

Inhibition of Methotrexate-Induced Chromosomal Damage by Vanillin and Chlorophyllin in V79 Cells

Channa Keshava,^{1,2} Nagalakshmi Keshava,^{1,2} Wen-Zong Whong,²
Joginder Nath,¹ and Tong-man Ong^{1,2*}

¹*Genetics and Developmental Biology Program, West Virginia University, Morgantown, West Virginia*

²*Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia*

Methotrexate (MTX), a chemotherapeutic agent used to treat cancer, produces cytogenetic damage and has a cytostatic effect in a variety of test systems. Several antigenotoxic agents have been studied in various *in vitro* and *in vivo* systems. However, data are limited regarding their ability to modulate MTX-induced genotoxicity. In the present study, vanillin (VA) and chlorophyllin (CHL) were used as antigenotoxic agents to study their ability to minimize the DNA damage caused by MTX. Exponentially growing V79 Chinese hamster lung cells were treated with MTX at five different concentrations (5–100 µg/ml) with S9 activation for 6 h and post-treated with two concentrations of either VA (50 or 100 µg/ml) or CHL (50 or 100 µg/ml) for 40 h. Cytochalasin B was added for the micronucleus (MN) assay along with antigenotoxic agents to evaluate MN in binucleated cells. Chromosomal aberrations were also evaluated in parallel cultures. Results indicate that MTX alone induced a dose-dependent decrease in the nuclear division index (NDI) and the mitotic index (MI). A significant increase in percent micronucleated binucleated cells (MNBN) and percent aberrant cells (Abs) was observed. Studies using VA as an antigenotoxic agent showed a decrease in the number of MNBN (26.3–83.1%) and Abs (16.0–87.5%) with the addition of either 50 or 100 µg VA/ml. The addition of CHL also significantly reduced the number of MNBN (53.0–91.5%) at both concentrations tested. Chromosomal aberrations were also significantly reduced (41.0–83.0). These

Results of a cooperative investigation of West Virginia Agriculture and Forestry Experiment Station and NIOSH. Published with the approval of the Director of West Virginia Agriculture and Forestry Experiment Station as Scientific Paper No. 2608.

*Correspondence to: Tong-man Ong, Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, 1095 Willowdale Road, Morgantown, WV 26505-2845.

studies indicate that both VA and CHL are capable of effectively minimizing MTX-induced chromosomal damage. *Teratogenesis Carcinog. Mutagen.* 17:313–326, 1997/98. © 1998 Wiley-Liss, Inc.

Key words: methotrexate; vanillin; chlorophyllin; antigenotoxic agents; antimutagens; micronucleus; chromosomal aberrations

INTRODUCTION

Cytotoxicity of methotrexate (MTX) is generally thought to be related to the inhibition of DNA synthesis, although the precise mechanism of cell death is not known. MTX restricts the synthesis of thymidylate and of purine nucleotides by inhibiting the enzyme dihydrofolate reductase (DHFR) and, to a lesser extent, thymidylate synthetase. In cells treated with MTX, a progressive accumulation of strand breaks in mature DNA (post-replicative DNA) was detected by Li and Kaminskas [1]. They postulated that strand breaks arising from spontaneous and normally repaired DNA lesions are not repaired due to a shortage of dTTP and purine nucleotides. In vitro cytogenetic as well as cytostatic activity of MTX has been studied in a variety of test systems. MTX was found to be a clastogenic agent in tumor cells and in cultured mammalian cells and a micronucleus (MN) inducer in human bone marrow erythroblasts [2–5]. MTX competitively binds to the enzyme DHFR thereby preventing its normal function of reducing dihydrofolate to tetrahydrofolate, an essential cofactor for nucleic acid synthesis. MTX is also a mitotic inhibitor that arrests the cell cycle in interphase and leads to prolongation of metaphase. Studies by Dalen et al. [6] have shown that the mitotic index (MI) was reduced drastically in Chang cells when MTX was administered at 0.05 µg/ml or higher. In vivo studies indicate that MTX-induced MN and the MN induction were enhanced after repeated treatments compared to single treatment in male mice [7,8]. Similar studies were conducted in bone marrow and peripheral blood cells and MN induction was enhanced by multiple treatments of MTX [9–11].

Vanillin (VA) is used as a flavoring agent in foodstuffs and has been demonstrated to inhibit mutagenesis in both bacterial and mammalian cell systems [12,13]. It has been suggested that VA modifies DNA replicative and/or DNA repair systems by enhancing the error-free repair leading to suppression of mutation in *Escherichia coli*. Imanishi et al. [14] have demonstrated the antimutagenic effect of VA in cultured Chinese hamster V79 cells in vitro. The frequency of 6-thioguanine (6-TG)-resistant mutations decreased by treatment with VA. This decrease was not due to cytotoxic effects on cellular growth or killing effects on damaged cells. Inouye et al. [15] have shown that MN induced by mitomycin C (MMC) were suppressed by post-treatment with VA. It has also been shown that the suppressing effect does not represent a delay in the formation of micronucleated polychromatic erythrocytes (MNPCEs) by the cytotoxic action of VA but rather its anticlastogenic effect in vivo. When VA was given to mice from 6 to 9 h after the injection of MMC, a significant reduction in the frequency of MNPCEs was observed. Sasaki et al. [16] have shown that induction of structural chromosomal aberrations (SCAs), especially the breakage types of aberrations, provoked by MMC was significantly suppressed by post-treatment with VA in cultured Chinese hamster ovary (CHO) cells. VA suppressed the cytotoxicity induced by H₂O₂ when cells were post-treated with VA after H₂O₂

treatment. It also suppressed the chromosomal aberrations induced by H₂O₂ [17]. Thus VA acted as an antigenotoxic agent in mammalian cells both in vitro and in vivo.

During the last decade there has been an increasing interest in chlorophyllin (CHL) as an antimutagenic agent. It is antimutagenic to a wide variety of individual mutagens, including aflatoxin B₁, polycyclic aromatic hydrocarbons, direct-acting compounds, and complex mixtures, e.g., cigarette smoke condensate [18]. CHL, a sodium-copper salt of chlorophyll, is known to be an antigenotoxic agent in several in vitro and in vivo test systems [19]. In the Salmonella test, chlorophyll was suggested to be the major component accounting for antimutagenicity. CHL was a strong antimutagen in *Drosophila melanogaster*, suppressing the somatic cell mutations induced by Trp-P-2. There are studies from many laboratories on the antimutagenic properties of CHL against individual mutagens as well as against mutagenic complex mixtures [20]. Our earlier studies have shown CHL to be a potential antimutagen in environmental and dietary complex mixtures [21] and antitransforming agents [22]. Studies by Sugiyama et al. [23] have shown the suppressive effect of CHL on the genotoxic action of the polycyclic mutagen MeIQx. It is thought that CHL inhibits the mutagenicity of promutagens through formation of a chemical complex with these agents.

Although there are several studies indicating that MTX can be used for cancer therapy, limited knowledge is available on its induction of cytogenetic damage in V79 cells. Despite a substantial body of data on the protective properties of VA and CHL against a wide variety of mutagens, little is known about the role of VA and CHL in MTX-induced cytogenetic damage. As a part of our study directed toward assessment of the cytogenetic effect of MTX and the antigenotoxic potential of VA and CHL, we investigated the protective effect of VA and CHL on MTX-induced chromosomal damage concurrently using MN and SCA assays in V79 cell cultures.

MATERIALS AND METHODS

Cell Line

V79 Chinese hamster lung fibroblast cell line was obtained from Dr. C.C. Chang (Michigan State University, East Lansing). The cell line was tested and confirmed to be negative for mycoplasma. The cells were grown in 75 cm² Falcon tissue culture flasks containing 15 ml Eagle's minimum essential medium (MEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U penicillin/ml medium, and 100 µg streptomycin/ml medium (Gibco). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ and were subcultured using trypsin-EDTA solution (Gibco) in phosphate-buffered saline (PBS).

Chemicals

MTX (Sigma, St. Louis, MO) was dissolved in 0.05 M NaOH and a 2.5 mg/ml stock solution was prepared. The stock was diluted immediately prior to use. Concentrations of 5, 10, 25, 50, or 100 µg MTX/ml medium were used in this study. The cell cultures, in duplicate, were treated with MTX for 6 h. These concentrations were based upon our preliminary findings as well as those previously published [24].

VA (Sigma) was dissolved in distilled water at a concentration of 4 mg/ml, stored at room temperature, and diluted with distilled water immediately prior to use. The concentration selection for VA (50 or 100 µg/ml) was based on studies

conducted by Sasaki et al. [16] and Inoye et al. [15]. After exposure to MTX, the cultures were post-treated with VA for 40 h, a post-treatment time established by our preliminary findings, to obtain a reasonable number of binucleated (BN) cells.

CHL (Sigma) was dissolved in distilled water at a concentration of 4 mg/ml and stored at 4°C. It was diluted with distilled water immediately prior to use. The concentration selection of 50 or 100 µg CHL/ml of medium was based on the studies conducted by Abraham et al. [25]. After exposure to MTX, cell cultures were post-treated with CHL for 40 h.

Cytochalasin B (CYB; Sigma) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2 mg/ml, stored at -20°C, and diluted with PBS immediately prior to use. A final concentration of 3 µg CYB/ml culture medium [26,27] was used. CYB was added simultaneously with the post-treatment of VA or CHL. To arrest cells at metaphase for chromosomal aberration studies, Colcemid (Gibco), at a concentration of 0.025 µg/ml culture medium, was prepared in Hank's balanced salt solution with phenol red (10 µg/ml) and was added 2 h prior to harvesting cells.

The S9 microsomal fraction was prepared according to the method of Ames et al. [28], from the liver tissue of adult male rats (Sprague-Dawley) pretreated with Aroclor 1254. The S9 mix was prepared immediately before use from 30% S9 fraction and appropriate cofactors. To each flask containing 5 ml culture medium, 0.5 ml of the S9 mix was added.

MN Assay

The MN assay was performed according to the procedure described by Krishna et al. [29]. Briefly, 1–2 million cells were seeded in each 25 cm² tissue culture flask with 5 ml culture medium. The cells were allowed to attach and grow for approximately 24 h. Duplicate cultures were included for each treatment group. MTX, at varying concentrations, along with S9 mix was added to the cultures and incubated for 6 h. Following treatment, the medium with S9 mix and MTX was aspirated and flasks were rinsed twice with PBS. VA or CHL, and CYB were added simultaneously to each culture flask and incubated for an additional 40 h. After post-treatment incubation, the medium was removed, flasks were rinsed with PBS, and cells, dislodged with trypsin-EDTA solution at 37°C, were collected and centrifuged at 1,000 rpm for 5 min. The supernatant was removed and the pellet resuspended in the remaining solution. The cells were treated with 5 ml hypotonic solution (75 mM KCl, dropwise) at 37°C for 3–5 min and recentrifuged. The supernatant was removed, the pellet was resuspended, and two drops of cell suspension per slide were carefully dropped onto pre-labeled clean dry slides angled at 45° and air dried for 5 min. The slides were then dipped in absolute methanol and air dried to fix the cells. The time between hypotonic treatment and fixing is very critical, as cells continue to swell and may burst if they are not fixed in a timely fashion. The slides were stained with Diff-Quik (McGraw Park, IL). For scoring, a Carl Zeiss (Oberkochen, Germany) microscope was used. For cell cycle analysis, 400 cells per treatment group were scored for the presence of one, two, or more than two nuclei per cell and the nuclear division index (NDI) was calculated. For the MN analysis, 1,000 BN cells per treatment group were analyzed. The morphological criteria for MN scoring in BN cells were similar to those reported by Countryman and Heddle [30] and Roberts et al. [31].

SCA Analysis

The SCA assay was performed according to the procedure of Galloway et al. [32]. Initial chemical treatments were similar to those for the MN assay. After 40 h post-treatment of VA or CHL, 0.025 μg of Colcemid/ml culture medium was added to each flask to arrest cells at metaphase. Cells were harvested 2 h after addition of Colcemid and collected into 15 ml conical tubes. Five milliliters of hypotonic solution (75 mM KCl) was added dropwise to each tube and the cells were incubated for 15 min at 37°C. Approximately five drops of freshly prepared fixative (3:1 methanol:acetic acid) were added to each tube. The cells were then centrifuged for 5 min at 1,000 rpm. The supernatant was decanted and the pellet was resuspended with the remaining solution. Five milliliters of fixative was added to each tube and incubated for 20 min at 4°C, then the cells were spun for 5 min at 1,000 rpm. This step was repeated twice. Two to four drops of cell suspension were dropped onto a cold, wet slide. Following air drying at room temperature for approximately 24 h (or overnight), cells were stained with 10% Gurr's Giemsa in Gurr's phosphate buffer (pH 6.8) and 100 metaphase cells in each treatment group were analyzed for SCAs. The types of SCAs were classified according to standard cytogenetic procedures. The following types of SCAs were recorded: chromatid gaps, chromatid breaks, chromatid deletions, fragments, minutes, triradials, quadriradials, complex rearrangements, chromosome gaps, chromosome breaks, acentric fragments, double minutes, dicentrics, and rings. For cytotoxicity, the MI was calculated based on the number of metaphase cells present in a total of 1,000 cells per treatment group. All slides were coded blindly before being scored.

Statistical Analysis

The MN and SCA data were analyzed by the χ^2 test. A sequential linear dose-trend test was also performed to compare the test groups with their respective controls. Correlation coefficients (r) were calculated for MN, SCA, NDI, and MI.

RESULTS

MTX-Induced Chromosomal Damage and Its Inhibition by VA

Studies were conducted using VA as an antigenotoxic agent to modulate MTX-induced genotoxicity. Two concentrations of VA (50 or 100 $\mu\text{g}/\text{ml}$) were used in this study. Five different concentrations of MTX (5, 10, 25, 50, or 100 $\mu\text{g}/\text{ml}$) were chosen to analyze the genotoxic effects of MTX using MN and SCA assays. The results of this study indicate that there was a significant decrease in NDI when the cells were exposed to MTX alone (Fig. 1A). NDI decreased from 3.4 in the control to 1.3 in the 100 μg MTX treatment group ($r = -0.79$). The multinucleated cells decreased dramatically with increase in MTX concentration as indicated by cell cycle kinetics (Table I). Percent micronucleated binucleated (MNB) cells increased significantly as the concentration of MTX increased (Fig. 1B, $r = 0.94$).

Inhibition studies using VA showed that NDI slightly increased with the addition of VA both at 50 and 100 $\mu\text{g}/\text{ml}$ at selected MTX concentrations. The total number of MNB cells decreased in experiments in which cells were treated with 50 μg of VA; the decrease was significant at concentrations of 10, 50, and 100 μg MTX/ml. With 100 μg VA/ml, a statistically significant ($P \leq 0.01$) decrease was

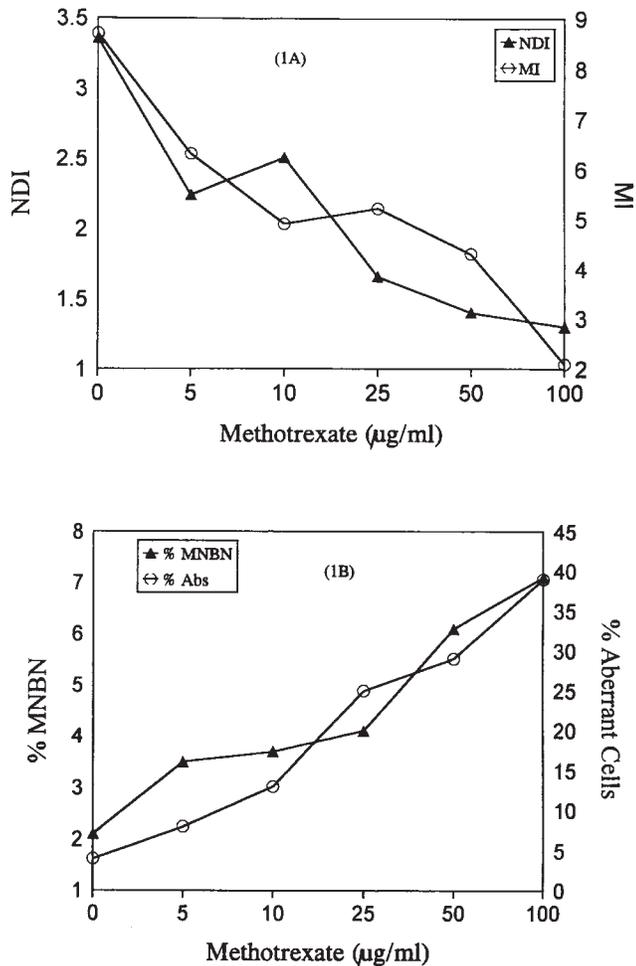


Fig. 1. Effect of MTX on cytogenetic damage in V79 Chinese hamster lung cells. **A:** Cytotoxicity as measured by NDI and MI. **B:** Clastogenicity as measured by percent MNBN and percent aberrant cells.

found in all concentrations of MTX tested. The percent MNBN inhibition ranged from 26.8 to 76.1% ($r = -0.87$) for 50 µg and from 48.7 to 83.1% ($r = -0.87$) for 100 µg of VA tested (Fig. 2A).

The SCA analysis showed that there was a significant decrease in MI with increase in MTX concentration (Table II, Fig. 1A). The correlation coefficient was -0.87 . The MI decreased from 8.7 in the control to 2.1 at 100 µg MTX/ml treatment. The total number of aberrant cells also increased significantly. There were more minutes and chromatid breaks compared to other types of aberrations. Percent aberrant cells also increased significantly ($P \leq 0.0001$) in a concentration-dependent manner ($r = 0.93$). The percent aberrant cells ranged from 4 in the control to 39 in cultures treated with 100 µg MTX/ml (Table II).

Addition of VA to the MTX-treated cultures at two different concentrations decreased aberrations. When 50 µg VA/ml was added, significant results were obtained at higher concentrations of MTX (50 or 100 µg/ml). However, when

TABLE I. MN Induced by MTX and Their Inhibition by VA in V79 Cells[†]

MTX	Concentration (µg/ml)		Cell cycle kinetics/400 cells				MNBN cells/1,000 BN cells				% MNBN cells	% MNBN inhibition
	+	VA	1N	2N	>2N	NDI ^a	1MN	2MN	>2MN	Total		
0	+	0	37	73	290	3.36	13	5	3	21	2.1	—
0	+	50	130	128	142	2.39	12	3	1	16	1.6	23.8
0	+	100	102	141	157	2.53	9	1	0	10*	1.0	52.4
5	+	0	152	124	124	2.24	28	4	3	35	3.5	—
5	+	50	107	114	179	2.63	15	5	3	23	2.3	34.3
5	+	100	107	136	157	2.52	11	1	2	14**	1.4**	60.0
10	+	0	98	152	150	2.51	30	3	4	37	3.7	—
10	+	50	129	123	148	2.42	19	2	0	21*	2.1*	43.2
10	+	100	120	116	164	2.52	16	3	0	19*	1.9*	48.7
25	+	0	166	220	14	1.66	33	6	2	41	4.1	—
25	+	50	185	209	6	1.57	23	5	2	30	3.0	26.8
25	+	100	187	205	8	1.57	12	1	0	13**	1.3**	68.3
50	+	0	257	134	9	1.40	46	10	5	61	6.1	—
50	+	50	205	184	11	1.54	18	3	1	22**	2.2**	63.9
50	+	100	180	214	6	1.58	17	3	1	21**	2.1**	65.6
100	+	0	292	103	5	1.30	51	11	9	71	7.1	—
100	+	50	266	129	5	1.36	15	1	1	17**	1.7**	76.1
100	+	100	242	150	8	1.44	10	1	1	12**	1.2**	83.1

[†]N = number of nucleus; BN = binucleated cells; MN = micronucleus; MNBN = micronucleated binucleated cells.

^aNDI = {1N + (2 × 2N) + (4 × >2N)}/400 cells scored.

*P < 0.05, **P < 0.01, significant values compared with their respective MTX concentrations using χ^2 test.

100 µg VA/ml was added, the number of aberrant cells significantly decreased for all MTX concentrations. Percent inhibition ranged from 16.0 to 53.8% in cells treated with 50 µg VA/ml and from 56.0 to 87.5% in cells treated with 100 µg VA/ml (Fig. 2B). These results clearly suggest that VA inhibits MTX-induced chromosomal damage.

MTX-Induced Chromosomal Damage and Its Inhibition by CHL

In the MN assay, MTX induced a concentration-dependent decrease in NDI (r = -0.98) (Table III). The NDI values decreased from 3.14 in the control to 1.9 at the highest concentration of MTX tested. The number of multinucleated cells decreased drastically as the concentration increased. The number of MNBN cells also increased with increase in MTX concentrations. Results of studies with CHL as an antimutagenic agent to MTX-induced damage indicated that the NDI decreased when compared to their respective MTX-treated groups. The number of multinucleated cells decreased dramatically and the number of mononucleated cells increased. However, CHL decreased the amount of chromosomal damage measured by MN formation at both concentrations tested (Table III, Fig. 3A). At 50 µg CHL/ml, a significant (P ≤ 0.01) reduction in percent MNBN cells was found in all MTX concentrations tested. Results were similar for the addition of 100 µg CHL/ml. The percent inhibition of MNBN ranged from 53.1 to 83.7 when 50 µg CHL/ml was added and from 75.0 to 91.6 with the addition of 100 µg CHL/ml. Percent inhibition was concentration dependent.

Results of SCA assay with MTX alone showed a concentration-related de-

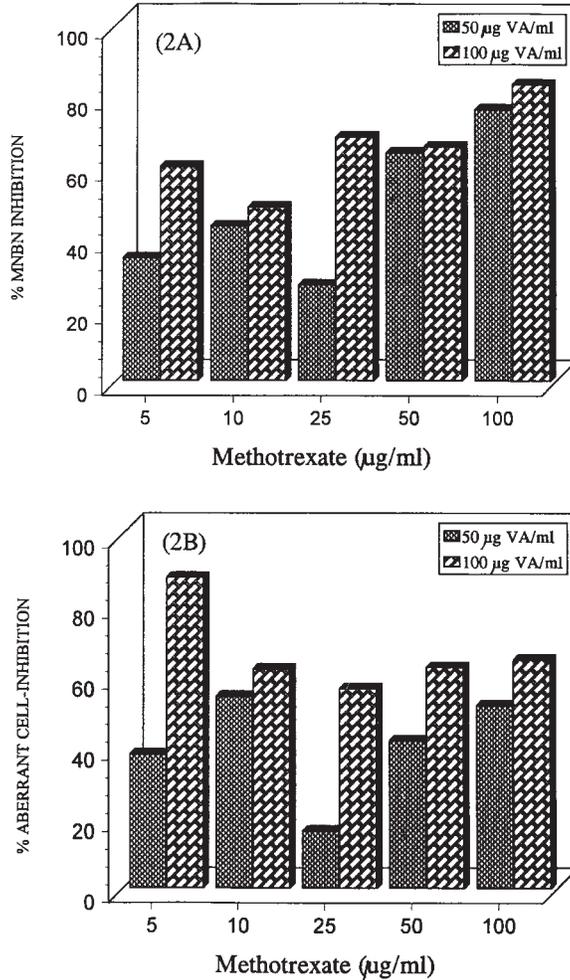


Fig. 2. Protective effect of VA on MTX-induced chromosomal damage in V79 Chinese hamster lung cells. **A:** Percent inhibition of MNBN cells. **B:** Percent inhibition of aberrant cells.

crease in MI (Table IV) ($r = -0.45$). Total aberrations/100 metaphase cells increased with increase in MTX concentration ($r = 0.98$). There were more minutes and chromatid breaks than other types of aberrations. Percent aberrant cells also increased significantly with increase in concentration. There were 8 aberrant cells/100 metaphases in the control, and this number increased to 33 in cells treated with 100 µg MTX/ml.

MI increased slightly at 10 and 100 µg MTX/ml when treated with CHL at both concentrations. Total aberrations decreased in all concentrations of MTX tested coupled with both concentrations of CHL. Percent reduction was significant at all concentrations (except 5 µg) of MTX with the addition of 100 µg CHL/ml (Table IV, Fig. 3B). These data clearly show the protective effect of CHL on MTX-induced chromosomal damage.

TABLE II. Chromosomal Aberrations Induced by MTX and Their Inhibition by VA in V79 Cells

Concentration (µg/ml)	Aberrations/100 metaphase cells ^b														Aberrations/100 metaphase cells (without gaps)	Abs ^c inhibition (%)		
	Chromatid type							Chromosome type										
MTX + CHL MI ^a	tg	tb	td	f	m	tr	qr	cr	sg	sb	af	dm	d	r				
0 + 0	8.7	0	2	0	0	1	0	0	0	2	0	0	1	0	0	4	4	—
0 + 50	6.2	1	1	0	0	2	0	0	0	0	0	0	0	0	0	3	2	50.0
0 + 100	3.4	1	1	0	0	1	0	0	0	0	0	0	0	0	0	2	2	50.0
5 + 0	6.3	1	5	1	0	1	0	0	0	0	0	0	0	0	1	8	8	—
5 + 50	4.1	1	1	0	0	2	0	0	0	2	1	0	0	1	0	5	5	37.5
5 + 100	2.3	2	0	0	0	1	0	0	0	0	0	0	0	0	0	1	1*	87.5
10 + 0	4.9	1	5	1	0	3	0	0	0	2	1	0	4	1	0	15	13	—
10 + 50	3.6	3	2	0	0	2	0	0	0	2	1	0	1	1	0	7	6	53.8
10 + 100	3.4	1	3	0	0	1	0	0	0	2	1	0	1	0	0	8	5*	61.5
25 + 0	5.2	8	24	3	2	8	2	3	1	9	6	1	1	0	0	51	25	—
25 + 50	3.6	7	12	2	3	9	2	0	1	4	1	1	3	1	3	38	21	16.0
25 + 100	3.5	2	4	3	2	10	1	0	0	1	0	0	4	0	0	21	11**	56.0
50 + 0	4.3	5	5	3	2	20	0	0	0	2	4	0	5	7	3	49	29	—
50 + 50	3.3	2	2	1	0	14	0	0	0	1	1	1	2	2	0	23	17*	41.4
50 + 100	3.4	2	2	0	1	6	0	0	0	2	0	0	3	1	0	13	11**	62.1
100 + 0	2.1	21	28	6	2	18	4	0	0	11	6	2	5	6	2	79	39	—
100 + 50	2.9	12	13	0	1	9	1	0	1	7	2	0	2	2	0	31	19**	51.3
100 + 100	2.7	4	4	1	0	4	0	0	0	3	1	0	0	3	0	13	14**	64.1

^aMI, number of metaphase cells × 100/1,000 cells scored.

^btg, chromatid gap; tb, chromatid break; td, chromatid deletion; f, fragment; m, minute; tr, triradial; qr, quadriradial; cr, complex rearrangements; sg, chromosome gap; sb, chromosome break; af, acentric fragment; dm, double minute; d, dicentric; r, ring.

^cAbs = aberrant cells.

P* < 0.05, *P* < 0.01, significant values compared with their respective MTX concentrations using χ^2 test.

DISCUSSION

Studies on MTX-Induced Genotoxicity

Significant reduction in NDI was observed in MTX-treated cells. This reduction can be explained by the fact that MTX inhibits synthesis of thymidylate, purines, and glycine due to its inhibitory effect on the enzyme DHFR in vivo [4]. Induction of MN by MTX has been observed in several studies. Kasahara et al. [10] have shown that the frequencies of micronucleated reticulocytes increased in a dose-dependent manner. The present study produced similar results with V79 cell lines. Single treatment of MTX increased MN slightly and multiple injections of MTX not only induced more MN but also severely reduced the percentage of polychromatic erythrocytes when compared to single injection [9]. It is reasonable to assume that the reduction of DHFR activity occurred due to intracellular accumulation of MTX. It has been suggested that MTX enters bone marrow cells, binds to DHFR, and completely inhibits the activity of this enzyme. The continuous inhibition of DHFR activity might cause an imbalance in the dNTP pools due to the storage of thymidylate and purine nucleotides and, as a consequence lead to DNA lesions [1]. Another possible mechanism, especially in the case of a single dose of MTX, may be that the decrease in the deoxyribonucleotide triphosphate (dNTP) pool is supplemented by a salvage pathway. However, because insufficient dNTP pool remains, the DNA lesions induced by MTX genotoxicity present themselves as MN. Several in vivo studies

TABLE III. MN Induced by MTX and Their Inhibition by CHL in V79 Cells[†]

Concentration ($\mu\text{g/ml}$)			Cell cycle kinetics/400 cells				MNBN cells/ 1,000 BN cells				% MNBN cells	% MNBN inhibition
MTX	+	CHL	1N	2N	>2N	NDI	1MN	2MN	>2MN	Total		
0	+	0	57	86	257	3.14	22	4	2	28	2.8	—
0	+	50	117	152	131	2.36	11	2	0	13	1.3	53.6
0	+	100	110	141	149	2.47	9	1	1	11*	1.1*	60.7
5	+	0	86	69	145	3.01	44	13	7	64	6.4	—
5	+	50	109	143	148	2.47	20	7	3	30*	3.0*	53.1
5	+	100	121	138	141	2.40	12	2	2	16*	1.6*	75.0
10	+	0	89	71	240	2.97	61	20	7	88	8.8	—
10	+	50	140	167	93	2.12	15	4	0	19*	1.9*	78.4
10	+	100	311	81	8	1.26	9	1	0	10*	1.0*	88.6
25	+	0	88	87	225	2.90	66	17	9	92	9.2	—
25	+	50	170	131	99	2.07	10	1	4	15*	1.5*	83.7
25	+	100	304	87*	9	1.29	6	2	1	9*	0.9*	90.2
50	+	0	119	76	205	2.73	69	19	7	95	9.5	—
50	+	50	165	165	70	1.94	15	3	0	18*	1.8*	81.1
50	+	100	291	96	13	1.34	6	2	0	8*	0.8*	91.6
100	+	0	136	216	48	1.90	48	18	33	99	9.9	—
100	+	50	154	159	87	2.05	18	1	2	21*	2.1*	78.8
100	+	100	352	48	0	1.12	9	1	0	10*	1.0*	89.9

[†]Abbreviations are defined in Table I.

* $P < 0.01$, significant values compared with their respective MTX concentrations using χ^2 test.

have shown that multiple doses of MTX-induced a dose-related increase in MN [7–11]. The multiple-dose effect on the induction of MN by MTX might be explained by the intracellular accumulation of the drug resulting in an imbalance or decrease in the dNTP pool generated by DHFR enzyme inhibition.

It is well established that MTX is a mitotic inhibitor which arrests cell cycle in interphase and leads to prolongation of metaphase in several cell types. Prolongation or complete block of the metaphase stage of the cell cycle was demonstrated after the addition of three different concentrations of MTX, namely 0.05, 3.2, and 204 μg to Chang cells in vitro [6]. In growing cells which enter the S phase of the cell cycle, deoxyribonucleoside triphosphate is needed for both DNA replication and DNA repair. Thus, strand breaks most likely occurred in mature DNA molecules as a result of defective repair caused by the restriction of supply of dTTP and of purine nucleotides. The need for constant repair in mature DNA molecules arises from a relatively frequent occurrence of DNA lesions. It should not be surprising that insufficient DNA repair due to decreased availability of deoxyribonucleoside triphosphates would result in DNA strand breaks.

Studies using VA as an antigenotoxic agent showed that VA significantly reduced both MN and SCA induced by MTX. These results are in agreement with other studies [16,17]. Similar studies were conducted using folic acid and VA as antigenotoxic agents to prevent the chromosomal damage induced by radiation [33,34]. Although the exact mechanism of anticlastogenicity of VA is still unknown, Sasaki et al. [35] have suggested that the anticlastogenic effect of VA may be due to enhanced repair of DNA strand breaks. Ultraviolet light (UV)-induced breakage type aberrations were suppressed by VA in G2 phase, which may support the correlation between anticlastogenicity of VA and post-replicative repair. DNA strand breaks

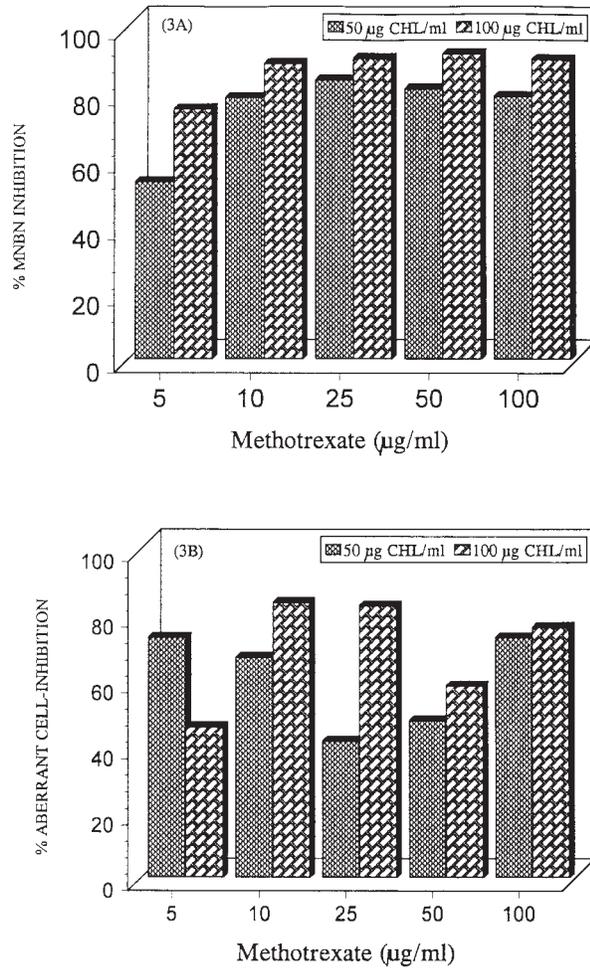


Fig. 3. Protective effect of CHL on MTX-induced chromosomal damage in V79 Chinese hamster lung cells. **A:** Percent inhibition of MNBN cells. **B:** Percent inhibition of aberrant cells.

appear to be poorly repaired by MTX-treated cells. Imanishi et al. [14] reported that the frequency of 6-TG-resistant mutations was decreased by post-treatment with 0.1 mM VA during the expression time in cultured Chinese hamster V79 cells. VA has also been shown to inhibit the genotoxic effect of chemicals. These studies are in agreement with the present study where there is a significant decrease in both MN and SCA.

The anticlastogenic effect of VA may be due to its ability to promote the rejoining process of DNA strand breaks in which DNA polymerase β may act [17]. In mammalian cells, key enzymes involved in DNA repair are polymerases α and β . Polymerase β acts throughout the cell cycle and the anticlastogenic activity of VA is correlated with pre- and post-replicative repair of double strand breaks and single strand breaks. A reduction in frequencies of MN and SCA seen in the present study by VA, therefore, may be due to the promotion of DNA rejoining.

The mechanisms by which CHL and other porphyrins exert their antimutagenic

TABLE IV. Chromosomal Aberrations Induced by MTX and Their Inhibition by CHL in V79 Cells[†]

Concentration ($\mu\text{g/ml}$)			Aberrations/100 metaphase cells													Aberrations/100 metaphase cells (without gaps)	Abs inhibition (%)	Abs inhibition (%)
MTX	+ CHL	MI	Chromatid type						Chromosome type									
			tg	tb	td	f	m	tr	qr	sg	sb	af	dm	d	r			
0	+	0	9.8	0	0	0	10	0	0	0	0	0	3	0	1	14	8	—
0	+	50	5.4	2	4	0	0	4	0	0	0	0	2	1	1	12	10	25.0
0	+	100	1.3	0	0	0	0	2	0	0	0	0	0	0	1	5	5	37.5
5	+	0	6.0	1	2	0	1	14	0	0	0	0	4	1	0	22	11	—
5	+	50	3.1	1	0	0	0	4	0	0	0	0	0	0	0	4	3*	72.7
5	+	100	1.3	8	1	0	0	6	0	0	2	0	0	1	0	8	6	45.5
10	+	0	5.0	1	2	0	0	16	0	0	2	0	0	3	1	22	12	—
10	+	50	5.4	4	1	0	0	1	0	0	0	0	0	2	2	6	4*	66.7
10	+	100	5.7	3	0	0	0	3	0	0	1	0	0	0	0	3	2*	83.3
25	+	0	5.0	3	4	0	1	19	0	0	2	0	0	6	1	31	17	—
25	+	50	4.0	7	3	0	0	4	0	0	1	0	0	2	2	13	10	41.2
25	+	100	3.1	4	0	0	0	6	0	0	4	0	0	0	2	8	3*	82.4
50	+	0	4.9	2	4	0	0	18	0	0	3	0	0	2	3	27	19	—
50	+	50	5.0	6	4	4	0	2	0	0	7	0	0	0	2	12	10	47.4
50	+	100	4.6	4	0	0	1	7	0	0	3	0	0	1	0	9	8*	57.9
100	+	0	4.8	18	38	6	3	12	2	1	11	3	3	3	2	76	33	—
100	+	50	6.9	4	3	0	0	7	0	1	0	1	0	0	0	12	9**	72.7
100	+	100	4.7	4	2	1	0	7	0	0	1	0	0	0	0	10	8**	75.8

[†]Abbreviations are defined in Table II.

* $P < 0.05$, ** $P < 0.01$, significant values compared with their respective MTX concentrations using χ^2 test.

activities are not entirely clear at present. Scavenging of radicals or suppression of metabolic activation has been suggested as two possible mechanisms [18]. CHL is known to inhibit the mutagenicity of a variety of compounds. It has been shown to inhibit mutagenicity seen in *Salmonella typhimurium* induced by a variety of complex mixtures. Most of the studies in the literature support the hypothesis that CHL acts through complex formation with promutagens or ultimate mutagens. The antimutagenicity of CHL with respect to Benzo[a]pyrene and its metabolites can best be explained by such complex formation. CHL is also known to be an antioxidant. In terms of its in vitro antimutagenic activity, it has been found to be more effective than many of the well-known antioxidants such as retinol β -carotenes, ascorbic acid, and α -tocopherol [36]. The results of the present investigation show that CHL has an in vitro protective effect. One cannot rule out the possibility of CHL exerting its protective effect by inhibiting the activation process, in addition to other mechanisms. Scavenging of radicals and/or interaction with the active group of mutagenic compounds may be responsible for its antimutagenic activity.

REFERENCES

1. Li J, Kaminskas E: Accumulation of DNA strand breaks and methotrexate cytotoxicity. Proc Natl Acad Sci USA 81:5694–5698, 1984.
2. Mondello C, Giorgi R, Nuzzo F: Chromosomal effects of methotrexate on cultured human lymphocytes. Mutat Res 139:67–70, 1984.
3. Benedict WP, Banerjee A, Gardner A, Jones PA: Induction of morphological transformation in mouse C3H/10T1/2 clone 8 cells and chromosomal damage in hamster A (T1)C1-3 cells by cancer chemotherapeutic agents. Cancer Res 37:2202–2208, 1977.

4. McBurney MW, Whitmore GF: Mechanism of growth inhibition by methotrexate. *Cancer Res* 35:586–590, 1975.
5. Polyzos A, Tsavaris N, Giannopoulos N, Archimandritis A, Papachristodoulou A, Kosmidis P: Biochemical modulation of fluorouracil: Comparison of methotrexate, folinic acid, and fluorouracil versus folinic acid and fluorouracil in advanced colorectal cancer. A randomized trial. *Cancer Chemother Pharmacol* 38:292–297, 1996.
6. Dalen H, Oftebro R, Engeset A: The effect of methotrexate and leucovorin on cell division in Chang cells. *Cancer* 18:41–48, 1965.
7. Yamamoto KI, Kikuchi Y: Studies on micronuclei time response and on the effects of multiple treatment of mutagens on induction of micronuclei. *Mutat Res* 90:163–173, 1981.
8. Hayashi M, Sofuni T, Morita T: Simulation study of the effects of multiple treatments in the mouse bone marrow micronucleus test. *Mutat Res* 252:281–287, 1991.
9. Kasahara Y, Nakai Y, Miura D, Yagi K, Hirabayashi K, Makita T: Mechanisms of induction of micronuclei and chromosome aberrations in mouse bone marrow by multiple treatments of methotrexate. *Mutat Res* 280:117–128, 1992.
10. Kasahara Y, Wakata A, Nakai Y, Yuno K, Miura D, Yagi K, Hirabayashi K, Makita T: The micronucleus test using peripheral blood reticulocytes from methotrexate-treated mice. *Mutat Res* 278:145–151, 1992.
11. Kasahara Y, Nakai Y, Miura D, Kanatani H, Yagi K, Hirabayashi K, Takahashi Y, Izawa Y: Decrease in deoxyribonucleotide triphosphate pools and induction of alkaline labile sites in mouse bone marrow cells by multiple treatments with methotrexate. *Mutat Res* 319:143–149, 1993.
12. Ohta T: Modification of genotoxicity by naturally occurring flavorings and their derivatives. *Crit Rev Toxicol* 23:127–146, 1993.
13. Ferguson LR: Antimutagens as cancer chemopreventive agents in the diet. *Mutat Res* 307:395–410, 1994.
14. Imanishi H, Sasaki YF, Matsumoto K, Watanabe M, Ohta T, Shirasu Y, Tutikawa K: Suppression of 6-TG-resistant mutations in V79 cells and recessive spot formation in mice by vanillin. *Mutat Res* 243:151–158, 1990.
15. Inouye T, Sasaki YF, Imanishi H, Watanabe M, Ohta T, Shirasu Y: Suppression of mitomycin C-induced micronuclei in mouse bone marrow cells by post treatment with vanillin. *Mutat Res* 202:93–95, 1988.
16. Sasaki YF, Imanishi H, Ohta T, Shirasu Y: Effect of vanillin on sister-chromatid exchanges and chromosome aberrations induced by mitomycin C in cultured Chinese hamster ovary cells. *Mutat Res* 191:193–200, 1987.
17. Tamai K, Tezuka H, Kuroda Y: Different modifications by vanillin in cytotoxicity and genetic changes induced by EMS and H₂O₂ in cultured Chinese hamster cells. *Mutat Res* 268:231–237, 1992.
18. Waters MD, Stack HF, Jackson MA, Brockman HE, De Flora S: Activity profiles of antimutagens: In vitro and in vivo data. *Mutat Res* 350:109–129, 1996.
19. Sarkar D, Sharma A, Talukder G: Chlorophyll and chlorophyllin as modifiers of genotoxic effects. *Mutat Res* 318:239–247, 1994.
20. Arimoto S, Fukuoka S, Itome C, Nakano H, Rai H, Hayatsu H: Binding of polycyclic planar mutagens to chlorophyllin resulting in inhibition of the mutagenic activity. *Mutat Res* 287:293–305, 1993.
21. Ong T, Whong W-Z, Stewart J, Brockman HE: Chlorophyllin: A potent antimutagen against environmental and dietary complex mixtures. *Mutat Res* 173:111–115, 1986.
22. Wu ZL, Chen JK, Ong T, Brockman HE, Whong W-Z: Antitransforming activity of chlorophyllin against selected carcinogens and complex mixtures. *Teratogen Carcinog Mutagen* 14:75–81, 1994.
23. Sugiyama C, Shinoda A, Hayatsu H, Negishi T: Inhibition of 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline-mediated DNA-adduct formation by chlorophyllin in *Drosophila*. *Jpn J Cancer Res* 87:325–328, 1996.
24. Pienkowska K, Koziarowska J: Influence of folinic acid (leucovorin) on the cytostatic and cytogenetic effects of methotrexate. *Folia Med Cracov* 27:239–244, 1986.
25. Abraham SK, Sarma L, Kesavan PC: Role of chlorophyllin as an in vivo anticlastogen: Protection against gamma-radiation and chemical clastogens. *Mutat Res* 322:209–212, 1994.
26. Fenech M, Morley AA: Measurement of micronuclei in lymphocytes. *Mutat Res* 147:29–36, 1985.
27. Wakata A, Sasaki M: Measurement of micronuclei by cytokinesis-block method in cultured Chi-

- nese hamster cells: Comparison with types and rates of chromosome aberrations. *Mutat Res* 190:51–57, 1987.
28. Ames B, McCann J, Yamasaki E: Methods for detecting carcinogens and mutagens with the *Salmonella/mammalian-microsome* mutagenicity test. *Mutat Res* 31:347–364, 1975.
 29. Krishna G, Kropko ML, Theiss JC: Use of the cytokinesis-block method for the analysis of micronuclei in V79 Chinese hamster lung cells: Results with mitomycin C and cyclophosphamide. *Mutat Res* 222:63–69, 1989.
 30. Countryman P, Heddle JA: The production of micronuclei from chromosome aberrations in irradiated cultures of human lymphocytes. *Mutat Res* 41:321–332, 1976.
 31. Roberts CJ, Morgan GR, Holt PD: A critical comparison of the micronucleus yield from high and low LET irradiation of plateau phase cell population. *Mutat Res* 160:237–242, 1986.
 32. Galloway SM, Aardema MJ, Ishidate M Jr, Ivett JL, Kirkland DJ, Mortia T, Mosesso P, Sofuni T: Report from working group on in vitro tests for chromosomal aberrations. *Mutat Res* 312:241–261, 1994.
 33. Keshava C, Nagalakshmi R, Ong T, Nath J: Inhibitory effect of folic acid on radiation-induced micronuclei and chromosomal aberrations in V79 cells. *Mutat Res* 352:123–134, 1996.
 34. Nath J, Keshava C, Ong T: Protective effect of vanillin on radiation-induced micronuclei and chromosomal aberrations in V79 cells. *Environ Mol Mutagen* 27(Suppl 27):51, 1996.
 35. Sasaki YF, Imanishi H, Watanabe M, Ohta T, Shirasu Y: Suppressing effect of antimutagenic flavorings on chromosome aberrations induced by UV-light or X-rays in cultured Chinese hamster cells. *Mutat Res* 229:1–10, 1990.
 36. Ong T, Whong W-Z, Stewart J, Brockman HE: Comparative antimutagenicity of 5 compounds against 5 mutagenic complex mixtures in *Salmonella typhimurium* strain TA 98. *Mutat Res* 222:19–25, 1989.