Genotoxicity Study of Illegal Drug MDMA and Its Nitroso Derivative N-MDMA by Micronucleus and Chromosomal Aberration Tests using Chinese Hamster Lung Fibroblast Cell Line

Hayato YOSHIOKA¹, Hidesuke SHIMIZU¹, Yoshimitsu TOYAMA^{1,2},
Yuichi MIYAKOSHI¹, Yuji SUZUKI¹ and Ryo TAKAGI¹

¹Department of Public Health and Environmental Medicine, The Jikei University School of Medicine, Tokyo, Japan

²Criminal Investigation Laboratory, Metropolitan Police Department, Tokyo, Japan

Abstract

Objectives: An increase in incidence of the illegal use of tablets containing 3,4-methylenedioxymethamphetamine hydrochloride (MDMA) has recently become a widespread social problem. MDMA ingested orally reacts with nitrite in the stomach and is synthesized into *N*-nitroso-3,4-methylenedioxymethamphetamine (N-MDMA). The aim of this study is to investigate the genotoxic effects of MDMA and N-MDMA on the basis of the results of an *in vitro* micronucleus (MN) test and an *in vitro* chromosomal aberration (CA) test using a Chinese hamster lung fibroblast cell line (CHL/IU).

Methods: Tablets containing MDMA obtained from the Regional Bureau of the Ministry of Health, Labor and Welfare were purified, and N-MDMA was synthesized from MDMA in our laboratory. To evaluate the effects of MDMA and N-MDMA, the MN test established by our laboratory and the CA test in accordance with the guidelines for toxicity studies of drugs recommended by the Ministry of Health, Labor and Welfare were performed.

Results: In the MN test, no increased frequency of MNs was not found for MDMA. On the other hand, an apparently increased frequency of MNs was observed for N-MDMA. In the CA test, no CA was found for MDMA, but CA was observed for N-MDMA apparently.

Conclusion: N-MDMA genotoxicity was observed in the MN and CA tests. However, no MDMA genotoxicity was observed.

Key words: MDMA, N-MDMA, genotoxicity, micronucleus test, chromosomal aberration test

Introduction

3,4-Methylenedioxymethamphetamine hydrochloride (MDMA) is a hallucinogen with a chemical structure similar to methamphetamine (MA). MDMA was synthesized as an anorexigenic agent for the first time by Merck Pharmaceuticals in 1912, and a patent for it was obtained. However, it was considered to have a harmful influence on the human body and was not manufactured at that time. MDMA began to be circulated on the market around 1970 and had been prescribed

as a therapeutic drug by psychiatrists from the beginning of the 1970s to the 1980s. MDMA had been used as a therapeutic agent for posttraumatic stress disorder (PTSD) mainly in the US until 1985, but the use of this drug thereafter became social problems (1, 2). Finally, MDMA was regulated in the United States in 1985 and in Japan in 1991, and most countries now strictly regulate its use (3).

The amount of MDMA confiscated in Japan in 2005 was about fivefold that confiscated over the five preceding years (2). Tablets containing MDMA are sometimes mixed with illegal drugs such as ketamine, and unexpected acute intoxication can thus be caused as a result of drug interactions (4). Cases of MDMA tolerance and dependence have also been reported (5), and there is an urgent need to prevent MDMA abuse.

Secondary and tertiary amines react with the nitrous acid found in medicines and food additives to form *N*-nitroso compounds in the stomach. *N*-Nitroso compounds induce genotoxic activity according to some mutagenicity tests and

Received Dec. 12, 2006/Accepted Mar. 14, 2007

Reprint requests to: Hayato YOSHIOKA

Department of Public Health and Environmental Medicine, The Jikei University School of Medicine, 3-25-8 Nishishinbashi, Minato-ku, Tokyo 105-8461, Japan

TEL: +81(3)3433-1111 (ex. 2266), FAX: +81(3)5472-7526

E-mail: y8810@jikei.ac.jp

carcinogenicity (6). Numerous animal studies have demonstrated the carcinogenicity of *N*-nitroso compounds (7–9). MDMA is a secondary amine. Therefore, tablets containing MDMA are considered to react with nitrous acid to form *N*-nitroso-3,4-methylenedioxymethamphetamine (N-MDMA) in the stomach.

The aim of this study is to confirm the possibility of developing health problems due to MDMA abuse. The neurotoxicity of MDMA has been widely reported (10–12), but the genotoxicity of this drug has not yet been previously studied. We investigated the risk of carcinogenesis induced by MDMA in this study.

The micronucleus (MN) and chromosomal aberration (CA) tests have been used as short-term screening assays for the detection of mutagens and carcinogens (13–15). To evaluate the effects of MDMA and its nitroso derivative N-MDMA, the MN and CA tests were performed using a Chinese hamster lung fibroblast cell line (CHL/IU).

Methods

Chemicals

Eagle's minimum essential medium (MEM), Dulbecco's phosphate-buffered saline (PBS), dimethylsulfoxide (DMSO), and glucose-6-phosphate (G-6-P) were obtained from Sigma-Aldrich Co., (St. Louis, MO, USA). Hydroxyethylpiperazinylthanesulfonic acid (HEPES) buffer solution, calf serum and trypsin were obtained from Invitrogen (Carlsbad, CA, USA). Nicotinamide adenine dinucleotide phosphate (NADP) disodium salt was obtained from Roche Diagnostics (Indianapolis, IN, USA). Benzo(a)pyrene (BaP) was obtained from Wako Pure Chemical Industries Ltd., (Osaka, Japan). Mitomycin C (MMC) was obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Giemsa solution was obtained from Merck (Darmstadt, Germany). All the chemicals used in this experiment were of analytical grade.

Cells

CHL/IU cells were obtained from the National Institute of Health Science (Tokyo, Japan). The modal chromosome number of this cell line is 25. The cells were maintained in Eagle's MEM supplemented with 10% heat-inactivated (56°C for 30 minutes) calf serum without antibiotics at 37°C in a humidified atmosphere containing 5% CO₂. The doubling time of the CHL/IU cells was estimated to be 17–18 hours under the culture conditions.

Purification of MDMA

MDMA was purified in our laboratory by the following method. Tablets containing MDMA were kindly given to us by the Kanto-Shinetsu Regional Bureau of Health and Welfare. Crushed tablets were subjected to extraction using CHCl₃, refined with acetonitrile, and finally dehydrated. CHCl₃ residues were extracted under alkaline condition. The resulting CHCl₃ layer was transferred, and HCl was added to it. The aqueous water layer was dehydrated with N₂ gas at 60°C and finally crystallized. A trifluoroacetyl derivative was detected as trifluoroacetyl MDMA by gas chromatography/mass spectrom-

etry (GC/MS). The absence of any impurities was confirmed by thin-layer chromatography, GC/MS, and an examination using a spectrometry in our laboratory.

Preparation of N-MDMA

Ando and Morita synthesized *N*-nitrosomethamphetamine (NMA) from MA (16). N-MDMA was synthesized using their method of NMA synthesis. MDMA and NaNO₂ were reacted under acidic condition induced by acetic acid. Then, CHCl₃ was added and the organic phase was evaporated in vacuo. The yield was 94%, the mass spectrum (EI) result was *m/z* 135, 77, 162, 56, 192, 105, 222(M)⁺ and the mass spectrum (CI) result was *m/z* 223(M+1)⁺, 163, 135. H-NMR chemical shifts (90 MHz, CDCl₃) were 1.42 ppm (d, 3H, -CH₃), 2.92 ppm (s, 3H, N-CH₃), 2.92 ppm (d, 2H, C₆H₃-CH₂-), 4.87 ppm (m, H, -CH-), 5.90 ppm (s, 2H, -O-CH₂-O-), and 6.65 ppm (m, 3H, C₆H₃).

Preparation of S9 mix

Rat S9 was obtained from Kikkoman Co. (Noda, Japan). Rat S9 was the S9 fraction of a liver homogenate of male Sprague-Dawley rats (6 weeks old, 215–260 g body weights) treated with phenobarbital sodium and 5,6-benzoflavone. Hamster S9 was prepared in our laboratory by the method of Ames et al. (17). Hamster S9 was the supernatant fraction obtained from the 9,000×g centrifugation of a liver homogenate of male Syrian hamsters (6 weeks old, 90–100 g body weights) treated with phenobarbital sodium and 5,6-benzoflavone. Each S9 mix contained 200 µl of 20 mM HEPES buffer solution (pH 7.2), 5 µmol of MgCl₂, 33 µmol of KCl, 5 µmol of G-6-P, 4 µmol of NADP and 300 µl of the S9 fraction in a total volume of 1 ml (18, 19). All the components except for S9 and the HEPES buffer solution were sterilized by filtration. Each S9 mix was prepared just before use.

MN and CA tests

An in vitro MN test using CHL/IU cells was established by our laboratory and improved by a collaborative validation study under the auspices on the Japanese Ministry of Health, Labor and Welfare (20, 21). In this study, the MN test was performed for MDMA and N-MDMA using this method. Cultures were incubated in triplicate for each dose. The number of micronucleated cells of 1000 cells was counted using a brightfield microscope at 400× magnification. Micronucleated cells were identified using the following criteria: the cells counted should have a normal cell morphology with clear cytoplasmic borders, the diameter of the micronuclei should not be larger than one-third of the nucleus, and bi- or poly-nucleated cells should not be scored. We used 3 dishes at each dose. One thousand cells were observed per dish, and 3000 cells were observed in all.

The CA test was performed in accordance with the guidelines for toxicity studies of drugs recommended by the Ministry of Health, Labor and Welfare with slight modifications (22). CHL/IU cells at a concentration of 1.0×10⁵ cells per 5 ml of medium were plated on 60-mm-diameter Petri dishes. After being incubating for 48 hours, the CHL/IU cells were exposed to MDMA or N-MDMA for 24 or 48 hours (24, 48 hours; continuous treatment). For a short-term treatment, the CHL/IU cells were exposed to MDMA or N-MDMA for 6 hours with or

without S9 mix (rat or hamster S9 mix) followed by a recovery period of 18 or 42 hours (6+18R, 6+42R hours; short-term treatment). The cells were treated with 0.2 µg of colcemid for 2 hours before trypsinization. After trypsinization, the cells were counted, treated with hypotonic solution (75 mM KCl), and fixed with methanol containing 25% acetic acid. Finally, the cells were spread on clean slides in high humidity. Staining was performed using the same method used for the MN test. The number of cells with CAs was recorded on 100 well-spread metaphases at 600× magnification. The aberrations were classified into 5 groups: gap (g), chromatid break (ctb), chromatid exchange (cte), chromosome break (csb), chromosome exchange (cse), and fragmentation and pulverization (others). Breaks less than the width of a sister chromatid were considered as gaps. The number of polyploidy cells was also recorded. We used 2 dishes at each dose. One hundred metaphases were observed per dish, and 200 metaphases were observed in all.

In accordance with the guidelines for toxicity studies of drugs, the maximum exposure dose of the test chemical was decided over three levels while referring to the 50% growth inhibition ratio. If cytotoxicity was not observed until 10 mM, a concentration of 10 mM (MDMA 2300 µg/ml, N-MDMA 2210 µg/ml) was decided as maximum dose (23).

Statistical procedures

In the MN test, the frequencies of MNs in the MDMA-treated and N-MDMA-treated cells were compared with those in the concurrent negative controls using Fisher's exact test. The dose-response relationship was evaluated using the Cochran-Armitage trend test (24, 25). P-values of less than 0.05 were considered to be statistically significant.

In the CA test, all experimental groups were judged to be as follows: negative if less than 4.9% of the population displayed aberrations, suspicious if between 5.0 and 9.9% of the population had aberrations, and positive if more than 10.0% of the population had aberrations. Gaps were not counted as aberrant cells.

Results

1) MN test

No significant increase in the frequency of MNs was observed after the continuous treatment with MDMA for 24, 48, or 72 hours or the short-term treatment with MDMA for 6+18R, 6+42R or 6+66R hours (Table 1). The frequencies of the MNs in the cells treated with N-MDMA are shown in Table 2. After the continuous treatment with N-MDMA for 24, 48, or 72 hours, the frequencies of MNs were found to increase in a dose-dependent manner. The highest frequencies of MNs were 24.0%, 23.7%, and 19%, respectively. After the short-term treatment with N-MDMA for 6+18R, 6+42R or 6+66R hours, the frequencies of MNs treated with N-MDMA were found to increase significantly in a dose-dependent manner with and without S9 mix. The highest frequencies of MNs were 78.0%, 66.0% and 37.3% in the absence of S9 mix, and 56.0%, 32.3%, and 24.3% in the presence of S9 mix, respectively.

2) CA test

No CA was observed in the MDMA groups after the continuous treatment for 24 or 48 hours or the short-term treatment for 6+18R or 6+42R hours (data not shown). After the continuous treatment with N-MDMA for 24 or 48 hours, the number of CAs were found to increase in a dose-dependent manner (Table 3). After the short-term treatment with N-MDMA for 6+18R or 6+42R hours without S9 mix at a concentration of 900 µg/ml, marked cytotoxicity was observed. A number of CAs were observed in the absence and presence of S9 mix (Table 4). After 6 hours of short-term treatment with an 18 hour recovery period at a concentration of 800 µg/ml, the highest percentages of aberrant cells with and without rat S9 mix were 18% and 23%.

Similar increases in the number of CAs were observed in the short-term treatment with N-MDMA with and without hamster S9 mix (Table 5). After the short-term treatment for 6+18R at a concentration of 800 µg/ml, the number of aberrant cells with hamster S9 mix was 1.6-fold higher than that with rat S9 mix. Dose-dependency was observed in all treatments. No polyploidy was observed in the MDMA and N-MDMA groups in either treatment.

Discussion

We conducted a genotoxicity study of MDMA and N-MDMA by the MN and CA tests using CHL/IU cells. We observed increased frequencies of MNs ($p < 0.05$) and CAs in cells (positive judgment) exposed to N-MDMA. However, MDMA itself showed negative results in these test systems. The frequencies of MNs and CAs induced by N-MDMA were higher in the short-term treatment than in the continuous treatment. In addition, genotoxicity was more frequently detected in the short-term treatment than in the continuous treatment (22).

In the short-term treatment, genotoxicity was observed both with and without S9 mix. The genotoxic effects without S9 mix thus suggested the possibility that intracellular enzymes of CHL/IU cells have the ability to metabolize N-MDMA into a mutagen. Therefore, N-MDMA might also be metabolized and be changed into a mutagen in humans.

After the short-term treatment with N-MDMA at a concentration of 900 µg/ml for 6+18R or 6+42R hours without S9 mix, the frequency of MNs increased significantly, but CA was not appreciated in the CA test. There were no metaphase cells, and it was not possible to judge even though the cells existed, because only the metaphase cells were analyzed by the CA test.

In the continuous treatment, CHL/IU cells were exposed to MDMA or N-MDMA for 24, 48 or 72 hours continuously without changing the culture medium. On the other hand, in the short-term treatment, the CHL/IU cells were exposed to MDMA or N-MDMA for 6 hours either with or without S9 mix and then they were cultured for 18, 42 or 66 hours with fresh culture medium. The cytotoxicity in the short-term treatment with and without S9 mix was lower than that in the continuous treatment, because of the short time exposure to the chemicals. Therefore, the maximum concentrations of MDMA and N-MDMA in the short-term treatment were higher than those in the continuous treatment.

Table 1 Micronucleus test on CHL/IU cells treated with MDMA

Treatment time (hours)	S9 mix	Concentration (µg/ml)	Growth ratio (%)	Micronucleated cells (‰)	Fisher's p-value	Trend p-value
24	–	0	100.0	9.7		p=0.3622
	–	500	62.2	9.0	0.8934	
	–	700	55.9	8.0	0.5815	
	–	900	40.7	8.7	0.7868	
	–	1100	25.0	8.0	0.5815	
	–	1300	21.3	7.7	0.4866	
	–	MMC (0.02)		27.0	p<0.0001	
48	–	0	100.0	7.3		p=0.5549
	–	100	72.3	8.0	0.8825	
	–	200	61.3	10.0	0.3297	
	–	300	51.5	9.0	0.5666	
	–	400	32.0	8.3	0.7700	
	–	500	21.1	9.0	0.5666	
	–	MMC (0.02)		74.0	p<0.0001	
72	–	0	100.0	7.7		p=0.7417
	–	100	74.2	8.7	0.7746	
	–	200	52.9	9.3	0.5742	
	–	300	44.4	8.0	1.0000	
	–	400	25.2	10.7	0.2784	
	–	500	11.5	7.7	1.0000	
	–	MMC (0.01)		147.7	p<0.0001	
6+18R	–	0	100.0	7.7		p=0.2854
	–	285	96.6	7.0	0.8800	
	–	575	95.9	7.0	0.8800	
	–	1150	80.3	6.3	0.6428	
	–	2300	55.7	9.7	0.4866	
	–	MMC (0.04)		27.0	p<0.0001	
6+18R	+	0	100.0	11.0		p=0.3545
	+	285	100.7	8.0	0.2870	
	+	575	92.9	11.3	1.0000	
	+	1150	90.7	8.3	0.3558	
	+	2300	63.6	8.3	0.3558	
	+	BaP (10)		44.0	p<0.0001	
6+42R	–	0	100.0	8.0		p=0.4606
	–	285	97.5	6.7	0.6504	
	–	575	88.9	10.3	0.4166	
	–	1150	79.4	10.7	0.3475	
	–	2300	45.0	9.0	0.7789	
	–	MMC (0.04)		26.3	p<0.0001	
6+42R	+	0	100.0	9.7		p=0.5005
	+	285	93.3	11.3	0.6128	
	+	575	91.4	11.0	0.7021	
	+	1150	75.6	9.0	0.8934	
	+	2300	45.4	12.3	0.3865	
	+	BaP (10)		41.7	p<0.0001	
6+66R	–	0	100.0	9.3		p=0.1636
	–	285	100.6	10.7	0.6975	
	–	575	95.9	7.0	0.3897	
	–	1150	70.3	8.3	0.7829	
	–	2300	53.6	6.7	0.3104	
	–	MMC (0.04)		22.0	p<0.0001	
6+66R	+	0	100.0	11.3		p=0.4537
	+	285	98.1	7.7	0.1828	
	+	575	101.3	8.0	0.2348	
	+	1150	93.8	7.7	0.1828	
	+	2300	60.5	8.3	0.2953	
	+	BaP (10)		48.0	p<0.0001	

R: Treatment with MDMA for 6 hours followed by recovery for 18, 42 or 66 hours.

MMC: Mitomycin C, B(a)P: Benzo(a)pyrene.

Table 2 Micronucleus test on CHL/IU cells treated with N-MDMA

Treatment time (hours)	S9 mix	Concentration ($\mu\text{g/ml}$)	Growth ratio (%)	Micronucleated cells (‰)	Fisher's p-value	Trend p-value
24	–	0	100.0	8.7		p<0.001
	–	200	78.9	9.7	0.7868	
	–	300	70.0	15.3	0.0237	
	–	400	59.7	23.0	p<0.0001	
	–	500	46.5	24.0	p<0.0001	
	–	600	21.4	12.3	p<0.0001	
	–	MMC (0.02)		29.5	p<0.0001	
48	–	0	100.0	9.7		p<0.001
	–	50	73.8	9.0	0.8934	
	–	100	64.7	13.7	0.1857	
	–	150	56.3	12.0	0.4546	
	–	200	41.6	23.7	p<0.0001	
	–	300	32.8	17.7	p<0.0001	
	–	MMC (0.02)		74.0	p<0.0001	
72	–	0	100.0	9.0		p<0.001
	–	50	83.8	10.7	0.6011	
	–	100	76.7	11.7	0.3717	
	–	150	64.0	13.3	0.1399	
	–	200	48.2	12.7	0.2121	
	–	300	35.3	19.0	0.0013	
	–	MMC (0.01)		42.3	p<0.0001	
6+18R	–	0	100.0	10.3		p<0.001
	–	500	83.8	10.0	1.0000	
	–	600	75.9	29.0	p<0.0001	
	–	700	64.4	33.0	p<0.0001	
	–	800	52.6	78.0	p<0.0001	
	–	900	25.0	40.3	p<0.0001	
	–	MMC (0.04)		44.0	p<0.0001	
6+18R	+	0	100.0	7.7		p<0.001
	+	500	92.2	8.0	0.8850	
	+	600	85.9	14.0	0.0241	
	+	700	73.5	27.7	p<0.0001	
	+	800	40.4	56.0	p<0.0001	
	+	900	16.9	24.0	p<0.0001	
	+	BaP (10)		35.3	p<0.0001	
6+42R	–	0	100.0	10.0		p<0.001
	–	500	93.3	10.7	0.8986	
	–	600	86.5	19.0	0.0047	
	–	700	71.2	45.0	p<0.0001	
	–	800	26.8	48.0	p<0.0001	
	–	900	15.7	66.0	p<0.0001	
	–	MMC (0.04)		37.0	p<0.0001	
6+42R	+	0	100.0	7.3		p<0.001
	+	500	88.4	11.3	0.1391	
	+	600	83.4	17.3	0.0006	
	+	700	65.4	22.0	p<0.0001	
	+	800	21.5	32.3	p<0.0001	
	+	900	11.8	31.7	p<0.0001	
	+	BaP (10)		34.0	p<0.0001	
6+66R	–	0	100.0	10.3		p<0.001
	–	500	91.9	9.7	0.8969	
	–	600	83.7	19.3	0.0052	
	–	700	58.5	37.3	p<0.0001	
	–	800	22.8	36.7	p<0.0001	
	–	900	11.6	28.3	p<0.0001	
	–	MMC (0.04)		46.0	p<0.0001	
6+66R	+	0	100.0	10.7		p<0.001
	+	500	94.1	13.0	0.4741	
	+	600	90.7	18.0	0.0171	
	+	700	78.2	21.0	0.0018	
	+	800	40.4	24.3	p<0.0001	
	+	900	22.7	17.0	0.0461	
	+	BaP (10)		35.0	p<0.0001	

R: Treatment with N-MDMA for 6 hours followed by recovery for 18, 42 or 66 hours.

MMC: Mitomycin C, B(a)P: Benzo(a)pyrene.

Table 3 Chromosomal aberration test on CHL/IU cells treated with N-MDMA; continuous treatment

Group	Time (h)	S9 mix	Concentration (µg/ml)	Growth ratio (%)	No. of metaphases	Cells with structural chromosome aberrations						No. of cells with aberrations (%)	Judge	Polyploidy %	Judge
						gap	ctb	cte	csb	cse	others				
Solvent Test substance	24	–	0	100	200	0	1	1	0	0	0	1	–	0	–
	24	–	200	70.1	200	0	0	2	0	0	0	1	–	0	–
	24	–	300	61.7	200	0	1	2	0	0	0	1.5	–	0.5	–
	24	–	400	46.8	200	0	4	9	1	0	0	7	+/-	0.5	–
	24	–	500	42.2	200	0	1	17	1	0	1	9.5	+/-	0.5	–
	24	–	600	25.4	200	0	2	2	0	0	2	2	–	0.5	–
MMC	24	–	0.08		200	0	5	63	0	0	0	29.5	+	0	–
Solvent Test substance	48	–	0	100	200	0	1	0	0	0	0	0.5	–	0	–
	48	–	50	82.9	200	0	1	3	0	2	0	3	–	0	–
	48	–	100	67.5	200	0	0	4	1	1	0	3	–	0	–
	48	–	150	41.7	200	0	2	9	1	0	0	6	+/-	0	–
	48	–	200	45.1	200	0	2	7	1	3	0	6.5	+/-	0	–
	48	–	300	34.4	200	0	5	14	0	3	0	10.5	+	0	–
MMC	48	–	0.04		200	2	14	83	0	2	0	46	+	0	–

MMC: Mitomycin C.

Table 4 Chromosomal aberration test on CHL/IU cells treated with N-MDMA; short-term treatment with and without rat S9 mix

Group	Time (h)	S9 mix	Concentration (µg/ml)	Growth ratio (%)	No. of metaphases	Cells with structural chromosome aberrations						No. of cells with aberrations (%)	Judge	Polyploidy %	Judge
						gap	ctb	cte	csb	cse	others				
Solvent Test substance	6+18R	–	0	100.0	200	0	0	1	0	0	0	0.5	–	0	–
	6+18R	–	500	74.2	200	1	1	2	0	0	0	1.5	–	0	–
	6+18R	–	600	70.0	200	0	5	10	0	1	0	8	+/-	0	–
	6+18R	–	700	53.8	200	2	2	26	0	2	0	13.5	+	0	–
	6+18R	–	800	47.4	200	1	11	45	0	0	3	22.5	+	0	–
	6+18R	–	900	3.3	Tox										
MMC	6+18R	–	0.08		200	2	8	45	0	0	0	26	+	0	–
Solvent Test substance	6+18R	+	0	100.0	200	1	0	1	0	0	0	0.5	–	0	–
	6+18R	+	500	105.7	200	1	2	4	0	1	0	3.5	–	0.5	–
	6+18R	+	600	90.7	200	1	2	9	0	2	0	6.5	+/-	0.5	–
	6+18R	+	700	68.8	200	1	4	16	0	4	0	12	+	0	–
	6+18R	+	800	35.6	200	0	6	26	1	4	0	18	+	0.5	–
	6+18R	+	900	18.1	200	0	4	12	0	0	0	8	+/-	0.5	–
B(a)P	6+18R	+	10		200	0	4	28	1	4	0	18.5	+	0	–
Solvent Test substance	6+42R	–	0	100.0	200	0	0	0	0	0	0	0	–	0	–
	6+42R	–	500	85.6	200	0	0	0	0	0	0	0	–	0	–
	6+42R	–	600	84.1	200	1	0	4	0	2	0	3	–	0	–
	6+42R	–	700	67.7	200	0	2	9	0	2	0	6	+/-	0	–
	6+42R	–	800	43.1	200	0	3	19	0	1	2	10.5	+	0.5	–
	6+42R	–	900	2.6	Tox										
MMC	6+42R	–	0.08		200	0	6	32	0	3	0	20.5	+	0	–
Solvent Test substance	6+42R	+	0	100.0	200	0	1	0	0	0	0	0.5	–	0	–
	6+42R	+	500	69.4	200	0	1	1	0	0	0	1.5	–	0	–
	6+42R	+	600	59.8	200	0	1	3	1	0	0	2.5	–	0	–
	6+42R	+	700	43.5	200	0	2	6	0	1	0	4.5	–	0	–
	6+42R	+	800	21.9	200	0	4	9	2	1	2	8	+/-	0	–
	6+42R	+	900	9.1	200	0	1	11	1	4	1	8.5	+/-	0	–
B(a)P	6+42R	+	10		200	0	5	16	0	3	0	11	+	0	–

Tox: Cytotoxicity was observed and cells showing chromosomal aberrations were not available to be counted.

R: Treatment with N-MDMA for 6 hours followed by recovery for 18 or 42 hours.

MMC: Mitomycin C, B(a)P: Benzo(a)pyrene.

Table 5 Chromosomal aberration test on CHL/IU cells treated with N-MDMA; short-term treatment with and without hamster S9 mix

Group	Time (h)	S9 mix	Concentration (μg/ml)	Growth ratio (%)	No. of metaphases	Cells with structural chromosome aberrations						No. of cells with aberrations (%)	Judge	Polyploidy %	Judge
						gap	ctb	cte	csb	cse	others				
Solvent Test substance	6+18R	–	0	100.0	200	0	0	2	0	0	0	1	–	0	–
	6+18R	–	500	82.3	200	0	0	1	0	0	0	0.5	–	0	–
	6+18R	–	600	74.8	200	0	2	9	1	0	0	5.5	–	0	–
	6+18R	–	700	52.3	200	0	11	40	0	0	0	23	+	0	–
	6+18R	–	800	37.6	200	1	17	51	1	1	0	25	+	0	–
	6+18R	–	900	2.1	Tox										
MMC	6+18R	–	0.08		200	0	11	53	0	0	0	29.5	+	0	–
Solvent Test substance	6+18R	+	0	100.0	200	0	0	1	0	0	0	0.5	–	0	–
	6+18R	+	500	90.5	200	0	1	0	0	0	0	0.5	–	0	–
	6+18R	+	600	79.7	200	3	3	6	0	0	0	4.5	–	0	–
	6+18R	+	700	60.1	200	1	11	24	1	1	0	15.5	+	0	–
	6+18R	+	800	42.4	200	0	14	55	4	1	0	28.5	+	0	–
	6+18R	+	900	20.1	200	0	6	33	0	2	1	18	+	0.5	–
B(a)P	6+18R	+	10		200	0	5	23	0	0	0	14	+	0	–
Solvent Test substance	6+42R	–	0	100.0	200	0	1	0	0	0	0	0.5	–	0	–
	6+42R	–	500	88.6	200	0	1	0	0	0	0	0.5	–	0	–
	6+42R	–	600	83.2	200	1	0	2	0	0	0	1	–	0	–
	6+42R	–	700	70.1	200	1	1	10	0	1	0	5.5	+/-	0	–
	6+42R	–	800	33.7	200	0	2	21	0	0	0	11	+	0.5	–
	6+42R	–	900	1.7	Tox										
MMC	6+42R	–	0.08		200	0	5	41	1	0	0	23.5	+	0	–
Solvent Test substance	6+42R	+	0	100.0	200	0	0	0	2	0	0	1	–	0	–
	6+42R	+	500	73.4	200	1	0	0	0	0	0	0	–	0	–
	6+42R	+	600	62.6	200	0	0	2	2	1	0	2.5	–	0	–
	6+42R	+	700	59.5	200	0	2	6	0	1	0	4.5	–	0	–
	6+42R	+	800	29.6	200	0	2	7	0	0	0	4.5	–	0	–
	6+42R	+	900	8.6	200	1	3	4	1	0	2	4	–	0	–
B(a)P	6+42R	+	10		200	0	4	17	0	1	0	11	+	0	–

Tox: Cytotoxicity was observed and cells showing chromosomal aberrations were not available to be counted.

R: Treatment with N-MDMA for 6 hours followed by recovery for 18 or 42 hours.

MMC: Mitomycin C, B(a)P: Benzo(a)pyrene.

Radiation and various mutagenic chemicals induce CAs. CAs are detected during metaphase. Radiation induces chromosome-type aberrations; however, chemical substances generally induce chromatid-type aberrations. These phenomena may be explained by the facts that damage to DNA induced by a chemical substance is still not immediately reflected and that CAs appear only after DNA undergoes a synthetic period (26). In this study, many chromatid-type aberrations were found to occur due to exposure to the chemical substance (N-MDMA), and the ratio of exchange-type chromatid aberrations out of all chromosomes or chromatid aberrations increased. The appearance of exchange-type chromatid aberrations has been often reported to be found after exposure to carcinogens (27, 28). On the other hand, MNs commonly arise from acentric chromosomal fragments or chromosomes that are not included in one of the daughter nuclei at cell division, and the frequency of MNs is considered to reflect genotoxic damage to cells (13, 29).

Many *N*-nitroso compounds have been demonstrated to be carcinogens (9). The metabolism of such compounds is considered to occur as follows. *N*-nitroso compounds are metabolized to an alkyl diazonium ion, which may damage DNA, and an aldehyde by the monooxygenase of the cytochrome P450

system. The alkyl diazonium ion is then metabolized to an alkyl carbonium ion that alkylates DNA. Finally, the alkylation of DNA induces mutation and carcinogenicity (30). In this study, the increased frequencies of MNs and CAs were only observed in N-MDMA treatment. Because N-MDMA was thought to pass through a metabolic pathway similar to that of known mutagenic *N*-nitroso compounds, the metabolites could cause DNA damage. The results of the *in vitro* MN test and the CA test using CHL/IU cells show good correlations with those of a microbial mutation test (Ames test) and carcinogenicity (20). N-MDMA carcinogenicity is thus suspected because N-MDMA genotoxicity was confirmed in this study.

Hamster S9 mix is generally considered to be better for detecting genotoxicity than rat S9 mix regarding the *N*-nitroso compound in the Ames test (31, 32). Shimizu et al. reported that the mutagenic activity of *N*-nitroso compounds (*N*-nitrosomethamphetamine) showed a fivefold increase for the use of hamster S9 mix compared with the use of rat S9 mix during the Ames test (33). In this study, the frequency of CAs was 1.6 times higher with hamster S9 mix than with rat S9 mix. This suggests that the genotoxicity of *N*-nitroso compounds increases in mammalian cell line tests using hamster S9 mix.

It is considered that N-MDMA is easily produced from MDMA under acidic conditions in the presence of nitrite. In this study, we synthesized N-MDMA from tablets containing MDMA, and N-MDMA thus appeared to be strongly genotoxic. We demonstrated that *N*-nitrosoketamine (NKT) shows strong genotoxicity using the MN test (19). However, until now, the effects of the *N*-nitroso compounds of the drug have not been studied extensively regarding drug abuse. Various secondary and tertiary amines of general medicines are regulated by the Ministry of Health, Labor and Welfare. Concerning the inges-

tion of illegal drugs, investigating the effects of not only the drug itself but also its *N*-nitroso compounds on human health is very important. There is widespread MDMA abuse among young people overwhelmingly because tablets containing MDMA can be easily obtained. The incidence of teratosis has been reported to increase when MDMA is abused by females during pregnancy (34). The findings of this genotoxicity study of N-MDMA lead us to conclude that young people should be strictly prohibited from taking tablets containing MDMA.

References

- (1) NIDA-NIH-U.S. Department of Health & Human Services. MDMA (Ecstasy): NIDA INFO FACTS, March. 2005. p. 1–3.
- (2) National Police Agency, Government of Japan. White Paper on Police 2006. 2006. p. 158–165. (Article in Japanese)
- (3) World Health Organization. Expert Committee on Drug Dependence. Twenty-Second Report. 1985. p. 24–25.
- (4) Makino Y, Tanaka S, Kurobane S, Nakauchi M, Terasaki T, Ohta S. Profiling of illegal amphetamine-type stimulant tablets in Japan. *J Health Science*. 2003;49:129–137.
- (5) Cami J, Farre M, Mas M. Human pharmacology of 3,4-methylenedioxymethamphetamine (“Ecstasy”): psychomotor performance and subjective effects. *J Clin Psychopharmacol*. 2000;20:455–466.
- (6) Nishimura Y. Recent abuse of 3,4-methylenedioxymethamphetamine (“Yaoto-wang”, “Ecstasy”). *Seishin Shinkeigaku Zasshi*. 2002;104:819–833. (Article in Japanese)
- (7) Johanlyn L, Jean LC. Methylenedioxymethamphetamine (MDMA, Ecstasy) neurotoxicity: cellular and molecular mechanisms. *Brain Res Review*. 2003;42:155–168.
- (8) Morton J. Ecstasy: pharmacology and neurotoxicity. *Curr Opin Pharmacol*. 2005;5:79–86.
- (9) Druckrey H, Preussmann R, Ivankovic S, Schmahl D, Afkham J, Blum G, et al. Organotrope carcinogene Wirkungen bei 65 verschiedenen *N*-Nitroso-Verbindungen an BD-Ratten. *Z Krebsforschung*. 1967;69:103–201.
- (10) Cardesa A, Pour P, Althoff J, Mohr U. Vascular tumors in female mice after intraperitoneal injection of dimethylnitrosamine. *JNCI*. 1973;51:201–208.
- (11) Magee PN, Barnes JM. The production of malignant primary hepatic tumors in the rat by feeding dimethylnitrosamine. *Br J Cancer*. 1956;10:114–122.
- (12) Toth B, Magee PN, Shubik P. Carcinogenesis study with dimethyl-nitrosamine administered orally to adult and subcutaneously to new born BALB/C mice. *Cancer Res*. 1964;24:1712–1721.
- (13) Heddle LA, Hite M, Kierkhart B, Mavourin K, MacGregor JT, Newell GW, et al. The induction of micronuclei as a measure of genotoxicity: a report of the US Environmental Protection Agency Gene-Tox Program. *Mutat Res*. 1983;123:61–118.
- (14) Sofuni T (Ed). *Data Book of Chromosomal Aberration Test in Vitro*. Life-science Information Center; 1998. (Article in Japanese)
- (15) Ishidate M Jr., Odashima S. Chromosome test with 134 compounds on Chinese hamster cell in vitro—a screening for chemical carcinogens. *Mutat Res*. 1977;48:337–354.
- (16) Ando H, Morita M. Possibility of in vitro and in vivo formations of *N*-nitrosomethamphetamine. Reports of National Research Institute of Police Science. 1983;36:16–20. (Article in Japanese)
- (17) Ames BN, McCann J, Yamasaki E. Method for detecting carcinogens and mutagens with the *Salmonella*/mammalian-microsome mutagenicity test. *Mutat Res*. 1975;31:347–364.
- (18) Matsuoka A, Hayashi M, Ishidate M Jr. Chromosomal aberration tests on 29 chemicals combined with S9mix in vitro. *Mutat Res*. 1979;66:277–290.
- (19) Toyama Y, Shimizu H, Suzuki Y, Miyakoshi Y, Yoshioka H. Genotoxic effects of *N*-nitrosoketamine and ketamine as assessed by in vitro micronucleus test in Chinese hamster lung fibroblast cell line. *Environ Health Prev Med*. 2006;11:120–127.
- (20) Li J, Suzuki Y, Shimizu H, Fukumoto M, Okonogi H, Nagashima T, et al. In vitro micronucleus assay of 30 chemicals in CHL cells. *Jikeikai Med J*. 1993;40:69–83.
- (21) Matsushima T, Hayashi M, Matsuoka A, Ishidate M, Miura KF, Shimizu H, et al. Validation study of the in vitro micronucleus test in a Chinese hamster lung cell line (CHL/IU). *Mutagenesis*. 1999;14:569–580.
- (22) Japanese Ministry of Health and Welfare. Guidelines for Toxicity Studies of Drugs. 1999.
- (23) Scott D, Galloway SM, Marshall RR, Ishidate M Jr., Brusick D, Ashby J, et al. Genotoxicity under extreme culture conditions. A report from ICPEMC Task Group 9. *Mutat Res*. 1991;257:147–205.
- (24) Cochran WG. Some methods for strengthening the common 2 tests. *Biometrics*. 1954;10:417–451.
- (25) Armitage P. Test for linear trends in proportions and frequencies. *Biometrics*. 1955;11: 375–386.
- (26) Savage JRK. Classification and relationship of induced chromosomal structural changes. *J Med Genet*. 1976;13:103–122.
- (27) Ishidate M Jr., Sofuni T, Yoshikawa K. Chromosomal aberration tests in vitro as a primary screening tool for environmental mutagens and/or carcinogens. *GANN Monograph on Cancer Research*. 1981;27:95–108.
- (28) Sasaki MS. Chromosome Aberration Formation and Sister Chromatid Exchange in Relation to DNA Repair in Human Cells. Plenum Press; 1980. p. 285–314.
- (29) Duncan AMV, Heddle JA, Blakey DH. Mechanism of induction of nuclear anomalies by γ -radiation in the colonic epithelium of the mouse. *Cancer Res*. 1985;45:250–252.
- (30) Tjalve H. The tissue distribution and the tissue specificity of bioactivation of some tobacco-specific and some other *N*-

- nitrosamines. Crit Rev Toxicol. 1991;21:265–294.
- (31) Araki A, Muramatsu M, Matsushima T. Comparison of mutagenicities of N-nitrosamines on *salmonella typhimurium* TA 100 and *Escherichia coli* WP2 UVRA/PKM101 using rat and hamster liver S9. Gann. 1984;75:8–16.
- (32) Raineri R, Poliey JA, Andrews AW, Pienta RJ, Lijinsky W. Greater effectiveness of hepatocyte and liver S9 preparations from hamsters than rat preparations in activating N-nitroso compounds to metabolites mutagenic to salmonella. JNCI. 1981;67:1117–1122.
- (33) Shimizu H, Takemura N, Ando H, Morita M, Machida K. Mutagenic activity of N-nitrosomethamphetamine and N-nitrosoephedrine. Cancer Lett. 1983;21:63–68.
- (34) McElhatton PR, Bateman DN, Evans C, Pughe KR, Thomas SHL. Congenital anomalies after prenatal ecstasy exposure. Lancet. 1999;354:1441–1442.