Assessment of Genotoxic Potential of Elk River Chemical Contaminants Using In Vivo Micronucleus and Bacterial Mutagenicity Assays

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Abstract

In January 2014, thousands of gallons of a mixture of coal-washing chemicals were accidentally spilled into the Elk River upstream of the water intake facility in Charleston, WV, contaminating tap water of ~300,000 residents for several days. Chemical analyses revealed the major constituent of the spilled liquid was a mixture of chemicals referred to as crude 4-methylcyclohexanemethanol (cMCHM: ~90% MCHM plus several low abundance chemicals); several minor constituents were also identified, including propylene glycol phenyl ether (PPH) and its closely-related dipropylene analog (diPPH). Limited toxicity information was available for most of the spilled chemicals. To generate data to aid in risk assessment, they were nominated for testing to the National Toxicology Program (NTP) in July 2014, with an emphasis on short-term tests due to the transient nature of the exposure that occurred. Included in the overall toxicological profiling effort, genotoxicity potential was assessed with bacterial mutagenicity and *in vivo* peripheral blood erythrocyte micronucleus assays. cMCHM, MCHM, PPH, and diPPH were tested for micronucleus induction in male rats following 5 days of exposure and for mutation induction in bacteria; These four chemicals plus other minor constituents were also tested for bacterial mutagenicity. In both the micronucleus and bacterial mutation assays, the top dose for each chemical was limited by toxicity. No evidence of chromosomal damage was noted for the four chemicals tested in the micronucleus assay; one chemical, dimethyl 1,4-cyclohexanedicarboxylate was mutagenic in the bacterial assay. The current study illustrates the vital role that NTP can play in responding rapidly to an environmental contamination event. Supported by NIEHS/NTP contract HHSN273201300009C.

Introduction

When several thousand gallons of a chemical mixture used in coal production spilled into the Elk River near Charleston, WV, the National Toxicology Program (NTP) was tasked with developing, within one year, a toxicological evaluation of the contaminants to be used as an aid to public health decision-makers[1]. At the time of the spill, the Centers for Disease Control and Prevention (CDC) released drinking water advisory levels for the major spill components: 1 ppm for MCHM[2] and 1.2 ppm for PPH[3]. However, data used to develop these advisory levels was limited to a few toxicological studies conducted by the chemical manufacturer. In addition, although initial internal NTP studies suggested little concern for lasting adverse effects given the transient, low-level exposures, data supporting this claim was lacking. The major goals for NTP testing of the Elk River contaminants were to reduce uncertainty surrounding the advisory levels, determine the potential for adverse effects to sensitive life stages, and to develop toxicological profiles for minor spill components[4]. Tests that were performed on spill chemicals are listed in Table 1. Included in this initial test battery was assessment of the potential for genotoxicity using a bacterial mutation assay and an in vivo assay for chromosomal damage as measured by micronucleus induction. These tests assess the ability of a chemical to cause mutations and/or chromosomal damage, indicating a potential to cause cancer or heritable genetic changes. Results of the genotoxicity testing and a summary of other short-term testing of the Elk River contaminants are presented.

Table 1: NTP Studies on Elk River Spill Chemicals

Test Article [Abbreviation, CASRN*]	Studies: Rat Prenatal Developmental Toxicity (Teratology)	Mouse Dermal Irritation and Hypersensitivity	5-Day Rat Toxicogenomic/In Vivo Micronucleus	Bacterial Mutagenicity	Zebrafish Developmental	Nematode Toxicity	High Throughput Screening	Structure-Activity Relationship Analysis	Notes
4-Methylcyclohexanemethanol [MCHM, 34885-03-5]	X	X	X	X	X	X	X	X	a
Dipropylene glycol phenyl ether [DiPPH, 51730-94-0]			X	X	X	X		X	b
Propylene glycol phenyl ether [PPH, 770-35-4]			X	X	X	X	X	X	b
1,4-Cyclohexanedimethanol [CHDM; 105-08-8]				X	X	X	X	X	b
2-Methylcyclohexanemethanol [2MCHM, 2105-40-0]				X	X	X		X	b
4-(Methoxymethyl)cyclohexanemethanol [MMCHM, 98955-27-2]				X	X	X		X	b
Dimethyl 1,4-cyclohexanedicarboxylate [DMCHDC, 94-60-0]				X	X	X	X	X	b
Methyl 4-methylcyclohexanecarboxylate [MMCHC, 51181-40-9]				X	X	X		X	b
Technical product ["crude MCHM"]		X	X	X	X	X			c

*Chemical Abstracts Service Registry Number

[a]Major constituent of the spilled liquid (>50% of the spilled chemical mixture).

[b]Minor constituent of the spilled liquid (<10% of the spilled chemical mixture).

[c]The commercial product present in the leaking tank; a mixture of MCHM, MMCHM, MMCHC, DMCHDC, CHDM, and methanol.

Materials and Methods

Bacterial Mutagenicity Assay:

Samples of the chemicals involved in the Elk River spill were provided by the NTP. Test chemicals were handled and stored in accordance to their MSDS and/or provided literature. Each chemical was tested in a bacterial reverse mutation assay, including a range-finder and two independent mutation assays, in three test strains: *Salmonella typhimurium* strains TA98 and TA100, and *Escherichia coli* WP2 *uvrA* pKM101. Bacterial cultures were exposed to 5-7 doses of the test chemical, or to positive and negative controls, in triplicate, with and without S9 mix containing 10% phenobarbital/benzoflavone-induced rat liver S9. The test substance was pre-incubated with the bacteria at 37˚ C for 20 minutes, then mixed with top agar containing the appropriate amino acid (histidine/biotin for *Salmonella* strains; tryptophan for *E. coli*) and poured onto the surface of a minimal glucose agar plate. Plates were incubated at 37 ± 1 ˚ C for 48 ± 2 hrs. The number of revertant colonies was counted using the Sorcerer plate counter and Ames Study Manager software (Perceptive Instruments, Surrey, UK).

In Vivo Micronucleus Assay:

ILS received EDTA-stabilized blood samples from adult male Sprague-Dawley rats following administration of the test chemicals orally once daily for 5 consecutive days. The blood samples were obtained 24 hours after the fifth treatment. Micronucleus formation in 20,000 reticulocytes and up to 1 X 10^6 mature erythrocytes per animal was determined by flow cytometry using the MicroFlow^{Plus}® Kit from Litron Laboratories (Rochester, NY) according to manufacturer's protocol. The percentage of reticulocytes among total erythrocytes was also determined as a measure of bone marrow toxicity.

Other Short-Term Tests:

For information about other NTP testing of the West Virginia Elk River chemicals, including test descriptions and testing status update, please visit the following website:

http://ntp.niehs.nih.gov/results/areas/wvspill/studies/index.html

Bacterial Mutagenicity Assay Results for Selected Elk River Chemicals

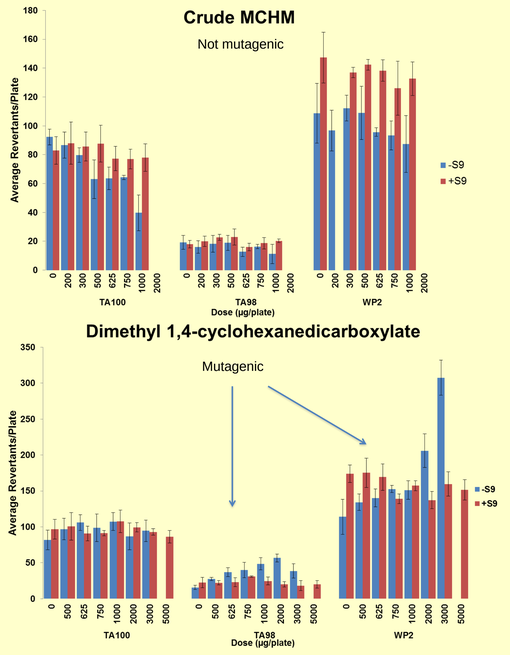

Table 2: *In Vivo* Micronucleus Assay Data for Selected Elk River Chemicals

Crude MCHM

Dose (mg/mL)	No. of Animals	MN-PCE/1000[b]	Pairwise *P*-value[c]	MN-NCE/1000[b]	Pairwise *P*-value[d]	Percent PCE[b]	Pairwise *P*-value[c]
0	6	0.67 ± 0.05		0.09 ± 0.01		2.401 ± 0.21	
0.1	6	0.69 ± 0.10	0.5030	0.13 ± 0.02	0.5233	2.369 ± 0.10	1.0000
1	6	0.64 ± 0.08	0.5866	0.11 ± 0.02	1.0000	2.363 ± 0.11	1.0000
10	5	0.77 ± 0.10	0.5636	0.16 ± 0.05	0.6209	2.724 ± 0.16	1.0000
100	5	0.60 ± 0.05	0.5834	0.13 ± 0.03	1.0000	2.088 ± 0.10	0.6949
300	6	0.71 ± 0.10	0.5698	0.24 ± 0.08	0.0893	2.297 ± 0.11	0.6787
500	6	0.67 ± 0.10	0.5777	0.22 ± 0.05	0.0307	2.313 ± 0.14	0.6838
		P = 0.515[e]		*P* = 0.005[f]		*P* = 0.383[e]	

MCHM

Dose (mg/mL)	No. of Animals	MN-PCE/1000[b]	Pairwise *P*-value[c]	MN-NCE/1000[b]	Pairwise *P*-value[c]	Percent PCE[b]	Pairwise *P*-value[d]
0	6	0.64 ± 0.07		0.06 ± 0.00		2.451 ± 0.25	
0.1	6	0.51 ± 0.09	1.0000	0.05 ± 0.00	1.0000	2.468 ± 0.19	0.8830
1	6	0.60 ± 0.09	1.0000	0.04 ± 0.01	1.0000	2.570 ± 0.22	0.9584
10	6	0.58 ± 0.04	1.0000	0.08 ± 0.02	1.0000	2.452 ± 0.12	0.9812
100	6	0.63 ± 0.06	1.0000	0.08 ± 0.02	0.9349	2.670 ± 0.16	0.9890
300	6	0.69 ± 0.16	1.0000	0.09 ± 0.02	0.4535	2.316 ± 0.36	0.9923
500	6	0.75 ± 0.08	1.0000	0.08 ± 0.01	1.0000	2.737 ± 0.25	0.5448
		P = 0.133[f]		*P* = 0.016[f]		*P* = 0.785[e]	

[a]Statistical tests and *P*-values were taken from the NTP database report.

[b]Mean ± Standard Error

[c]Pairwise comparison with the Control group; significant at $P \leq 0.025$ by William's test.

[d]Pairwise comparison with the Control group; significant at P <= 0.025 by Dunn's test.

[e]Dose-related trend; significant at $P \leq 0.025$ by linear regression.

[f]Dose-related trend; significant at $P \leq 0.025$ by Jonckheere's test.

Table 3: Summary of NTP Testing of Selected Elk River Chemicals

NTP Test	Test Result: MCHM	Crude MCHM	DMCHDC
Rat Prenatal Developmental Toxicity (Teratology)	Decreased fetal weight at top 2 doses Fetal malformations (extra cervical and lumber ribs; decreased fusion of cartilage to sternum; incomplete ossification of sternebrae) at top dose	Not Tested	Not tested
Mouse Dermal Irritation and Hypersensitivity	Mild skin irritation at top 2 doses No indication of hypersensitivity	Mild skin irritation Evidence of hypersensitivity	Not tested
5-Day Rat Toxicogenomic*	Weak toxicogenomic response Slight increase in liver weight at top 2 doses Increased serum triglycerides at top dose	Weak toxicogenomic response Slight increase in liver weight at top 2 doses Increased serum triglycerides at top dose	Not tested
In Vivo Micronucleus*	Negative	Negative	Not tested
Bacterial Mutagenicity	Negative	Negative	Positive in *S. typhimurium* TA98 and *E. coli* WP2 *uvra* pKM101 without S9
Zebrafish Developmental	Positive for photomotor effect	Negative	Positive for structural abnormalities
Nematode Toxicity	Negative	Negative	Negative
High Throughput Screening	Inactive	Not tested	Inactive
Structure-Activity Relationship Analysis	Positive prediction for eye and skin irritation and for developmental toxicity	Results not yet available	Results not yet available

*5-day rat toxicogenomic and in vivo micronucleus assays were conducted using the same animals

Results

•Genotoxicity

- DMCHDC was mutagenic in bacteria without metabolic activation. Mutagenicity was observed at 625 µg/plate in *S. typhimurium* TA98 and 2000 µg/plate in *E. coli* WP2 *uvrA* pKM101; the other chemicals tested did not induce mutations in bacteria.
- The 3 chemicals tested for micronucleus induction *in vivo* were all negative.

•Other tests[1,4]

- MCHM was positive for developmental toxicity in rats (200 and 400 mg/kg/day) and zebrafish (photomotor response; ~4.5 ppm); DMCHDC induced structural abnormalities in zebrafish at dose levels of ~13 ppm.
- MCHM, cMCHM, and PPH induced weak toxicogenomic responses and mild clinical chemistry changes in rats after 5 days of dosing at a maximum tolerated dose.
- MCHM and cMCHM induced mild skin irritation and cMCHM induced skin sensitization at a dose of 20%.

Conclusions

- Genotoxicity was observed only with one of the minor contaminants, DCMHDC; the effect was eliminated with the addition of a metabolic activation source.
- Although there is some evidence that MCHM and crude MCHM can cause developmental toxicity and dermal irritation/sensitivity, the doses required to induce these effects were quite high.
- The likelihood of long-term effects is still low; the data generated to date supports the adequacy of the advisory levels established at the time of the spill.

References

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