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OFFICE OF
PESTICIDES AND TOXIC SUBSTANCES

7/13/82

MEMORANDUM

DATE:

SUBJECT: Caswell No.: 849A. Mutagenicity Studies With
Thiabendazole, Active Ingredient of "Mertect 340-F":
Review and Evaluation.

EPA Reg. No. 618-75 Action Code 570

Accession No. 247112

TO: Mr. John H. Lee, PM
Registration Division (TS 797-C)

FROM: Irving Maues, Ph.D., Geneticist
Toxicology Branch
Hazard Evaluation Division (TS-769)

Action Requested: Review and assessment of mutagenicity studies with thiabendazole (TBZ), chemically: 2-(4-thiazolyl) benzimidazole), a.i. of Mertect 340-F (Merck, Sharp and Dohme), part of a report dated June 3, 1977 and submitted under Accession No. 247112.

Studies Reviewed: The studies were performed with TBZ lot number F291764, reported to be in excess of 98.6% pure, and included the following types of tests conducted by the laboratories indicated (see Table I, page following, for summary):

- (i) Gene mutation in bacterial strains of Salmonella typhimurium (Ames) and/or Escherichia coli --- at the Institute of Environmental Toxicology (IET), Tokyo, Japan (Dr. Yasuhiko Shirasu); U.S. Department of Agriculture (USDA), Western Regional Research Center, Albany, CA (Dr. James Mac Gregor); and the Merck Institute for Therapeutic Research (MITR), West Point, PA.

- (ii) Mammalian cytogenetics (chromosomal damage) in mammalian cell cultures, and in rats and mice --- at IET and MITR.
- (iii) Primary DNA damage/repair in Bacillus subtilis --- at IET.

Evaluation of Studies: Table I is a summary of reported results and TB evaluations, following which detailed assessments of the individual studies are presented. Studies have been judged either acceptable (A) or not acceptable (NA). On the basis of this evaluation, TBZ is probably not mutagenic in microbial cells, but the reported negative results in animal cytogenetic studies are considered inconclusive because of deficiencies in study protocols. [A detailed report of a second in vitro cytogenetic study (WI-38) indicated in summary statements of the submission as having been performed at IET, was not included in Acct. #247112.]

Recommendations: TB recommends:

- (i) Provide TB with the missing in vitro mammalian cytogenetics study in WI-38 cells, said to have been performed by IET under MSD Report # 76-9815C, for review.
- (ii) Repeat the inadequate animal cytogenetics studies, or provide information capable of resolving the deficiencies found, which led to the assessments indicated in Table I and detailed reviews (such as LD50's, metabolic data, etc.)
- (iii) As an alternative to (ii), discuss with TB other short-term animal tests which would satisfy the same issues (such as tests for sister-chromatid exchanges, unscheduled DNA synthesis).

TABLE I: Summary of Mutagenicity Studies with Thiabendazole (TBZ), Reported June 3, 1977, Submitted under Accession No. 247112.

Study Type	Test System	MSD Report No. (6/3/77)	Study Lab*	Reported Result	TB Evaluation**
<u>In vitro</u> microbial gene mutation:	<u>S. typhimurium</u> (Ames)	[None]	USDA	NEG	NA
	--(Ames)--	[None]	MITR	POS/NEG***	A
	--(Ames)--	76-9813C	IET	NEG	A
	<u>E. coli</u> WP-2	[None]	IET	NEG	A
Host-mediated assay:	Mice/ <u>S. typhimurium</u> G46	76-9814C	IET	NEG	A
<u>In vitro</u> mammalian cytogenetics:	Human embryonic fibroblasts (#1162;46,XY)	76-9815C	IET	NEG	A
	WI-38 Cells	76-9815C	IET	NEG	[****]
<u>In vivo</u> mammalian cytogenetics:	Rat bone marrow	76-9816C	IET	NEG	NA
	Mouse dominant lethal (C3H/HeCR)	76-9817C	IET	NEG	NA
	---[Same]---(CFIS)	76-7030	MITR	NEG (500 mpk)	NA
	Mouse micronu- cleus test (CD-1)	76-8038	MITR	NEG (500 mpk)	NA
Primary DNA damage/repair	Rec-assay (<u>B.</u> <u>subtilis</u> HL7/M45)	76-9813C	IET	NEG	A

* Study Lab: USDA, United States Department of Agriculture, Western Regional Research Center, Albany, CA (Dr. James Mac Gregor).

MITR, Merck Institute for Therapeutic Research, West Point, PA

IET, Institute of Environmental Toxicology, Tokyo, Japan
(Dr. Yasuhiro Shirasu).

3

** TB Evaluation: A, acceptable; NA, not acceptable.

*** Positive result in M188 only; shown to be due to lot impurity.

II. Review of Individual Studies Constituting MSD Report:
"Thiabendazole Mutagenic Evaluation, June 3, 1977."
(Accession No. 247112).

- A. "Mutagenicity Testing on Thiabendazole (TBZ) in Microbial Systems." Y. Shirasu, M. Moriya and K. Kato (Institute of Environmental Toxicology, Tokyo, Japan): (1) Rec-assay; (2) reverse mutation tests in E. coli WP2 and S. typhimurium TA strains (Ames procedure); (3) host-mediated assay with S. typhimurium G46 in mice.

Test Substance: Thiabendazole (TBZ), 2-(4-thiazolyl) benzimidazole, 98.86%, dissolved in DMSO.

Procedures:

1. Rec Assay: To test primary DNA damaging potential of TBZ, cultures of the recombination-proficient strain H17 and the recombination-deficient H45 were streaked side-by-side (but not touching) onto B-II agar plates, and the streak origins covered with TBZ-soaked filter paper discs. Lengths of inhibition zones were measured after overnight incubation (37°C). Kanamycin and Mitomycin-C (MC) served as negative and positive controls, respectively.
2. Reverse Mutation Tests: To examine TBZ's potential for direct mutagenicity in bacteria (by reversion to prototrophy), cultures of five histidine-requiring (his-)strains of S. typhimurium (the Ames's strains TA 1535, 1537, 1538, 98, and 100) and E. coli WP2 hcr- were plated onto minimal agar with test compound (0.1 ml in DMSO) and the number of revertent colonies compared to DMSO controls after 2 days' incubation. (Preliminary toxicity tests were performed to determine testing range of TBZ concentrations.) The following mutagens served as positive controls: 2-AF [2-(2-furyl)3-(5-nitro-2-furyl) acrylamide] for the E. coli strain, and for TA 98 and TA 100; beta-propiolactone for TA 1535; 9AAc [9-aminoacridine] for TA 1537; and 2-NF [2-nitrofluorene] for TA 1538. All solutions and bacterial suspensions were tested for contamination.

A complete set of tests (as above) was also run with a mammalian metabolic activation (MA) system, consisting of the 900' x g supernatant

of liver (microsomal) homogenate (S-9) from an Aroclor 1254-induced adult male S-D rat, combined with appropriate regenerating (NADPH-phosphate-salt) co-factors, added (as 0.5 ml complete S-9 mix) to each treated minimal plate. 2-AAanth [2-aminoanthracene] was used as a positive control substance requiring metabolic activation for mutagenic activity in all strains.

3. Host-mediated Assay (HMA): Following preliminary toxicity tests, seven male ICR mice per group were dosed orally (by gastric intubation) with either 300 or 1000 mg/kg TBZ (in 5% gum arabic) daily for 5 days (total dosage, 1500 and 5000 mg/kg, respectively).

Six mice were given equal volumes of gum arabic (negative control), and a fourth group of 6 animals received a single oral dose of dimethylnitrosamine (DMN, 50 mg/kg). Immediately after the last dose, each mouse was inoculated with a 2 ml suspension (8.4×10^8 cells/ml) of S. typhimurium G46 (his-) i.p. All animals were killed three hours after treatment, peritoneal fluid removed, and both bacterial survival and histidine revertents determined after incubating minimal plates (in triplicate) for 2 days.

A comparison reverse mutagenicity test using the same bacterial strain (G46) was also conducted. in vitro.

Results and Conclusions:

1. Rec-assay: As indicated in tabulated results, no inhibition zones at all were observed in TBZ-treated plates (8 levels, from 2-1000 μ g) of either the H17 or the M45 strain, whereas 0.1 μ g MC induced marked differential inhibition (9 mm), and 10 μ g kanamycin caused equal zones of inhibition (i.e., no differential toxicity).

Thus TBZ was considered negative for differential toxicity, a measure of bacterial primary DNA damage/repair.

2. Reverse Mutation Tests: Compared to increases in number of revertent colonies caused by positive control direct mutagens appropriate

to each strain (= 10 to 1000 X control); concentrations of 100, 500, 1000, and 2500 ug TBZ/plate induced no more revertants than found in non-activated control (DMSO) plates.

In tests with MA, TBZ at concentrations of 10, 100, and 1000 ug was also without effect on colony counts, compared to the marked mutagenicity shown by 2AA (30-220X negative control).

It was concluded that TBZ was not mutagenic in the Ames Assay and E. coli reverse mutation tests with/without MA.

3. Host-mediated Assay (HMA): Mean number of revertants in G46 bacteria withdrawn from orally treated mice (5 daily doses of 300 or 1000 mg/kg TBZ) was not significantly different from the gum arabic controls (mean revertants = 0.41 and 0.37, vs. 0.45), whereas the DMN-treated group showed a significant mean increase (250 X negative control). The direct in vitro exposure of G46 cultures to concentrations of 100, 500, 1000, and 2000 ug TBZ/pl was also negative, compared to the positive response (15X control) of this strain to the mutagen beta-propiolactone.

EVALUATION: All the above microbial mutagenicity tests conducted with TBZ are considered well controlled, and the procedures are considered to have generated valid results, i.e., negative for gene mutation tests in S. typhimurium and E. coli and for primary DNA damage in B. subtilis. Hence these studies are acceptable.

- B. 1. Cytogenetic Studies with Thiabendazole in Cultured Human Fibroblasts. Y. Shirasu, H. Tezuka, R. Hemmi, and N. Murakami (Institute of Environmental Toxicology, Tokyo, Japan).

Test Material: Thiabendazole (TBZ), 2-(4-thiazolyl) benzimidazole, 98.6%+, dissolved in and diluted with DMSO (0.5% final concentration).

Procedures: Following a preliminary toxicity study, exponentially growing sub-cultures (8th passage) of diploid human fibroblasts (# 1162; 46, X/Y) from an abortus were exposed to TBZ at 2, 10, 25, or 50 ug/ml for either 3 hours or 24 hours. Crystallization of TBZ was observed microscopically at the highest exposure (50 ug).

Cultures treated with 0.1 ug mitomycin-C (MC) for 24 hours served as positive controls. Cells blocked in the metaphase stage of mitosis were accumulated by the addition of 0.2 mg/ml colchicine 3 hours before harvest of cultures.

After standard slide preparation, 200 metaphases per culture were scored for chromosomal aberrations according to standardized criteria. The assay was replicated once. Statistical analysis of the results of these observations was by chi-square.

Results and Conclusions: No statistically significant increase in frequencies of breaks, gaps, exchanges, or acentric fragments over control values (2.0% and 1.5%) were found at any concentration of TBZ (0.5% to 4% for all concentrations in both assays). In contrast, the positive control MC produced 13% cells with aberrations. It was concluded that TBZ does not have potential to induce chromosome aberrations in cultured human embryo fibroblasts under these experimental conditions.

EVALUATION: Protocol, controls and procedures fulfill all the criteria for an adequate assay, and the results of this study are acceptable.

2. Cytogenetic Studies With TBZ in Rat Bone Marrow Cells. Y. Shirasu, H. Tezuka, R. Hemmi, and N. Murakami (Tox. Div., Institute of Environmental Toxicology, Tokyo, Japan).

Test Material: (Same as B.1.).

Procedures: Groups of 5 juvenile (8-week) male Wistar rats were given TBZ (suspended in 0.2% CMC-water) by oral intubation at doses of 100, 300, or 1000 mg/kg as a single dose, or 30, 100, or 300 mg/kg daily for 5 days. Dose selection was based upon preliminary toxicity studies indicating an acute oral LD₅₀ of 3,330 mg/kg. Mitomycin-C (MC, 2.5 mg/kg, i.p., once) served as the positive control (4 rats). All animals received 2 mg/kg colchicine i.p. to arrest mitosis of bone marrow cells, which were processed for cytogenetic analysis by standardized methods. Fifty metaphases were scored per animal (50+ in the MC group), and statistical significance assessed by Fisher's Exact Test.

Results and Conclusion: No differences in incidence of chromosome aberrations (CA) were found between control slides and those from any TBZ dose level (for either acute or 5-day treatment), whereas MC-treated animals showed 35% of cells with CA (breaks for the most part, % tid/100 = 62.7%).

It was concluded that TBZ manifested no clastogenic (chromosome-breaking) potential in this test.

EVALUATION: No clinical effects were reported in treated mice, and one wonders whether any were observed. There were also no measures of any effects on mitosis or cell division (such as mitotic delay, mitotic indices, etc.). Therefore, it is doubtful whether a sufficient dosage of TBZ was administered. The results, consequently, are inconclusive, and the study is judged not acceptable.

3. "Dominant Lethal Studies with Thiabendazole in Mice." Y. Shirasu, S. Teramoto, and M. Kaneda (Tox. Div., IET).

Test Substance: [As above in B.1., but as a water suspension in 5% gum arabic.]

Procedures: (Four) Groups of 10 week-old male C3H/He/Cr mice were dosed daily by oral intubation with 200 or 600 mg/kg TBZ or the gum arabic suspension (solvent negative control) for 5 days (15 males/group), or given a single i.p. injection of 300 mg/kg EMS (positive control group of 10 males). Preliminary acute toxicity testing had established the oral LD₅₀ for TBZ at 3100 mg/kg for this strain of mice. Each male was then mated for one week with 2 untreated virgin mice, and re-mated weekly for 6 additional mating periods with pairs of fresh females. Females were sacrificed 12-13 days after copulation, and uterine contents examined. Group mean numbers of corpora lutea (cl), implants, live embryos, early and late embryonic deaths (ED) were calculated for each week's mating. Induced dominant lethal (DL) percentages were then calculated according to Rohrborn, as:

$$1 - \frac{\text{Number live embryo/pregnancy (test)}}{\text{Number live embryo/pregnancy (control)}} \times 100$$

Estimates of pre- and post-implantation losses were based upon total numbers of cl's and implants, and of implants and live embryos, respectively.

Results and Conclusions: No signs of toxicity were observed in TBZ-treated males, and no gross pathological lesions were apparent at autopsy. Percent pregnancies in TBZ-treated groups were said to be comparable to the gum arabic control for all the mating weeks, although the tabulation in the report indicates a significant decrease ($p < 0.05$) in fifth-week matings at 200 mg/kg (to 77%). A significant difference in pregnancy rate was noted for EMS-treatment (positive control group) in both week-2 matings (to 40%), and week-4 (to 80%). No significant decreases were reported in TBZ-treated groups for measurement of cl, implants, or live embryos throughout the 7 mating weeks; an increase in number of live embryos at week-4 of TBZ-treated groups reflected higher number of cl. By contrast, EMS treatment induced significant decreases in mean number of live embryos at both weeks 1 and 2, and of implants at week 2, resulting in DL rates of 20% and 62.2%, respectively.

Finally, no increase in embryonic death occurred at any stage of development after mating with TBZ-treated males, whereas EMS induced significant pre-implantation losses at week-2 (41.8%, vs 12.5% for control), and post-implant losses at weeks 1 and 2 (26.8% and 58.5%).

It was concluded that TBZ does not induce dominant lethal mutations at any stage of spermatogenesis (through 6 weeks of mating following treatment), whereas EMS caused the expected DL's in spermatozoa (week-1) and late spermatids (week-2).

EVALUATION: Both the procedures and controls appear to have been adequate to generate valid results. It is not clear, however, why a 5-day schedule of TBZ-compound administration was chosen; multiple dosing at 600 mg/kg (high dose) daily may not add up to the acute LD₅₀ reported in preliminary toxicity testing (= 3100 mg/kg). Therefore, an insufficient dosage is likely--due to factors of absorption, metabolic turnover, and/or inadequate transport to gonads. The positive control, on the other hand, was active because it was injected i.p. at a dose level known to produce DL's in mice. The lack

of any toxicity in TBZ-treated animals also indicates insufficient dosage schedules. Hence, this study is judged not acceptable as a comprehensive assay of TBZ to induce DL's in mice.

- C. "Mutagenicity Tests of Thiabendazole in the Ames Salmonella typhimurium Reverse Mutation Test"
James McGregor, USDA Western Regional Research Center, (Albany, CA), May 28, 1976.

The "report" consist of a cover letter and a single tabulation of values (revertents per plate) from cultures of Salmonella strains TA 1535, TA 1537, TA 98, and TA 100, (presumably) treated with TBZ at concentrations of 10, 100, 1000, 2000, and 5000 ug/plate, in the absence and presence of metabolic activation provided by liver S-9. The highest dosage (5000 ug) exceeded the solubility limit, since precipitated compound was reported in the top agar.

Strain-specific positive controls were run for both activated and non-activated assays, to which cultures these responded appropriately (4 to 100 times spontaneous reversion). In contrast, values for TBZ treatment were within the range of negative controls.

EVALUATION: This summary report is unacceptable, since no protocol is given.

- D. Thiabendazole Mutagenic Evaluation, Merck Institute for Therapeutic Research, West Point, PA and Rahway, NJ, June 3, 1977, consisting of the following assays:
(1) Mutagenic Study in the Mouse Using the Micronucleus Test, TT# 76-8038; (2) Mutagenic (Subacute Dominant Lethal) Study in the Mouse, TT# 76-703-0; and (3) Microbial Mutagenicity Studies (Ames Test) with Salmonella typhimurium [no report number].

1. Micronucleus Test (MT), TT# 76-8038.

Procedures: Thiabendazole (TBZ, identified as lot # F291764, but not otherwise characterized as to composition or purity) was given orally as a suspension in 0.5% aqueous methylcellulose (400 cps) to juvenile (5-week old) CD-1 mice (8 males, 8 females per group) in split doses (acute) totalling 125, 250, and 500 mg/kg. A group of 14 males and 14 females served as negative controls, receiving the methylcellulose at the same volume. A positive control (3 males, 3 females) received a split i.p. injection of

methyl methanesulfonate (MMS) totalling 90 mg/kg. All animals were killed 6 hours after dosing, and bone marrow prepared for microscopic evaluation on glass slides which were coded for reading in blind/random fashion.

Results and Conclusions: No toxic signs were observed in any TBZ-treated mouse. No differences in the number of polychromatic erythrocytes (PCE) containing micronuclei were found between mice from either sex treated at any dosage of TBZ and negative controls. The number of micronuclei in normoblastic erythrocytes (NBE) did not show any relationship to TBZ treatment. Mice given ip MMS responded as expected with a highly significant ($p < 0.001$) increase in micronuclei in PCE's compared to controls.

It was concluded that TBZ showed no effect [induction of micronuclei] in this assay.

EVALUATION: The MT is rapid, easily scored, and conducted in vivo (whole animals). On the other hand, it is also decidedly insensitive, compared to in vivo cytogenetics assays. Whereas the positive response to ip MMS is evidence for transport to, and an effect upon, target cells in bone marrow, there is no assurance that the oral dose schedules of TBZ employed was sufficient, even for transport. To avoid this contention would have meant (at the least) some incidental toxicity at the highest dosage, increasing the dose to an LD₂₅ if necessary; and/or extending the period of sampling to at least the regenerative cycle in the bone marrow erythroid series; and/or a 5 to 7 day course of treatment.

Hence this study is inadequate to demonstrate potential for micronuclei induction, and thus the results are not acceptable.

2. Dominant Lethal Test (DLT) in Mice, TT# 76-703-0.

Procedures: Lot # F291764 TBZ was administered orally (gavage) to male adult CF₁S mice for 5 consecutive days at dose levels of 125, 250, and 500 mg/kg/day (10 mice per group) as a suspension in 0.5% methycellulose. Two controls were run concurrently, both receiving the vehicle in the same volume (10 ml/kg) as TBZ-treated animals. On the afternoon of the last day of dosing, and weekly for 7 weeks thereafter,

treated males were mated to untreated females (1:1), females renewed each week. On day 14 following observation of vaginal plug, the females were killed and their reproductive status recorded: Number of implants, early and late resorptions, live and dead fetuses.

Male-weight data were analyzed by Analysis of Variance (ANOVA), with significant comparisons determined by Dunnett's "t" test. Reproductive parameters (number of resorptions, dead fetuses per litter) were analyzed using an IBM-385 series computer programmed for non-parametric analyses.

Results: TBZ-treated mice lost weight at all dosage levels, but only at 500 mg/kg (highest dose) was this change significantly different from controls. There were no adverse effects on breeding performance of males at any dosage level of TBZ, especially no significant differences between treated and control groups in resorptions over the eight weeks of post-treatment breeding. Although weekly variations in average number of implants and live fetuses per litter occurred through the 8-week breeding, these were considered normal since they were seen both within and among control and TBZ-treated groups, without statistical significance.

It was concluded that no evidence of a dominant lethal effect was observed at dosage level of TBZ up to 500 mg/kg/day administered orally to male mice.

EVALUATION: Both the protocol and study parameters of this assay appear to have been adequate, and inspection of the values (tabulated for group summary and individual animal data fulsomely in 43 arrays) clearly support the lack of effect of TBZ for potential to induce dominant lethals at the dosage schedules employed. The marginal effect (loss of weight) in high-dose males (at 500 mg/kg/day) during days 1 to 3 treatment might be taken to indicate administration of a clinically "effective" (minimal effective) dose, such that confidence could be expressed in the resultant germinal parameter (dominant lethals). Unfortunately, this "clinical effect" might have been chance variation, since the same animals appear to recover after two further days treatment (days 3 to 5). If spurious, then an insufficient

treatment schedule is suspect, which casts doubt on the validity of the results, thus an adequate study was not performed. Further, no positive control was included in the study. Together, these deficiencies add up to an inconclusive assay, and an unacceptable conclusion for dominant lethality.

3. Ames Test (Salmonella typhimurium)

Procedures: With minor modifications, the Ames et al. (1975) procedure was followed in assaying TBZ (lot #F291764) mutagenicity, but only in the TA 98 strain of Salmonella typhimurium.

Two types of rat liver microsomal preparations (S-9) were used: One derived from rats pre-treated with phenobarbital (4 x 75 mg/kg/day, i.p.; killed day 5); and the standard Aroclor 1254 treatment (1 x 500 mg/kg, i.p.; killed 5 days later).

Results: Statistically significant increases in revertants over saline controls were found at the highest concentrations of 1,000 and 2,000 mcg/plate lot #F291764 (veterinary grade TBZ), but only in tests with activation (S-9) systems derived from phenobarb-treated rats. The same TBZ preparation gave negative results in activation assays containing Aroclor 1254-induced S-9, as well as when tester strains TA 1538 and TA 100 were used in the presence of either phenobarb or Aroclor S-9 (these data not reported here).

Two other preparations of TBZ, Lot #F147325 and Stock 6764 (produced for human use) also gave negative results (in TA 98) with or without phenobarb-induced S-9.

To test for an impurity in Lot F291764 (vet.) being responsible for the unique ("spurious") positive in TA 98, the veterinary grade of TBZ was fractionated (by ethanol phase separation) into "purified" TBZ and concentrated "impurity(s)", and the fractions re-tested. Purified TBZ gave negative results with TA 98 at 2000 mcg (and 1000 mcg) in phenobarb-activated plates, whereas the concentrated impurity fraction was positive at 20 mcg/plate and higher (in a dose-response fashion).

Although this active contaminant of Lot #F291764 (vet.) has not yet been identified, concentration and purification techniques have ascertained there are at least two mutagenic components in impurities removed from this preparation of TBZ.

[Attempts at characterizing production lots of TBZ for mutagenic activity are currently being pursued at MSD, with activity defined in terms of the Ames test using material activated by liver microsomal enzymes from CRCD male rats induced with phenobarbital and Salmonella typhimurium TA 98 as the tester strain. Recent sampling of lots from various sources is given in a tabulation.]

It was concluded that thiabendazole per se is not positive in the Ames test, whereas the extracted impurities are. Thus, thiabendazole itself does not have mutagenic activity, although two impurities which can be extracted from thiabendazole will show mutagenic activity in the in vitro bacterial system of Ames.

EVALUATION: The procedure was adequate to generate valid results, and supports the conclusion that the mutagenic activity of (some) lots of TBZ in Ames testing is due to an active impurity. The study is acceptable.