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Differentiation of the mechanism of micronuclei induced by cysteine and glutathione conjugates of methylenedi-*p*-phenyl diisocyanate from that of 4,4′-methylenedianiline

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Abstract

Methylenedi-*p*-phenyl diisocyanate (MDI) is widely used in the production of polyurethane products. Diisocyanates are reactive compounds, MDI can react under physiological conditions with various functional groups found on biological molecules resulting in conjugate formation or undergo non-enzymatic hydrolysis to form 4,4′-methylenedianiline (MDA). We have previously reported that addition of MDI directly to Chinese hamster lung fibroblasts (V79) cultures did not induce micronuclei (MN), but MDA, and the glutathione and cysteine conjugates of MDI (BisGS–MDI and BisCYS–MDI), induced a concentration-dependent increase in the frequency of MN. The conventional MN assay does not discriminate between MN produced by acentric chromosome fragments from those arising due to whole lagging chromosomes that were not incorporated into daughter nuclei at the time of cell division. The mechanism of MN induction from these potential MDI metabolites/reaction products was explored in the present study using immunofluorescent staining of kinetochore in MN of cytokinesis-blocked V79 cells. This assay discerns the presence of centromere within the MN to distinguish the MN containing centric chromosomes from those containing acentric fragments. Eighty five percent of MDA-induced MN were negative with respect to anti-kinetochore antibody binding (KC^-). This is consistent with an interaction between MDA and DNA resulting in chromosome breakage. However, BisGS–MDI and BisCYS–MDI induced a higher percentage of MN that were positively stained by the anti-kinetochore antibody (KC^+). These results suggest that the mechanism of MN formation induced by BisGS–MDI and BisCYS–MDI is mediated through disruption and/or by affecting the function of the mitotic spindle. This mechanism is distinctly different from the mechanism of MN induction by MDA. Published by Elsevier Science B.V.

Keywords: Micronucleus; Clastogens; Aneugens; Methylenedi-*p*-phenyl diisocyanate

Abbreviations: BNR, Brown Norway rat; CAs, chromosome aberrations; CYS, L-cysteine hydrochloride monohydrate; CME, L-cysteine methyl ester hydrochloride; Cyt B, cytochalasin B; GSH, glutathione; KC^-, kinetochore negative micronuclei; KC^+, kinetochore positive micronuclei; MDA, 4,4′-methylenedianiline; MDI, methylenedi-*p*-phenyl diisocyanate; BisCYS–MDI, cysteine conjugate of methylenedi-*p*-phenyl diisocyanate; BisCME–MDI, cysteine methyl ester conjugate of methylenedi-*p*-phenyl diisocyanate; BisGS–MDI, glutathione conjugate of methylenedi-*p*-phenyl diisocyanate; MDI–PPT, MDI–precipitate control; MIC, methyl isocyanate; MN, micronuclei; MNPCEs, micronucleated polychromatic erythrocytes; MMC, mitomycin C; NDI, Nuclear division index; PI, propidium iodide; SCEs, sister chromatid exchanges; TDI, toluene diisocyanate; VCR, vincristine sulfate

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1. Introduction

Methylenedi-*p*-phenyl diisocyanate (MDI) is an important industrial chemical used in the production of polyurethane products such as foams and wood binders. Isocyanates are known to induce pulmonary hypersensitivity reactions (asthma and hypersensitivity pneumonitis) and direct irritant toxic responses [1]. Genotoxic potential of aromatic diisocyanates has also been of concern. Many products may be formed, in vivo, by exposure to MDI, due to its reactivity towards functional groups found on biological molecules. MDI can react non-enzymatically under aqueous conditions to form 4,4′-methylenedianiline (MDA), react with thiols and amines under physiological conditions forming thiocarbamates and ureas, respectively, and react with hydroxyl groups in hydrophobic regions of proteins to form carbamates. Thiocarbamates are of particular interest due to their reversibility at physiological pH, resulting in liberation of free isocyanate [2]. It must be noted that thiocarbamates have not yet been identified from individuals or animals exposed to diisocyanates.

Macromolecular adducts can be formed by both MDI and MDA [3]. MDA is listed as a carcinogen in animals [4,5]. DNA double-strand breaks in leucocytes of a worker following MDI inhalation [6] and in cultured human lung epithelial cells have been reported [7]. Vock et al. concluded that MDI produced DNA double strand breaks by extragenomic mechanisms related to the process of cell death. MDI and methyl isocyanate (MIC) in a variety of in vivo and in vitro studies increased the frequencies of both sister chromatid exchanges (SCEs) and chromosomal aberrations (CAs) in Chinese hamster ovary cells and human lymphocytes, respectively [8,9]. Two genetic endpoints, CAs and SCEs, in peripheral lymphocytes were also significantly higher among human survivors following the MIC gas tragedy in India [10]. Following inhalation exposure of MIC to mice, high frequencies of SCEs and CAs in bone marrow and lung cells and peripheral blood micronucleated polychromatic erythrocytes (MNPCE) were observed [8,11]. Carcinogenicity studies of MDI exposure to rats have been negative, but an increase in pulmonary adenomas was noted [12].

These studies demonstrated that isocyanates can be genotoxic, but the mechanism by which they cause genetic damage is unclear. Previous studies in our laboratory showed that, inhalation exposure of Brown Norway rats (BNR) to MDI resulted in a concentration-related increase in the frequency of MNPCEs in bone marrow. In addition, the results from in vitro exposure of Chinese hamster lung fibroblasts (V79) to MDA, cysteine and glutathione conjugates of MDI (BisCYS–MDI and BisGS–MDI) suggested the genotoxic potential of possible MDI metabolites [13]. The formation of micronuclei (MN) is thought to be a consequence of either chromosome breakage (DNA single- and/or double-strand break) induced by clastogens and/or spindle dysfunction induced by aneugens. Detection of centromere containing MN can distinguish between the clastogen and aneugen. The aim of the present study was to further distinguish the mechanism of MN induction following exposure to MDA, BisGS–MDI, and BisCYS–MDI in cytokinesis-blocked V79 cell cultures using the anti-kinetochore immunofluorescence antibody assay [14].

2. Materials and methods

2.1. Chemicals

MDI, MDA and L-cysteine methyl ester hydrochloride (CME) were purchased from Aldrich (Milwaukee, WI). Acetonitrile (ACN), dimethyl sulfoxide (DMSO), acetone, and methanol were purchased from Fisher Scientific (Pittsburgh, PA). L-cysteine hydrochloride monohydrate (CYS), glutathione (GSH), fetal bovine serum (FBS), cytochalasin B (Cyt B), propidium iodide (PI), vincristine sulfate (VCR), mitomycin C (MMC), and *p*-phenylenediamine were obtained from Sigma (St. Louis, MO). Cell culture reagents were purchased from Gibco (Grand Island, NY). Anti-kinetochore antibody (human anti-centromere antibodies) and goat anti human IgG–FITC were purchased from Antibody Incorporated (Davis, CA).

2.2. Synthesis and characterization of cysteine and glutathione conjugates of methylenedi-p-phenyl diisocyanate

Details of the method used to make the required bis-thiocarbamate type adducts were based on previ-

ous work reported by Han et al. [15]. Addition of MDI in acetone to a two-fold molar excess of cysteine or glutathione in aqueous acetonitrile afforded a satisfactory route to the desired bis-thiocarbamate conjugates as described below. The reactions proceeded rapidly at room temperature and afforded the products in fair to good yield.

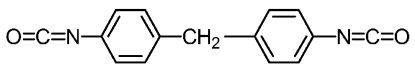
The insolubility of these adducts lead to their identification being based primarily upon comparison to solid state NMR data of the L-cysteine methyl ester conjugate of MDI (BisCME–MDI), which had been fully assigned by solution state NMR spectroscopy and further characterized by ESMS. Solution and solid state NMR spectra of BisCME–MDI showed formation of the thiocarbamate linkage was readily distinguishable by the appearance of a carbonyl signal at ~165 ppm. Solid state NMR spectra of the products obtained from the reactions of CYS and GSH with MDI showed the appearance of new carbonyl signals at 165.4 and 165.3 ppm, respectively. The presence of this characteristic carbonyl signal attributable to thiocarbamate formation, along with the remaining chemical shifts being consistent with the proposed bis-type adduct structures, provided the basis for the assignment of the respective *S*-linked conjugates of MDI. The chemical structures of MDI, MDA and the bis-type MDI adducts are shown in Fig. 1.

2.3. Preparation of BisCYS–MDI

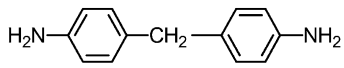
A solution of MDI (125 mg, 0.501 mmol) in acetone (2 ml) was added drop wise to a solution of L-cysteine hydrochloride monohydrate (176 mg, 1.00 mmol) in ACN/H_2O (7:3, 10 ml). The mixture was stirred at room temperature for 30 min. A white solid (104 mg, 42%) was collected by filtration washed successively with acetone and diethyl ether then dried under vacuum. Solid state NMR: δ 175.7 (CO_2H), 165.4 (SCO), 136.9 (C1 and C4), 130.9 (C2 and C6), 123.5 (C3 and C5), 52.1 (Cys–CH), 42.2 (CH_2), 33.3 (Cys–CH_2).

2.4. Preparation of BisGS–MDI

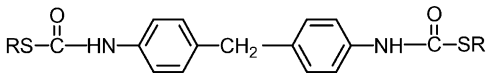
A solution of MDI (125 mg, 0.501 mmol) in acetone (2 ml) was added drop wise to a solution of glutathione (308 mg, 1.00 mmol) in ACN/H_2O (7:3, 10 ml). The conjugate was obtained in 87% (323 mg) using the method described above. Solid state NMR: δ 178.3–173.8 (Cys–CO, Glu–CONH, Gly–CO_2H and Glu–CO_2H), 165.3 (SCO), 136.9 (C1 and C4), 130.4 (C2 and C6), 120.7 (C3 and C5), 55.1 (Glu–CH), 52.7 (Cys–CH), 43.7 (Gly–CH_2 or CH_2), 41.8 (Gly–CH_2 or CH_2), 32.8–26.0 (2× Glu–CH_2 and Cys–CH_2).



MDI



MDA



RS^-	Conjugate
Cysteine	BisCYS-MDI
Cysteine methyl ester	BisCME-MDI
Glutathione	BisGS-MDI

Fig. 1. Chemical Structures of MDI, MDA and the bis-type MDI adducts.

2.5. Preparation of BisCME–MDI

A solution of MDI (251 mg, 1.00 mmol) in acetone (4 ml) was added drop wise to a solution of L-cysteine methyl ester hydrochloride (345 mg, 2.00 mmol) in ACN:H_2O (7:3, 10 ml). The solution was stirred at room temperature for 30 min. Precipitation was induced by the addition of diethyl ether and acetone. The white solid (337 mg, 65%) was collected by filtration, washed with diethyl ether and dried under vacuum. ^{1}H NMR: δ 7.41 (d, $J = 8.5$ Hz, 4H, H3 and H5), 7.13 (d, $J = 8.5$ Hz, 4H, H2 and H6), 4.40 (dd, $J = 6.2$ and 4.5 Hz, 2H, Cys–CH), 3.89 (s, 2H, CH_2), 3.86 (s, 6H, CO_2CH_3), 3.61 (dd, $J = 15.3$ and 4.5 Hz, 2H) and 3.41 (dd, $J = 15.3$ and 6.2 Hz, 2H, Cys–CH_2). ^{13}C NMR: δ 169.3 (s, CO_2CH_3), 165.3 (s, SCO), 139.0 (s, C4), 138.1 (s, C1), 130.5 (d, C2 and C6), 121.2 (d, C3 and C5), 54.7 (d, Cys–CH), 54.1 (q, CO_2CH_3), 41.7 (t, CH_2), 30.6 (t, Cys–CH_2). Solid state NMR: δ 169.1 (CO_2CH_3), 163.7 (SCO), 137.7.(C1 and C4), 129.8 (C2 and C6), 119.4 (C3 and C5), 54.1 (CO_2CH_3 and Cys–CH), 41.1 (CH_2), 30.7 (Cys–CH_2). ESMS: m/z 521 [$M + H^+$].

2.6. Instrumentation

Solid state NMR spectroscopy was performed at ambient temperature on a Bruker Advance DMX 300 NMR spectrometer equipped with a double resonance (^{13}C–^{1}H) CP/MAS probe. 100–200 mg samples were packed in 7 mm zirconia rotors and spun at 4.6–5.6 kHz. ^{13}C NMR spectra were obtained at 75.468 MHz using conventional cross polarization techniques with spinning side-band suppression and high power ^{1}H decoupling (cptossb pulse sequence, 60 kHz B1 fields, 4 ms contact time). Typically, 12 k scans were signal-averaged with 4 s recycle delays, and 0.25 Hz line broadening was applied for sensitivity enhancement. Hexamethylbenzene was used as an external chemical shift reference (17.3 ppm relative to TMS).

Solution state NMR spectra were also recorded on a Bruker DMX-300 spectrometer. ^{1}H (300.13 MHz) and ^{13}C (75.47 MHz) NMR spectra were recorded in CD_3OD. Chemical shifts were referenced internally with TMS using the following standard references for deuterated methanol: ^{1}H, 3.31 ppm; and ^{13}C, 49.15 ppm. Full NMR assignments were made using COSY, HETCORR, HMBC and NOE experiments.

Mass spectra were recorded on a Micromass Q-Tof-2 mass spectrometer, using 1% acetic acid/methanol as the solvent.

2.7. Chemical preparation and in vitro exposure

MDA, BisCYS–MDI and BisGS–MDI were prepared immediately prior to use in dry DMSO at concentrations of, 6.25, 12.5, 25 and 50 mg/ml. The exposure concentration levels were selected from our prior study [13] at final concentrations of 62.5 125, 250, and 500 μg/ml. A maximum of 150 μl of each test solution/suspension was added to 15 ml of culture medium.

A MDI precipitate control (MDI–PPT) was prepared utilizing the method described in the preparation of the *S*-linked conjugates of MDI. MDI (0.5 mM) was added drop wise into 10 ml 70% ACN, and resultant precipitate collected. Dry DMSO was used as a solvent control. Cysteine and GSH (500 μg/ml) were used as thiol controls.

VCR and MMC were dissolved in phosphate buffered saline (PBS), to make stock solutions of 60 and 40 μg/ml, respectively.

2.8. Cell line and culture

The Chinese hamster lung fibroblast (V79) cell line was supplied by Dr. C.C. Chang (Michigan State University, East Lansing, MI). Cells were maintained in 75 cm^2 flasks with 15 ml Minimum Essential Medium (MEM, Gibco, Grand Island, NY), 10% FBS, 2 mM L-glutamine, 100 U penicillin/ml, 100 μg streptomycin/ml and sub-cultured every 3–4 days by treatment with trypsin–EDTA solution (Gibco) in PBS. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 for all experiments.

Approximately, 2×10^5 V79 cells/ml per slide were seeded directly onto pre-sterilized slides, and allowed to adhere for 2 h before addition of 12 ml of MEM. Exposures were carried out 24 h after adhering the cells to the slide. Culture medium was replaced with PBS (to avoid media constituents which could react with an isocyanate), followed by addition of the appropriate test solution/suspension. After 3 h the cells were rinsed thoroughly with PBS and incubated with fresh MEM.

Cyt B was added at a final concentration of 4 μg/ml and cultures incubated for an additional 20 h. DMSO, MDI–PPT, GSH, CYS, VCR, and MMC were run concurrently as solvent, MDI, thiol, kinetochore positive (KC^+) and kinetochore negative micronuclei (KC^-) controls, respectively. Duplicate exposures were run for each concentration and 2 experiments for each test compound was performed.

2.9. Anti-kinetochore immunofluorescent antibody staining assay

The procedure used for slide preparation and immunofluorescent staining has been described by Channarayappa et al. [16]. Cultures were terminated after incubation with Cyt B by rinsing with PBS, and treated with hypotonic 0.075 M KCl. After fixation of the cells in cold methanol (−20°C) for 15 min, the cells were air dried at room temperature for a few minutes. Cell-cycle kinetic analysis (nuclear division index, NDI) to determine the cytotoxicity of chemical exposures was performed on two slides/exposure. Slides for immunofluorescent staining of kinetochore in binucleated cells were rinsed with PBS, treated with Tween 20–PBS, and cells were labeled with anti-kinetochore antibody and goat anti-human IgG antibody (FITC). Propidium iodide (PI) was used to stain the cells followed by treatment with anti-fade solution. Slides were kept in the dark at 4°C for up to 3 weeks until scoring was completed.

2.10. Scoring and criteria

A Zeiss microscope equipped with an IVF1 epi-fluorescence condenser was used to score the slides with an excitation wavelength of 480 nm, a band width of 20 nm and a barrier filter at 520 nm. Slides were randomized and coded prior to scoring to allow blinded scoring of each slide. The MN stained an orange/red color under epi-fluorescence. The kinetochore appeared yellow/green against the orange/red nucleus, while the cytoplasm appeared faint green. Duplicate cultures were run for each experiment. The number of micronuclei at each dose level was determined by scoring 2000 binucleated cells per culture and two experiments were run with a total of 4000 cells counted per treatment at a magnification of 1000×. Cytotoxicity was evaluated using the NDI parameter. NDI was determined by scoring the number of cells with one to four nuclei in 1000 V79 cells per group: $\mathrm{NDI} = [(M_1 + (2 \times M_2) + (3 \times M_3) + (4 \times M_4)]/N$.

2.11. Statistical analysis

Frequency of MN was calculated per 1000 cells. Statistical analysis was performed using the Cochran and Armitage trend test and variance *t*-test for group comparisons. Percentages of KC^+ and KC^- were compared by the Chi-square test. The statistical analysis of NDI was performed using the *t*-test. All statistical comparisons are made in reference to the DMSO control cultures.

3. Results

MN induction frequency in DMSO, MDI–PPT, and thiol controls were in a range from 11 to 20‰. The percentage of KC^+ was consistent from experiment to experiment (26–43%) for all of the above controls. All reagent controls were negative (compared to the DMSO vehicle control) for induction of MN at concentrations up to the highest concentration of 500 μg/ml. The positive KC^+ control, VCR, and KC^- control, MMC, were used to verify the ability of the assay to distinguish between MN with or without kinetochore (Table 1). MDA induced a concentration-dependent increase in the frequency of MN from 57.3‰ at 62.5 μg/ml to 80.5‰ at 500 μg/ml ($P < 0.01$). About 36% of MN in DMSO control cells were KC^+; however, MN induced by MDA had significantly fewer KC^+ ($P < 0.01$). No significant decrease in NDI was noted in the cultures treated with MDA (Table 1).

A significant concentration-related increase in MN frequency was obtained following BisGS–MDI exposure in the concentration range from 62.5 to 500 μg/ml. BisGS–MDI induced an almost 2 to 3.5-fold increase in the frequency of MN cells. Immunofluorescent kinetochore staining of MN was positive for up to 76% of the micronucleated cells (Table 2). As reported previously [13], a precipitate could be observed within the medium and cell cytoplasm at higher exposure concentrations, but these precipitates were easily distinguishable from MN by morphology and staining characteristics. The number of KC^+ in-

Table 1
Frequency of MN and percentage of KC^+ in cytokinesis-blocked V79 cells after MDA treatment

Chemical treatment	Concentration (μg/ml)	Number of MN cells (‰)[a]			KC^+ (%)	NDI[b] ± S.D.
		Mean[c] ± S.D.	KC^+ ± S.D.	KC^- ± S.D.		
DMSO	Vehicle	17.3 ± 4.6	6.3 ± 2.5	11.0 ± 2.9	36	2.1 ± 0.1
VCR	0.04	113.5 ± 6.4**	104.5 ± 7.8**	9.0 ± 1.2	92**	2.3 ± 0.0
MMC	0.04	115.0 ± 12.7**	24.0 ± 1.4**	91.0 ± 14.1**	21**	1.9 ± 0.0
MDA	62.5	48.3 ± 2.2**	7.8 ± 2.6	49.5 ± 2.6**	14**	2.0 ± 0.1
MDA	125	67.8 ± 10.9**	7.3 ± 2.9	60.5 ± 8.7**	11**	2.0 ± 0.1
MDA	250	74.8 ± 14.7**	15.0 ± 3.7	59.8 ± 18.2**	20**	1.9 ± 0.1
MDA	500	80.5 ± 9.7**	13.8 ± 5.9	66.8 ± 4.9**	17**	1.9 ± 0.1

[a] The results shown are the means of two experiments based on 4000 cells scored.
[b] Nuclear division index $= [M_1 + (2 \times M_2) + (3 \times M_3) + (4 \times M_4)]/N$, where M_1–M_4 are the number of cells with one to four nuclei and N is the total number of cells (2000).
[c] A concentration–response relationship. Trend test $Z = 12.174$; $P < 0.01$.
** $P < 0.01$ by Chi-square test and variance t-test.

Table 2
Frequency of MN and percentage of KC^+ in cytokinesis-blocked V79 cells following BisGS–MDI exposure

Chemical treatment	Concentration (μg/ml)	Number of MN cells (‰)[a]			KC^+ (%)	NDI[b] ± S.D.
		Mean[c] ± S.D.	KC^+ ± S.D.	KC^- ± S.D.		
DMSO	Vehicle	19.8 ± 0.9	6.3 ± 0.5	13.5 ± 1.3	32	1.9 ± 0.1
VCR	0.04	104.7 ± 9.1**	90.0 ± 16.1**	14.7 ± 4.0	86**	2.3 ± 0.2**
GSH	500	19.5 ± 0.7	6.5 ± 0.7	13.0 ± 1.4	33	2.0 ± 1.9
BisGS–MDI	62.5	35.3 ± 3.2**	24.0 ± 4.1**	11.3 ± 0.9	68**	1.8 ± 0.1
BisGS–MDI	125	48.3 ± 2.2**	33.8 ± 2.2**	14.5 ± 3.1	70**	1.9 ± 0.1
BisGS–MDI	250	53.5 ± 5.1**	38.0 ± 8.1**	15.5 ± 3.7	71**	1.9 ± 0.1
BisGS–MDI	500	70.0 ± 2.1**	53.3 ± 2.1**	16.8 ± 3.9	76**	1.9 ± 0.1

[a] The results shown are the means of two experiments based on 4000 cells scored.
[b] Nuclear division index $= [M_1 + (2 \times M_2) + (3 \times M_3) + (4 \times M_4)]/N$, where M_1–M_4 are the number of cells with one to four nuclei and N is the total number of cells (2000).
[c] Z-value by trend test $= 5.698$; $P < 0.01$.
** $P < 0.01$ by Chi-square test and variance t-test.

Table 3
Frequency of MN and percentage of KC^+ in V79 cells following exposure to BisCYS–MDI

Chemical treatment	Concentration (μg/ml)	Number of MN cells (‰)[a]			KC^+ (%)	NDI[b] ± S.D.
		Mean[c] ± S.D.	KC^+ ± S.D.	KC^- ± S.D.		
DMSO	Vehicle	15.3 ± 1.0	4.8 ± 0.9	10.5 ± 0.6	31	2.0 ± 0.1
VCR	0.04	74.5 ± 9.9**	61.5 ± 15.0**	13.0 ± 6.7	83**	1.9 ± 0.1
Cysteine	500	11.5 ± 0.7	3.0 ± 0.0	8.5 ± 0.7	26	1.9 ± 0.1
BisCYS–MDI	62.5	37.3 ± 4.2**	28.0 ± 2.7**	9.3 ± 1.7	75**	1.9 ± 0.1
BisCYS–MDI	125	48.5 ± 3.1**	35.0 ± 1.4**	13.5 ± 1.7	72**	1.9 ± 0.1
BisCYS–MDI	250	51.8 ± 7.9**	37.8 ± 4.3**	14.0 ± 4.2	73**	1.9 ± 0.1
BisCYS–MDI	500	63.5 ± 7.6**	48.0 ± 4.9**	15.5 ± 3.3	76**	1.9 ± 0.1

[a] The results shown are the means of two experiments based on 4000 cells scored.
[b] Nuclear division index $= [M_1 + (2 \times M_2) + (3 \times M_3) + (4 \times M_4)]/N$, where M_1–M_4 are the number of cells with one to four nuclei and N is the total number of 2000 cells.
[c] Z-value by trend test $= 5.453$; $P < 0.01$.
** $P < 0.01$ by Chi-square test and variance t-test.

Table 4
Frequency of micronuclei in V79 cells following exposure to MDI–PPT

Chemical treatment	Conc. (μg/ml)	Number of MN cells (‰)[a]			KC^+ (%)	NDI[b] ± S.D.
		Mean ± S.D.	KC^+ ± S.D.	KC^- ± S.D.		
DMSO	Vehicle	12.5 ± 3.0	4.0 ± 1.2	8.5 ± 1.9	32	1.8 ± 0.1
MDI–PPT	62.5	13.1 ± 2.2	5.3 ± 1.9	7.8 ± 0.5	41	1.8 ± 0.1
MDI–PPT	125	14.6 ± 1.7	6.3 ± 0.5	8.3 ± 1.7	43	1.7 ± 0.1
MDI–PPT	250	13.0 ± 3.4	4.5 ± 1.0	8.5 ± 2.4	35	1.7 ± 0.1
MDI–PPT	500	16.3 ± 1.7	5.8 ± 1.0	10.5 ± 2.4	36	1.8 ± 0.1

[a] The results shown are the means of two experiments based on 4000 cells scored.

[b] Nuclear division index $= [M_1 + (2 \times M_2) + (3 \times M_3) + (4 \times M_4)]/N$, where M_1–M_4 are the number of cells with one to four nuclei and N is the total number of 2000 cells.

creased four to nine-fold over that of untreated cells at concentrations from 62.5 to 500 μg/ml.

BisCYS–MDI also produced a concentration-related increase in MN frequency in the concentration range studied, with more than 70% being KC^+ (Table 3). Precipitates were noted, similar to those observed following BisGS–MDI treatment. There were no significant differences between BisGS–MDI, BisCYS–MDI and the controls with respect to the NDI (Tables 2 and 3).

Addition of MDI to the reaction solvent (70% ACN/H_2O) produced a white precipitate. As reported in Table 4, this precipitate did not induce MN, alter the percentage of KC^+, or cause significant cytotoxicity.

4. Discussion

MDI aerosol exposure was previously demonstrated to induce MNPCEs in the bone marrow of BNR and the non-enzymatic reaction products, MDA, BisGS–MDI, and BisCYS–MDI, may be potential genotoxic metabolites [13]. Systemic genotoxicity, however, was not seen in a similar MDI exposure study of Brown Norway rats [17]. The present study was undertaken to identify if the genotoxic mechanisms of MDA and the *S*-linked adducts of MDI, BisCYS–MDI and BisGS–MDI could be distinguished. The conventional MN assay [18], is useful for the screening of chromosomal damage, but it does not allow discrimination between the induction by clastogens (chemicals that interact with DNA forming chromatid or chromosomal breakage, e.g. MMC) and/or by aneugens (chemicals that disturb the mitotic apparatus causing aneuploidy, e.g. VCR). An immunocytochemical assay that labels centromere containing MN was employed to distinguish between clastogenic an aneugenic activity [14]. This assay is based on the fact that the centromere/kinetochore are morphologically distinct structures that can be visualized by fluorescent microscopy following immunocytochemical staining. Cytochalasin B was used to block cytokinesis and prevent artifactual loss of chromosomes. However, in the Cyt B-blocked binucleated cell assay, nuclei continue to divide while cells do not, so the number of cells undergoing mitotic cycles can be determined by quantifying the number of nuclei present within each cells as a NDI for cytotoxicity assessment. None of the compounds tested significantly altered the NDI indicating that they were not cytotoxic at the concentrations tested.

VCR and MMC were employed as positive controls to induce high percentages of KC^+ (83–92%) and KC^- (79%), respectively. It is known that different mechanisms of aneuploidy induction exist. VCR interferes with microtubule assembly by causing the precipitation of tubulin in an abnormal 3-D polymeric microtubule structure [19]. Vinblastine effectively induces chromosome malsegregation, and chromosome non-disjunctions [20].

MDA induced MN in V79 cells in a concentration-related manner, increasing MN three to five times over the DMSO vehicle control, and 85% of the MN were KC^- (Table 1). Thus, it appears that MDA-induced MN formation in V79 cells was due to induction of clastogenic chromosomal damage. Therefore, these MN were most likely derived from acentric chromosome fragments. Hemoglobin adducts and urine

metabolites of MDA have been found in rats exposed to MDI aerosols or treated with MDA suggesting that MDA was bioavailable after MDI exposure [21,22]. MDA in hydrolyzed plasma or urine can be used for biological monitoring of occupational dermal exposure [23]. MDA is an aromatic amine with a structure similar to that of benzidine and 4-aminobiphenyl. The biotransformation of these two compounds has been well documented [24] and they have been identified as human carcinogens [25]. MDA has been classified as a carcinogen in animals and is a suspected carcinogen in human [4].

The biological significance of aneuploidy following exposure to *S*-linked conjugates of MDI is of particular interest. Attempts to do anti-kineticore staining in our rat model did not provide satisfactory results. The resultant fluorescence was very weak and MN area much smaller than seen in the V79 cell culture studies. In addition, the systemic genotoxic affect of MDI was relatively weak. In vitro V79 culture studies were employed to investigate possible mechanisms of potential MDI metabolites/reaction products. It was observed that treatment of V79 cells with BisGS–MDI or BisCYS–MDI produced a significant increase in the frequency of MN and that the mechanism of MN induction was different from that of MDA. A high percentage of KC^+ was displayed in binucleated cells (Tables 2 and 3). This is consistent with the findings of Vock et al. [7], who suggested that MDI genotoxicity was via "epigenetic modes of action," and not likely to be as a result of reactivity toward DNA/chromosomes. Lange et al., [26] found TDI anti-sera binding to TDI-exposed human airway epithelial cultured cells was co-localized with tubulin on the cilia. This suggests a possible avidity of isocyanates for tubulin or tubulin associated proteins and is consistent with the findings of this study. In a separate study Lange et al. [27] reported the presence of BisGS–TDI adducts in human bronchoepithelial cell cultures exposed to TDI. A feasible mechanism for BisCYS–MDI and BisGS–MDI induction of aneuploidy is through regeneration of free isocyanate within the cell that could then form protein adducts. These adducts may effect microtubulin formation and/or alter the function of spindles during mitosis.

In conclusion, the present study examined, in vitro, the mechanisms of genotoxicity of the MDI reaction products/potential metabolites MDA, BisGS–MDI and BisCYS–MDI. It was found that MDA-induced MN by a distinctly different mechanism from that of BisCYS–MDI and BisGS–MDI. The *S*-linked conjugates of MDI are aneugens, but the exact mechanism by which they induce aneuploidy is not known. The formation of thiocarbamates and their subsequent toxicity at the intracellular level may be important in the development of isocyanate-induced diseases.

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