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Genetic Toxicology

In vitro studies on the genotoxicity of 2,4-dichloro-6-nitrophenol ammonium (DCNPA) and its major metabolite

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Abstract

2,4-dichloro-6-nitrophenol ammonium (DCNPA) is used as a herbicide. However, information on the potential health hazards of DCNPA is limited. In a previous study, we found that DCNPA is genotoxic to *Bacillus subtilis* and yeast. Further studies were performed to determine whether DCNPA and its major metabolite, 2,4-dichloro-6-aminophenol (DCAP), can induce reverse mutations in Salmonella, gene mutations at the HPRT locus, sister chromatid exchanges (SCEs) and micronuclei (MN) in V79 cells. Results show that DCNPA does not produce a positive response for any endpoint at concentrations tested. However, treatment of V79 cultures with DCAP caused a significant increase in SCEs and MN in a concentration-dependent manner. These results indicate that DCAP damages DNA and causes chromosomal aberrations in V79 cells. Therefore, DCNPA could pose potential health hazards to populations exposed to this herbicide.

Keywords: 2,4-Dichloro-6-nitrophenol ammonium; Herbicide; Ames test; Gene mutation; Sister chromatid exchange; Micronucleus assay

1. Introduction

2,4-dichloro-6-nitrophenol ammonium (DCNPA) is used in agriculture as a herbicide. The use of DCNPA is rapidly increasing because it leaves minimal residue in crops and soil, and because of its efficacy and competitive price. However, little information is available regarding the potential health hazards of this compound to exposed workers. Several studies on the genotoxicity/carcinogenicity of some DCNPA analogues have been reported. Trichlorophenols have been shown to induce chromosome aberrations (CAs) in Chinese hamster ovary (CHO) cells and in Chinese hamster lung fibroblasts (V79 cells) (Armstrong et al., 1993) and to cause tumors of the hematopoietic system in male rats and of the liver in male and female mice (NCI, 1979). A significant increase in the incidence of CAs and sister chromatid exchanges (SCEs) was observed in an Italian population exposed to pesticides, including nitro-organic herbicides and nitro-organic fungicides (Ferrari et al., 1991). Results of our previous studies with the *Bacillus subtilis* (*recA*$^-$) and yeast (*cyh*^{R})

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test systems showed that DCNPA damages DNA (Heng and Wang, 1992; Heng et al., 1993). However, whether DCNPA can cause gene mutations or chromosomal aberrations in mammalian cells is not known.

Metabolites in liver perfusate, blood and urine of rats treated with DCNPA by gavage have been analyzed. Results showed that of the 4 metabolites detected, only 2,4-dichloro-6-aminophenol (DCAP) was consistently present in a significant amount (Yu et al., in press). No data on the toxicity of DCAP have been reported in the literature. Therefore, these studies have been carried out to determine whether DCNPA and its major metabolite, DCAP, can induce mutations in *Salmonella*, gene mutations at the HPRT locus, SCEs and micronuclei (MN) in V79 cells.

2. Materials and methods

2.1. Chemicals and reagents

DCNPA and DCAP (> 98% purity) were obtained from the Institute of Sichuan Chemical Industry (Chengdu, China). 2,4,7-Trinitrofluorenone (TNF) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), chemicals used for positive controls, were purchased from Aldrich (Milwaukee, WI, USA). 6-Thioguanine and 5-bromo-2-deoxyuridine (BrdU) were purchased from Sigma (St. Louis, MO, USA).

2.2. Salmonella / microsomal assays

Salmonella tester strains TA98 and TA100 were kindly provided by Prof. B. Ames (University of California, Berkeley, CA, USA). The plate-incorporation and pre-incubation tests with and without S9 were performed as described by Maron and Ames (1983). The positive (TNF and MNNG) and solvent controls were run simultaneously. Results are expressed as the mean ± standard deviation (SD) of revertant colonies.

2.3. Cell line and culture

V79 cells were supplied by Dr. C.C. Chang (Michigan State University, East Lansing, MI, USA). The cells were cultured in 75 cm^2 tissue flasks containing 15 ml Eagle's Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum, 1 mM L-glutamine and 1% penicillin/streptomycin. Cells were cultured at 37°C with 5% CO_2 and 95–100% humidity.

2.4. Chemical treatment

Solutions of DCNPA and DCAP were prepared fresh prior to each treatment. DCNPA was dissolved in DMSO at the concentration of 40 mg/ml, and DCAP was dissolved in sterile distilled water at 30 mg/ml. Lower concentrations were made by diluting the original solution. Approximately 24 h after culture initiation, 0.1 ml test chemical solution was added to a dish for a final concentration of 50, 100, 200 or 400 μg DCNPA/ml and 50, 100, 200 or 300 μg DCAP/ml. Cells were exposed to chemicals for 3 h and then rinsed with phosphate-buffered saline (PBS). Solvent and positive (MNNG) controls were run with each trial. The same chemical treatment scheme was used for gene mutation, SCE and MN assays in V79 cells.

2.5. Cytotoxicity and gene mutation assay

The treated cells were trypsinized and resuspended. One hundred cells per treatment group were seeded into each of 6 (60 mm) dishes with 5 ml medium. After 7 d incubation, the colonies were stained and counted. The relative survival was determined as an indicator of cytotoxicity.

Induction of 6-thioguanine-resistant (TG^r) mutations at the HPRT locus was detected using the procedure of Bradley et al. (1981). Following the treatment, 2.5×10^5 cells per treatment group were seeded into a 100 mm dish with 10 ml medium and incubated 7 d to allow for expression. During expression, cells were subcultured every 2–3 d. At the end of the expression period, cells were harvested by trypsinization and counted. One hundred cells were reseeded into each of 6 dishes (60 mm) for detection of colony-forming efficiencies (CFE) after 7 d incubation, while 2.5×10^5 cells were reseeded into each of 6 dishes (100 mm) containing 10 ml selection medium with 10 μM 6-thioguanine for determination of TG^r mutant colonies after 10 d incubation. The colonies formed on the dishes were scored after

being fixed in absolute methanol and stained with 5% Giemsa in Sorenson's buffer. The mutant frequency (MF) was estimated by the following calculation:

2.6. SCE assay

After 3 h treatment, the medium of each dish was replaced with MEM containing 12.5 μM BrdU and incubated for an additional 30 h in the dark. This incubation time gave the highest number of cells in second division. Colcemid (0.15 μg/ml) was added for the last 2 h. Preparation and staining of slides were performed by standard procedures (Perry and Wolff, 1974). Fifty metaphase spreads with 20–23 chromosomes were scored per treatment group. Results are expressed as the mean number of SCEs per metaphase spread ± standard error (SE) and the variance of SCEs induced by DCNPA or DCAP were compared using Student's *t*-test. The relationship between chemical concentrations and SCEs was evaluated by a simple correlation analysis.

2.7. Micronucleus assay

Following chemical treatment, cells were incubated for 21 h and harvested by trypsinization. Cells were then resuspended in PBS at an optimal density and 65 μl cell suspension was pelleted onto a slide using a Cytospin® (Shandon Southern Products, Astmoor, Runcorn, Cheshire, England). The slides were fixed in absolute methanol and stained with Diff-Quik stain (American Scientific Products, Broadview Heights, OH, USA). Three thousand interphase cells per treatment group were scored for MN according to the criteria of Heddle et al. (1983). The frequency of micronuclei was calculated and Chi-square analysis was performed.

3. Results

3.1. Salmonella / microsomal assays

The Salmonella mutagenicity results in the plate-incorporation and pre-incubation tests without S9 are given in Table 1. TNF and MNNG, positive controls, markedly increased mutations in tester strains TA98 and TA100. Nevertheless, both DCNPA and DCAP failed to produce an increase in revertants even at the highest concentration tested. Results of the test with rat liver S9 are similar to those of test without S9 (data not shown). DCNPA and DCAP at the highest

Table 1
Results of the Ames Salmonella/microsomal assays for DCNPA and DCAP

Compound	Dose (μg/plate)	Number of revertant colonies (mean ± SD) [a]			
		TA98		TA100	
		plate-inco [b]	pre-incu [c]	plate-inco [b]	pre-incu [c]
DCNPA	0	25.2 ± 3.3	20.7 ± 1.9	111.4 ± 5.5	103.2 ± 12.8
	4	23.0 ± 2.0	20.3 ± 2.1	116.7 ± 7.5	107.0 ± 8.7
	20	24.2 ± 2.3	21.6 ± 2.1	113.8 ± 6.9	101.6 ± 14.8
	100	24.0 ± 3.2	18.6 ± 1.5	106.0 ± 4.5	97.0 ± 14.0
	500	15.6 ± 8.5	6.5 ± 3.9	60.4 ± 17.4	23.2 ± 11.0
DCAP	0	28.7 ± 3.1	24.2 ± 5.8	117.8 ± 16.2	109.2 ± 17.9
	4	28.3 ± 3.1	25.3 ± 2.3	126.0 ± 7.9	108.0 ± 8.7
	20	27.8 ± 3.9	24.0 ± 7.3	118.6 ± 11.7	115.8 ± 21.9
	100	29.2 ± 4.2	24.0 ± 7.6	121.4 ± 16.2	116.6 ± 29.4
	500	19.6 ± 3.8	15.6 ± 5.2	97.5 ± 2.1	5.5 ± 2.1
TNF	0.4	798.4 ± 21.0	855.5 ± 17.0		
MNNG	2.0			1042 ± 180.0	992.3 ± 203.0

[a] Average of 3 plates.
[b] Plate-inco, plate-incorporation test; [c] pre-incu, pre-incubation test.

concentration tested appeared to be toxic to bacterial cells, as demonstrated by a reduction in the number of revertants.

3.2. Cytotoxicity and gene mutation assay

Increasing concentrations of DCNPA and DCAP decreased the formation of viable V79 colonies and relative percent survivals (Fig. 1). The relative survival at the highest dose of DCNPA (400 μg/ml) and DCAP (300 μg/ml) was 19.6 and 17.3%, respectively. The toxic effect of DCAP to V79 cells seems to be greater than that of DCNPA.

Table 2 shows the frequency of TG^r mutations at the HPRT locus in V79 cells treated with various concentrations of DCNPA or DCAP. No significant increase in the frequency of TG^r mutations over spontaneous background was observed for any concentration of DCNPA or DCAP tested. Treatment of cells with 1 μg MNNG/ml produced an increase 30 times that of the solvent control.

3.3. SCE assay

The frequency of SCEs in V79 cultures treated with DCNPA and DCAP are shown in Table 3. The mean values and ranges of SCEs/cell following the treatment of DCNPA for all 4 concentrations (50, 100, 200, 400 μg/ml) are close to the baseline. Thus, DCNPA did not induce SCEs in V79 cells. In contrast, DCAP caused a dose-dependent increase in SCE frequencies ($r = 0.987$, $p < 0.01$). The increase was significantly higher than that of solvent control ($p < 0.01$). SCEs/cell in the highest concentration of DCAP tested (300 μg/ml) were more than twice the baseline.

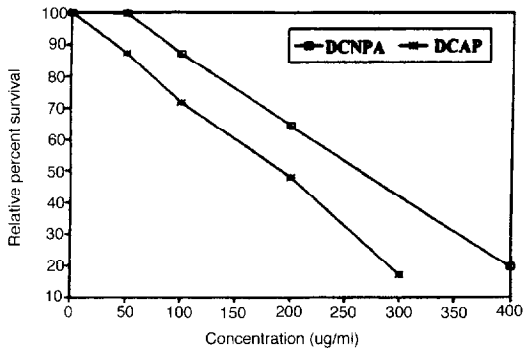

Fig. 1. Relative survival of V79 cells exposed to DCNPA and DCAP.

Table 2
Frequencies of 6-TG^r mutants in V79 culturestreated with DCNPA and DCAP

Compound	μg/ml	Colonies/plate	$\bar{x} \pm SD$	Survivors [a] ($\times 10^5$)	MF [b] ($\times 10^5$)
DCNPA	0	2.7	1.9	2.4	1.3
	50	1.6	1.5	2.4	1.0
	100	2.0	1.9	2.2	0.9
	200	2.2	1.6	1.9	1.2
	400	0.6	0.9	1.9	0.3
DCAP	0	2.2	1.9	1.5	1.5
	50	2.8	2.8	1.7	1.7
	100	1.6	1.3	2.0	0.8
	200	2.8	2.4	2.1	1.3
	300	1.4	0.9	1.7	0.8
MNNG	1	72.5	19.3	1.9	38.2

[a] Survivors = (number of cells plated) × CFE.
[b] Mutant frequency.

3.4. Micronucleus assay

The results of MN induction by DCNPA and DCAP in V79 cells (Table 4) was similar to that seen for SCEs. DCNPA did not cause a significant

Table 3
Frequencies of SCE in V79 cells treated with DCNPA and DCAP

Compound	μg/ml	Cells scored	SCEs per cell		
			Range	Mean ± SD	
DCNPA	0	50	2 ~ 12	6.3	2.76
	50	50	2 ~ 10	6.5	2.31
	100	50	3 ~ 12	6.4	2.04
	200	50	2 ~ 14	6.6	2.53
	400	50	3 ~ 14	6.5	2.64
DCAP	0	50	3 ~ 11	6.1	2.08
	50	50	2 ~ 14	6.1	2.25
	100	50	3 ~ 16	8.7 *	2.64
	200	50	4 ~ 30	10.4 *	4.80
	300	50	5 ~ 47	14.1 *	9.16
MNNG	1	50	41 ~ 82	69.1 *	15.07

* $p < 0.001$, compared to solvent control by Student's t-test.

Table 4
Frequencies of MN in V79 cells treated with DCNPA and DCAP

Compound	Micronucleated cells		
	μg/ml	Total [a]	% Cells
DCNPA	0	17	5.7
	50	16	5.3
	100	18	6.0
	200	16	5.3
	400	17	5.7
DCAP	0	16	5.3
	50	17	5.7
	100	18	6.0
	200	52	17.3 *
	300	64	21.3 *
MNNG	1	59	19.8 *

[a] Number of micronucleated cells in 3000 cells scored.
* $p < 0.005$, compared to solvent control.

increase of MN whereas DCAP did, in a dose-related manner. The frequencies of MN for DCAP concentrations of 200 μg/ml and 300 μg/ml were significantly higher ($p < 0.01$) than that observed for the solvent control.

4. Discussion

The studies reported here indicate that DCAP, unlike DCNPA, induced SCEs and MN in V79 cells. However, neither compound caused gene mutations in bacteria or in cultured mammalian cells. In these studies, tester strains and cells are sensitive to positive control substances and the spontaneous mutation frequencies are in the range historically acceptable in our laboratory. Although the highest concentration of DCNPA (400 μg/ml) was at the limit of solubility in DMSO, it gave a survival of approx. 20% in V79 cells (Fig. 1). This corresponds to the criteria of an upper limit of concentration recommended by Aaron et al. (1994).

Garrett et al. (1986) evaluated the genotoxic activity of 65 pesticides and suggested that pesticides with similar structures produced similar profiles of genotoxic activity, and that certain test systems might be more appropriate for some classes of pesticides. DCAP is a halogenated phenol with an amino-group. No data are currently available on its mutagenic/carcinogenic effects. A few studies on the genotoxic effects of related phenols have been carried out and have shown that chemicals, such as *o*-phenylphenol (Moriya et al., 1983; Tayama-Nawai et al., 1984; Lambert and Eastmond, 1994), 2,4,6-trichlorophenol (Haworth et al., 1983; Jansson and Jansson, 1992; Armstrong et al., 1993) and aminophenol (Holme et al., 1988) possess genotoxic characteristics similar to those shown here for DCAP. These characteristics include the positive responses in clastogenicity assays, but no response in gene mutation endpoints (in a mammalian cell line or Salmonella). Thus, some phenol compounds may cause chromosomal damage, if they are genotoxic, rather then gene mutations.

The micronucleus test is a method devised primarily for screening chemicals for clastogenic/aneuploidogenic effects (MacGregor et al., 1987). The SCE assay has been used widely as a sensitive indicator of genotoxicity; however, the mechanism of SCE formation and their biological significance are not clear (Tucker et al., 1993). Data accumulated over the last 20 years show that the majority of the SCE-inducing compounds also produce DNA damage (Latt et al., 1981; Lambert et al., 1986). MN and SCEs studies indicate that DCAP causes DNA damage and is clastogenic and/or aneuploidogenic to mammalian cells.

In our previous studies, DCNPA induced positive responses in the *B. subtilis* (*recA*$^-$) (Heng and Wang, 1992) and yeast (*cyh*^{R}) (Heng et al., 1993) test systems, that is, DCNPA is a DNA damaging agent according to the relevant test endpoints (Tanooka, 1977; Brusick, 1986). It is interesting that the positive responses were produced without applying any activation system. In contrast, the response became too weak to be detected in *B. subtilis* when rat liver S9 mix was used. In our present study, only DCAP, a metabolite of DCNPA, induced a clear dose-related increase of SCEs and MN. This difference may be due to biotransformation. It has been reported that liver S9 fractions do not necessarily reflect the metabolic activity of the whole organ (Tweats, 1993). Also the activation pathway of liver S9 fractions is limited (You et al., 1993), so it may be insufficient for activation of some classes of chemicals. The metabolism of DCNPA to DCAP

occurs by the reduction of a nitro-group. *B. subtilis* and yeast may be more effective than rat liver S9 mix in the reduction of DCNPA to DCAP. Unlike *Bacillus* and yeast, V79 cells seem to be unable to reduce DCNPA on their own.

It has been demonstrated that all the amines in benzidine derivatives are genotoxic in vivo causing chromosomal aberrations (You et al., 1993), and that potential carcinogenic effects of nitroquinoline *N*-oxide resulted from reduction of a nitro-group (Timbrell, 1993). Metabolism of DCNPA to DCAP by reduction of a nitro-group and deamination caused a critical change in toxicity. DCAP appeared to be more cytotoxic to V79 cells than its parent chemical (Fig. 1) and more genotoxic. Reduction of the nitro-group in DCNPA occurring in vivo may contribute to the clastogenic and/or aneuploidogenic activities of DCAP. Therefore, DCNPA poses genotoxic and potential carcinogenic hazards to the exposed population.

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