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Effect of tetrandrine on micronucleus formation and sister-chromatid exchange in both in vitro and in vivo assays

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Summary

The genotoxicity of tetrandrine, a drug potentially useful for the treatment of silicosis, was studied using the micronucleus and the sister-chromatid exchange (SCE) assay systems. Cultured Chinese hamster lung (V79) cells were used for the in vitro micronucleus and sister-chromatid exchange studies. Mouse bone marrow was used for the in vivo micronucleus assay and mouse spleen cells for the in vivo/in vitro sister-chromatid exchange analysis. The results show that SCE levels in V79 and in spleen cells were significantly elevated by treatment with tetrandrine at doses above 0.08 mg/ml and 100 mg/kg bw, respectively. Increased tetrandrine-induced SCE in vitro was metabolic activation dependent. Tetrandrine failed to induce micronuclei at any of the doses tested. A decrease of replicative index with an increase in the concentration of tetrandrine was found both in vitro and in vivo. These results indicate that tetrandrine is a weak indirect-acting genotoxicant.

Tetrandrine, one of the bisbenzylisoquinoline alkaloids extracted from Chinese herbaceous medicine Hang Fong Ji (*Stephania tetrand*), has been shown to inhibit the development of silicosis and to reduce fibrogenesis in experimental animals and silicotic patients (Li et al., 1982; Lu et al., 1983). It also possesses antitumor, antibacterial, antiinflammatory (aseptic), and hypotensive activities (Berezhinskaya et al., 1971; Vichkanova et al., 1973; Gralla et al., 1974; Kuroda et al., 1976; Liao, 1980). In China, tetrandrine has been used in clinical treatments of silicosis, high blood pressure and tumors (Deconti et al., 1975; Dong et al., 1982; Lu et al., 1983).

Toxicity studies of tetrandrine in animals and patients have revealed that this drug, at low concentrations, is non-toxic (Li et al., 1982; Seow et al., 1986). At high concentrations and/or after a long-term treatment, however, tetrandrine causes an acute hypotension, lymphoid tissue necrosis, severe hepatotoxicity, and nephrotoxicity (Gralla et al., 1974; Li et al., 1982; Lu et al., 1983; Zhu et al., 1985). Although several toxicity studies have been reported, information regarding the potential genotoxic and carcinogenic hazards of tetrandrine for treated patients is very limited.

The genotoxicity of tetrandrine has been studied in our laboratory by using the micronucleus and the sister-chromatid exchange (SCE) assay systems. Cultured Chinese hamster lung (V79) cells were treated in vitro with tetrandrine to study

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both genetic endpoints. Mouse bone marrows were used for the in vivo micronucleus assay while spleen cells from mice were used for the in vivo/in vitro SCE analysis. The results of these studies are presented in this report.

Materials and methods

Animals

Male CD_1 mice, 2–3 months old and weighing 25–30 g, were purchased from Charles River Breeding Laboratories (Wilmington, MA). Mice were housed in cages containing hardwood chip bedding and excelsior nesting material. A commercial Purina certified laboratory rodent chow (St. Louis, MO) and water were provided ad libitum during the period of animal holding and experimentation.

Cell line

The male Chinese hamster lung fibroblast cell line (V79) was obtained from Dr. C.C. Chang (Michigan State University, MI). In all experiments, cells were seeded into 75-cm^2 tissue culture flasks with 15 ml Eagle's minimal essential medium (MEM) supplemented with fetal calf serum (FCS) (10%) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) and were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Chemical preparation and treatment

Tetrandrine was provided by Dr. H. Jiang (Shanghai Institute of Industrial Hygiene and Occupational Disease, Shanghai, China). Tetrandrine was dissolved in distilled water to form a final concentration of 15 mg/ml. To accelerate dissolution, 2–3 drops of 1 N HCl were added and then adjusted to pH 6.5 with saturated NaOH solution.

Groups of animals were treated with a single intraperitoneal injection of tetrandrine (0, 50, 100 and 200 mg/kg bw). The doses selected for the study were based on available information (Dong et al., 1982). 5 and 3 animals per dose were used for the micronucleus and the SCE assays, respectively.

Cyclophosphamide (CPA), mitomycin C (MMC), and cytochalasin B (CYB) were purchased from Sigma Chemical Company (St. Louis, MO). Triethylenemelamine (TEM) was obtained from Polysciences Laboratory (Warrington, PA). CPA, TEM, and MMC, dissolved in phosphate-buffered saline (PBS, pH 7.0), were used as positive controls, while PBS or dimethyl sulfoxide (DMSO) was used as a negative control. CYB, used for cytokinesis block, was dissolved in DMSO and diluted further with Hanks' balanced salt solution.

In vitro sister-chromatid exchange assay

After incubation for 24 h, the cells were exposed to tetrandrine with or without S9 mix for 3 h and then washed 2 times with PBS. Fresh medium (15 ml) and 5-bromodeoxyuridine (BrdU, 12.5 μM) were added to each flask and the flasks were incubated for 40 h. Colcemid was then added and 3 h later the cells were harvested, subjected to a hypotonic treatment with 0.075 M KCl and fixations with methanol–acetic acid (3 : 1 v/v) at 4°C. The cells were dropped onto slides and air-dried. The method of staining for SCE analysis was a modified technique of Perry and Wolff (1974).

In brief, the slides were stained with the fluorescent dye Hoechst 33258 for 15 min, exposed to UV (15 W) for 15 min at a distance of 1 cm while immersed in Sørenson's buffer, then rinsed and stained with 7% Giemsa for 20 min.

In vitro micronucleus assay

Cytokinesis block by cytochalasin B for the micronucleus test (Fenech and Morley, 1986) was used for in vitro micronucleus studies. This procedure makes scoring of micronuclei more precise than by the conventional method because the enumeration of micronuclei is restricted to the binucleated cells. 3-day-old V79 cultures (approximately 3×10^6 cells in a 75-cm^2 flask) were treated with different concentrations (10–40 μg/ml) of tetrandrine in the presence or absence of S9. In the absence of activation, CYB (3 μg/ml) was added after 4 h incubation and the cells were incubated continuously for 24 h. For metabolic activation, the cells were washed twice with PBS after 2 h treatment with tetrandrine. 15 ml of growth medium containing 10% FCS and 3 μg CYB/ml was then added, and incubation was continued for 22 h. The cells were then harvested by trypsinization. After centrifugation ($285 \times g$, 5 min), the pellets were resuspended in 0.075 M KCl

for 10 min, centrifuged again, and slides prepared. The slides were fixed with methanol and stained with Diff-Quik solution I (Fisher Scientific, Orangeburg, NY) for 8 sec and solution II for 5 sec.

In vivo micronucleus assay

24 h after chemical treatment, the mice were sacrificed by cervical dislocation. Femora were removed and marrow was flushed from bones into 15-ml centrifuge tubes with 3 ml of FCS. The cell suspension was centrifuged at 285 × *g* for 6 min. The supernatant except for a few drops was removed. One drop of resuspended cells was placed onto each slide and then smeared. Dried slides were fixed in absolute methanol for 15 min, then placed into May–Gruenwald stain for 17 min. Slides were rinsed with distilled water and put in 5% Azure B Giemsa for 5–6 min, agitated in Gurr's buffer for 10–20 sec, then rinsed again and air-dried.

In vivo / in vitro sister-chromatid exchange assay

Spleens from the same animals used for micronucleus studies were used for the in vivo/in vitro SCE test. The methods of Krishna et al. (1986) for cell preparation and culturing were followed. Approximately 1.5×10^7 cells were cultured in a 25-cm^2 tissue culture flask containing 5 ml RPMI 1640 and necessary supplements. The flasks were then covered with aluminum foil, and incubated at 37 °C with 98% relative humidity. After 36 h incubation, colcemid (0.1 μg/ml) was added, and the cells were harvested 4 h later and were treated with hypotonic solution and fixed with methanol–acetic acid. The slide preparation and staining were the same as the in vitro SCE test.

Scoring

25 well-differentiated second division (M_2) cells, each containing at least 38 and 20 chromosomes in spleen and V79 cells from each culture, respectively, were scored for SCEs. Cell kinetic information was obtained by determining the number of the first, second, and third division cells in 100 consecutive metaphases. The replicative index (RI) was calculated as follows: $RI = (1M_1 + 2M_2 + 3M_3)/100$ (Schneider and Lewis, 1981). This value is indicative of compound toxicity for cells.

1000 binucleated cells were scored for the incidence of micronuclei (in vitro). The toxicity of tetrandrine was evaluated by assessing the percentage of mononucleated, binucleated, trinucleated, and quadrinucleated cells in 500 cells scored. 2000 polychromatic erythrocytes (PCEs) per animal were scored for the number of micronucleated PCEs (in vivo), and the ratio of PCEs/NCEs (normochromatic erythrocytes) was determined in the first 100 PCEs scored per animal.

TABLE 1

SISTER-CHROMATID EXCHANGES INDUCED BY TETRANDRINE IN V79 CELLS

Treatment doses (μg/ml)	SCEs/cell ± SE [a]		Replicative index [b]	
	−S9	+S9	−S9	+S9
80	8.92 ± 0.71	11.0 ± 0.98 *	2.12	2.16
160	10.04 ± 0.76	13.88 ± 1.22 **	2.06	1.94
320	– [c]	15.04 ± 1.53 **	– [c]	1.70
Solvent control	7.92 ± 0.91	8.24 ± 0.75	2.31	2.28
Mitomycin C				
0.02	19.32 ± 1.1 **		2.29	
Cyclophosphamide				
1.5		34.64 ± 2.02 **		1.83

[a] Total for 25 cells.
[b] 100 cells scored.
[c] Toxic.
* $p < 0.05$,
** $p < 0.001$.

TABLE 2

SISTER-CHROMATID EXCHANGES IN MOUSE SPLEEN CELLS IN VIVO/IN VITRO CONDITION FOLLOWING TREATMENT WITH TETRANDRINE

Treatment	Dose (mg/kg)	Animal	SCEs/cell ± SE	Replicative index
Tetrandrine	50	1	6.28 ± 0.41	
		2	6.0 ± 0.59	
		3	6.2 ± 0.60	
		Mean	6.16 ± 0.08	2.17
	100	1	6.88 ± 0.42	
		2	7.64 ± 0.44	
		3	7.64 ± 0.56	
		Mean	7.32 ± 0.26 *	1.95
	200	1	7.64 ± 0.48	
		2	8.4 ± 0.85	
		3	9.32 ± 0.76	
		Mean	8.45 ± 0.49 **	1.96
Solvent control (distilled water)		1	6.2 ± 0.54	
		2	5.72 ± 0.56	
		3	5.04 ± 0.39	
		Mean	5.65 ± 0.34	1.96
Positive control (cyclophosphamide)	15	1	33.32 ± 1.87	
		2	30.96 ± 1.71	
		3	29.52 ± 1.71	
		Mean	31.27 ± 1.11 **	2.01

* $p < 0.05$.
** $p < 0.01$.

TABLE 3

FREQUENCIES OF MICRONUCLEI IN CYTOKINESIS-BLOCKED CELLS OF V79 EXPOSED TO TETRANDRINE

Treatment	Doses (μg/ml)	Number of micronuclei/ 1000 binucleated cells		% cells			
		+ S9	− S9	+ S9		− S9	
				1N	≥ 2N	1N	≥ 2N
Tetrandrine	10	32	32	48.7	51.3	49.6	50.4
	20	45	43	58.5	41.5	74.9	25.1
	30	46	– [a]	61.9	38.1	–	–
	40	47	–	73.2	26.8	–	–
Solvent control (DMSO)		43	39	36.1	63.9	25.9	74.5
Positive control							
(triethylenemelamine)	2.0	–	142	–	–	86.1	13.9
(cyclophosphamide)	1.5	135	–	82.6	14.9	–	–

[a] Most of the cells treated with tetrandrine at dosages of 30 μg/ml and 40 μg/ml (− S9) were broken due to toxic effects and could not be scored.

Statistical analysis

To compare the SCE frequencies and RI from treated cells with control values, Student's *t*-test was used. The significance level was chosen at 0.05. No statistical analyses were carried out for the micronucleus test because no dose-related increase of micronuclei was observed.

Results

The results of tetrandrine-induced SCE in V79 cells are shown in Table 1. In the presence of activation by S9, an increase in SCE frequencies was observed as the concentration of tetrandrine increased, and the result is statistically significant at or above the dose of 80 μg/ml. Without metabolic activation, no significant increase in SCEs was found. The replicative index (RI) showed a slight cell-cycle delay. A dose–response relationship of SCE induced by tetrandrine was also observed in mouse spleen cells (Table 2). At the dose of 50 mg/kg bw, tetrandrine did not induce SCE. However, at higher doses (100 mg and 200 mg tetrandrine/kg bw), a significant increase in SCE frequences was noted.

The results of the micronucleus studies with tetrandrine in cytokinesis-blocked V79 cells and in mouse bone marrow are presented in Tables 3 and 4, respectively. Tetrandrine had no significant effect on micronucleus formation in both in vitro and in vivo assays. The effect of tetrandrine on cell cycle kinetics in CYB-blocked V79 cells is illustrated in Table 3. Cells treated with tetrandrine showed a toxic effect as demonstrated by a dose-related increase in the percentages of mononucleated cells and dose-dependent decrease in the percentages of binucleated cells. As to the in vivo micronucleus test, the ratios of PCEs/NCEs decreased slightly with an increase in the dose of tetrandrine (Table 4). In addition, most of the cells exposed to tetrandrine for 24 h at dosages of 30 and 40 μg/ml (without metabolic activation) were killed in the in vitro micronucleus test, and almost half of the animals died at the high dose (200 mg/kg bw) in the in vivo micronucleus and SCE studies.

TABLE 4

RESULTS OF MICRONUCLEUS ASSAY IN MOUSE BONE MARROW CELLS FOLLOWING IN VIVO EXPOSURE TO TETRANDRINE

Exposure dose (mg/kg)	Animal	MPCEs [a]/ 1000 PCEs	Ratio of PCEs/ NCEs [b]
Tetrandrine			
50	1	1.5	1.51
	2	1.0	1.45
	3	1.5	1.61
	4	1.5	0.82
	5	1.5	1.19
	Mean	1.4	1.32
100	1	2.0	1.00
	2	1.5	1.25
	3	1.5	0.98
	4	0.5	1.18
	5	3.0	1.56
	Mean	1.7	1.19
200	1	2.5	0.97
	2	2.5	1.33
	3	0.0	1.10
	4	2.5	0.85
	5	1.5	0.87
	Mean	1.8	1.02
Solvent control (phosphate-buffered saline)	1	1.5	1.12
	2	1.5	1.12
	3	3.0	1.17
	4	1.5	1.00
	5	0.5	1.64
	Mean	1.6	1.21
Positive control (triethylene-melamine, 0.5)	1	57.5	0.17
	2	76.0	0.18
	3	79.0	0.22
	4	60.5	0.21
	5	57.0	0.19
	Mean	66.0	0.19

[a] Micronucleated PCEs. 2000 PCEs per animal were scored.
[b] Based on 1000 PCEs.

Discussion

The frequencies of SCE induced by tetrandrine increased significantly in both in vitro and in vivo/in vitro assays. However, the increases in SCE frequencies were relatively small which indicates that tetrandrine is a weak genotoxic agent. Whong et al. (1989) also showed that tetrandrine induced a low frequency of gene mutations in *Salmonella typhimurium*.

Metabolic activation is necessary for SCE induced by tetrandrine in the in vitro test, since positive results were found in V79 cells only in the presence of S9 mix (rat liver homogenate). This

demonstrates that tetrandrine is an indirect-acting genotoxicant. The dependence on metabolic activation to express the genotoxic effect of tetrandrine was also noted in the Ames/Salmonella assay system (Whong et al., 1989).

Tetrandrine treatment did not induce micronucleus at any of the doses tested in both in vitro and in vivo assays. However, a significant increase in the frequency of micronuclei in mouse bone marrow cells treated with tetrandrine was reported by Dong et al. (1982). This discrepancy may be due to the different strains of mice used. In the current study, tetrandrine caused a slight but dose-related decrease in the ratio of PCEs/NCEs in mouse bone marrow and caused an obvious dose–response decrease in the percentage of CYB-blocked V79 binucleated cells. In addition, several animals died immediately following high-dose treatments, and nearly half of the animals died 24 h or 48 h after treatment. These results suggest that tetrandrine is toxic. It has been reported that tetrandrine inhibits DNA synthesis (Creasey, 1976) and delays or inhibits cell division including the final mitosis of the erythroblasts before nucleation, even the necrosis of some organs occurred directly (Li et al., 1982).

The different results obtained from the SCE and the micronucleus assays for tetrandrine reported here may be due to the difference in the sensitivity of the two assay systems. It is possible, however, that tetrandrine, under the condition tested, did not break chromosomes nor impair spindle apparatus but it caused DNA damages and/or perturbed DNA repair which lead to sister-chromatid exchanges (Latt et al., 1977; Howard-Flanders, 1981; Linnaimaa and Wolff, 1982).

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