxic metabolites.

Conclusion:

Oryzalin induced increased SCE's when administered intraperitoneally but a negative response orally.

Evaluation:

The studies appear to have been conducted with appropriate control measures to assure the generation of valid data. Therefore both the protocol and results (positive ip, negative po) are acceptable.

Two minor reporting deficiencies are noted:

1. Only the source, but not the strain of the animals is stated.

2. Cytotoxicity was not quantitated, merely characterized qualitatively as "depression in mitotic index", "accumulation of first division mitotic cells", and "reduction of second division mitotic cells".

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