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Genotoxicity and genotoxic enhancing effect of tetrandrine in *Salmonella typhimurium*

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Summary

Tetrandrine has been used for the treatment of silicosis in China. The potential genotoxic and carcinogenic hazards of this drug were studied using the Salmonella/histidine reversion assay and the SOS/Umu test. The results show that tetrandrine was weakly mutagenic to *Salmonella typhimurium* TA98 with metabolic activation and did not induce SOS response. However, tetrandrine increased the mutagenic activity of benzo[*a*]pyrene, trinitrofluorenone (TNF), 2-aminoanthracene (2AA), diesel emission particles, airborne particles, and cigarette smoke condensate by more than 100%; the activity of aflatoxin B_1 and fried beef was increased by over 75%. It also increased the 2AA and TNF-induced SOS response by more than 300%. These results indicated that tetrandrine was a weak promutagen inducing frameshift mutations and was a potent genotoxic enhancer. The mechanism for the genotoxic enhancement is not known. However, the fact that the increase in mutagenicity was noted only in TA98 and not in TA1538 suggested that the enhancement of genotoxicity by tetrandrine may result from an increase in error-prone DNA repair.

Silicosis is a chronic occupational lung disease found mainly in miners resulting from a long exposure to silica and related mine dust. The distinguishing characteristic of this disease is the formation of fibrosis in the lung. It is believed that the silica-induced fibrosis is due to the activation of biosynthesis of collagen in fibroblasts by a factor released by macrophages following the inhalation of silica (Heppleston and Styles, 1967; Burrell and Anderson, 1973; Aho and Kulonen, 1980). Presently, there is no reliable treatment for this occupational disease.

Tetrandrine, a plant alkaloid isolated from *Stephania tetrand* S. Moore, has been shown to have antitumor, antiinflammatory, and tuberculostatic activities (Berezhinskaya et al., 1971; Vichkanova et al., 1973; Kuroda et al., 1976). It has been used clinically in the treatment of high blood pressure, angina, tumors, and amoeba diarrhea in China (Lu et al., 1983). Studies with laboratory

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animals have shown that tetrandrine inhibited the development of silicosis and decreased fibrogenesis in silicotic rats (Yu et al., 1983; Zou and Gou, 1983). Clinical investigations have revealed that the treatment of patients with this drug significantly reduced pathological symptoms associated with silicosis (Q. Li et al., 1981, 1982; Lu et al., 1983). Although, at high concentrations, it showed marked nephrotoxic, hepatotoxic, and/or lymphotoxic effects in experimental animals (Gralla et al., 1974; T. Li et al., 1982) and caused disfunction and swelling of the liver in patients following long-term treatment (Lu et al., 1983), tetrandrine is potentially useful for the treatment of silicosis (Q. Li et al., 1981, 1982; Lu et al., 1983).

In this report, the potential genotoxic and carcinogenic hazards of tetrandrine were studied using two genetic endpoints, i.e., gene mutation and DNA damage. The Salmonella/histidine reversion assay and the SOS/*Umu* test were used for measuring gene mutations and DNA damage, respectively. Since certain non-genotoxic carcinogens, which have been postulated to act as tumor promoters, may enhance the mutagenicity and/or carcinogenicity of genotoxic carcinogens (Watanabe and Williams, 1978; Williams, 1981; Furuya et al., 1983; Dewdney and Soper, 1984; Purdy and Marshall, 1984), it is relevant to include studies on mutagenic modulation in genotoxicity testing. Therefore, the modulating effect of tetrandrine on the genotoxicity of other mutagens and carcinogens was also examined.

Materials and methods

Test chemicals

Tetrandrine (97% purity), provided by Dr. T. Hu (Shanghai Institute of Industrial Hygiene and Occupational Disease, Shanghai, China), was prepared in sterile water at pH 3 and further adjusted to pH 6.5 with saturated NaOH solution. Known mutagenic substances, i.e., trinitrofluorenone (TNF), 2-aminoanthracene (2AA), aflatoxin B_1 (AFB_1), benzo[*a*]pyrene (BaP), diesel emission particles (DEP), airborne particles (AP), cigarette smoke condensate (CSC), and fried beef (FB) were used for the modulation study of tetrandrine. 2AA and TNF were purchased from Aldrich Chemical Co. (Milwaukee, WI), AFB_1 from Calbiochem (San Diego, CA), and BaP from Sigma Chemical Co. (St. Louis, MO). The dietary (FB) and environmental (DEP, AP and CSC) complex mixtures were extracted with organic solvents. The procedures for the extractions and the sources of the mixtures have been described previously (Whong et al., 1986). All the mutagenic substances were dissolved in dimethyl sulfoxide (DMSO).

Tester strains

Salmonella typhimurium TA98, TA100, and TA1538 were used for the Salmonella/histidine reverse mutation assay, while *S. typhimurium* TA1535/pSK1002 was used for the SOS/Umu test. The tester TA1535/pSK1002 was derived by introducing plasmid pSK1002, which carried a *umuC–lacZ* fusion gene, into *S. typhimurium* TA1535 (Oda et al., 1985). The genetic characteristics of those testers have been reported previously (Ames et al., 1975; Oda et al., 1985).

Histidine reversion assay

The plate-incorporation test was employed for mutagenicity studies. The procedure followed that described by Maron and Ames (1983). For the mutagenicity study of tetrandrine, 0.1 ml each of an overnight culture of tester and tetrandrine were added to 2 ml of molten soft agar. In the controls, when tetrandrine was omitted from the soft agar, the solvent (H_2O) was added. S9 mix (0.5 ml) was also added for metabolic activation. After mixing, the soft agar was overlaid onto a Vogel–Bonner minimal glucose agar (VB) plate. The plates were incubated at 37 °C for 2 days. His^+ revertants on the plates were scored with an Artek counter (Model 880). In order to determine the effects of tetrandrine on the mutagenicity of known mutagens, 0.1 ml of the mutagen solution was also added to the soft agar which contained tester cells and tetrandrine. S9 was prepared from the livers of Aroclor 1254 (500 mg/kg body weight) pretreated male CD rats (Maron and Ames, 1983).

Simultaneous and sequential exposure of TA98 to tetrandrine and other mutagen

The different manners of exposing TA98 cells to mutagen and tetrandrine, i.e., concurrent, pre-, and post-treatment, were studied by using 2 treat-

ment mixtures. The His reversion suspension test, which determines mutation frequencies according to surviving cells, was carried out for the study. In the first treatment, each test tube contained 1.2 ml of TA98 overnight culture in the presence or absence of tetrandrine (187.5 μg/ml) and/or DEP extract (0.5 mg/ml). The mixtures were incubated at 37°C for 30 min with shaking. At the end of the incubation, cells were washed twice with sodium phosphate buffer (0.1 M, pH 7) by centrifugation, and resuspended in 1.2 ml phosphate buffer. Then, the cells in each tube were subjected to a second treatment which was the same as the first, with the exception of the different combinations of mixtures. In the concurrent treatment, cells were treated simultaneously with tetrandrine and DEP extract at the second treatment. In the pre-treatment with tetrandrine, tetrandrine and DEP extract were added to the first and second treatment mixtures, respectively. In the post-treatment with tetrandrine, DEP extract and tetrandrine were added to the first and second treatment mixtures, respectively. After washing the cells, 0.1 ml of the second treatment mixtures were added individually to 2 ml of molten soft agar and overlaid onto a VB (minimal) plate for determining revertants. For measuring populations, ~ 1000 cells from the second treatment after the proper dilutions were plated onto a VB plate supplemented with full strength histidine (10 μg/ml). The incubation and scoring of the plates were the same as that described for the histidine reversion assay.

SOS / Umu test

The procedures for the SOS/Umu test have been described previously (Whong et al., 1986). In brief, the overnight culture of tester cells (TA1535/pSK1002) was diluted 10 times with warm TGA medium and incubated to log-phase. The log-phase culture was further diluted 4 times with TGA medium (without metabolic activation) or with S9 mix (with metabolic activation). For the experiment, 0.3 ml of the diluted log-phase cultures and 10 μl of tetrandrine at different concentrations were mixed in a test tube. 10 μl of TNF (without metabolic activation) or 2AA (with metabolic activation) was also added to the mixture to study the effect of tetrandrine on the SOS functions induced by these two mutagens. Each mixture was diluted 10 times with TGA medium and further incubated for 2 h. After incubation, the cell density was measured at OD_{600}, and β-galactosidase activity was determined according to the combined method of Miller (1972), Oda et al. (1985), and Quillardet and Hofnung (1985) with minor modifications. A 100% or more increase in β-galactosidase activity above the control with a dose-related response was considered as a positive result.

Results

The mutagenic activity of tetrandrine was investigated using tester strains TA98 and TA100. A weak tetrandrine-induced mutation (~ 2.7 times the control) was found only in TA98 with metabolic activation (Table 1). With or without S9 activation, a drastic killing effect was observed when the doses of tetrandrine were more than 1.5 mg/plate.

The effect of tetrandrine on the mutagenicity of 8 known direct-acting mutagens and promutagens (TNF, 2AA, AFB_1, BaP, FB, CSC, DEP, and AP) is summarized in Table 2. Tetrandrine exerted a

TABLE 1

MUTAGENIC ACTIVITY OF TETRANDRINE IN *Salmonella typhimurium* [a]

Conc. of tetrandrine (mg/plate)	S9	His$^+$ revertants/plate	
		TA98	TA100
0	−	19	111
	+	37	149
0.188	−	20 (1.1) [b]	129 (1.2) [b]
	+	51 (1.4)	162 (1.1)
0.375	−	21 (1.1)	132 (1.2)
	+	66 (1.8)	154 (1.0)
0.75	−	18 (0.9)	134 (1.2)
	+	76 (2.1) [c]	162 (1.1)
1.5	−	28 (1.5)	86 (0.8)
	+	100 (2.7) [c]	179 (1.2)

[a] Positive control (2.5 μg 2AA/plate with S9): TA98, 1547 revertants/plate; TA100, 1796 revertants/plate.
[b] Revertant ratio (experimental/control).
[c] Positive response.

TABLE 2

ENHANCING EFFECT OF TETRANDRINE ON THE MUTAGENIC ACTIVITY OF MUTAGENS IN *Salmonella typhimurium* TA98

Mutagen	S9	His^+ revertants/plate					
		0 [a]	0.0947	0.188	0.375	0.75	1.5
Negative control	–	21	–	–	22	20	26
	+	35	–	45	44	59	..
Diesel emission (0.5 mg/plate)	–	788	–	1072 (37) [b]	1145 (46)	1734 (123)	2023 (160)
Airborne particle (10.45 mg/plate)	–	238	–	424 (86)	425 (86)	516 (128)	696 (208)
TNF (0.05 μg/plate)	–	560	–	1101 (100)	1251 (128)	1355 (148)	1606 (194)
2AA (1 μg/plate)	+	592	694 (18)	930 (59)	1224 (112)	1381 (137)	–
AFB_1 (40 ng/plate	+	705	909 (30)	995 (40)	1061 (52)	1241 (76)	–
BaP (5 μg/plate)	+	255	318 (29)	436 (78)	503 (109)	615 (153)	–
Fried beef (2.25 g/plate)	+	985	1153 (18)	1406 (43)	1433 (46)	1931 (97)	–
Cigarette condensate (0.0125 cigarette/plate)	+	676	921 (38)	954 (42)	1002 (49)	1479 (122)	–

[a] Concentration of tetrandrine (mg/plate).

[b] % enhancement = [(revertants induced by tetrandrine plus mutagen) − (revertants induced by mutagen)]/revertants induced by mutagen. Spontaneous revertants were subtracted.

dose-dependent enhancement of mutations induced by all the mutagens studied. The degree of enhancement ranged from ~ 80% (AFB_1) to 200% (AP). Further increases in tetrandrine concentrations resulted in decreasing mutagenic enhancements owing to toxic effects, reflecting considerably low colony densities on the plate (results not shown). The enhancing effect of tetrandrine on the mutagenic activity induced by various doses of the mutagens was also examined (Table 3). In this study, only one mutagen was selected for tests with (for FB) and without (for DEP) metabolic activation. The enhancement of mutagenic activity by tetrandrine was found to have an inverse relationship with the concentration of mutagens. Within the concentrations examined, both mutagens displayed ~ 2-fold higher tetrandrine-enhanced mutagenic activity at the lowest dose than at the highest dose tested. In the combined treatment with DEP and tetrandrine, a considerable decrease in the number of background colonies was observed as the concentration of DEP was increased. This indicated that there was also an enhancing effect of tetrandrine on DEP-induced toxicity. Table 4 shows the effect of concurrent, pre- and post-treatments with tetrandrine on the induced mutagenicity in *S. typhimurium* TA98. With the liquid-suspension test, a high enhancement of DEP mutagenesis (more than 3 times that of DEP alone) by tetrandrine was found in the concurrent treatment with DEP and tetrandrine and in the post-treatment with tetrandrine. Only a very slight increase in the DEP mutagenicity (~ 27%) was noticed when cells were treated with tetrandrine before DEP treatment.

To determine whether the error-prone DNA repair of cells is a contributor to the enhancing effect of tetrandrine on mutagenicity, TA98 and TA1538 were employed for a comparative mutagenicity study with tetrandrine. The only dif-

TABLE 3

ENHANCING EFFECT OF TETRANDRINE ON THE MUTAGENIC ACTIVITY OF MUTAGENS AT VARIOUS CONCENTRATIONS IN *Salmonella typhimurium* TA98

Mutagen		Conc. of tetrandrine (μg/plate)	S9	His$^+$ revertants/plate	% Enhancement [a]
Type	Conc.				
Diesel emission	0	0	−	13	
(μg/plate)	0	750	−	15 (1.2) [b]	
	62.5	0	−	188 (14.5)	
	62.5	750	−	622 (47.8)	246
	125	0	−	356 (27.4)	
	125	750	−	1086 (83.5)	212
	250	0	−	655 (50.4)	
	250	750	−	1624 (124.9)	150
	500	0	−	858 (66.0)	
	500	750	−	2025 (155.8)	138
Fried beef	0	0	+	30	
(g/plate)	0	750	+	63 (2.1)	
	0.14	0	+	176 (5.9)	
	0.14	750	+	534 (17.8)	223
	0.28	0	+	273 (9.1)	
	0.28	750	+	844 (28.1)	221
	0.56	0	+	541 (18.0)	
	0.56	750	+	1114 (37.1)	106
	1.12	0	+	738 (24.6)	
	1.12	750	+	1505 (50.2)	104

[a] % enhancement = [(revertants induced by tetrandrine plus mutagen) − (revertants induced by mutagen)]/revertants induced by mutagen. Spontaneous revertants were subtracted.

[b] Revertant ratio (experimental/control).

ference between these two testers was that TA98 contains an R factor, which results in an increase in error-prone repair. With the direct-acting mutagen, DEP, an obvious enhancement of induced mutagenicity by tetrandrine was observed in TA98, but not in TA1538 (Table 5).

The induction of DNA damage by tetrandrine was also investigated using the SOS/Umu test. As shown in Table 6, there was no tetrandrine induced SOS function observed at doses up to 150 μg and 50 μg/treatment with and without S9 activation, respectively. These concentrations were the highest doses tested without drastic killing. Studies were also conducted to see whether tetrandrine exerted enhancing effects on SOS functions induced by TNF (without activation) and 2AA (with activation). Results showed that tetrandrine also enhanced TNF- and 2AA-induced SOS responses in a dose-related manner. The highest tetrandrine-enhanced SOS responses were more than 3 times greater for 2AA and 4 times greater for TNF (Table 7) than those induced by both compounds without tetrandrine, respectively.

Discussion

The aim of the present study was to determine the genotoxicity of tetrandrine and its modulating effects on the genotoxicity of environmental and occupational mutagens. Results from the study indicated that although tetrandrine was a weak mutagen, it was a potent genotoxic enhancer. The results of tetrandrine being mutagenic only to TA98 (a frameshift mutant) with metabolic activation suggested that this drug was an indirect-acting mutagen that induced frameshift mutation. However, it has been reported by other investigators that tetrandrine was inactive in the histidine reversion assay system (Dong et al., 1982). We found that the negative result obtained by them was due

TABLE 4

EFFECT OF TREATMENT MANNER WITH TETRANDRINE ON THE MUTAGENICITY OF DIESEL EMISSION IN *Salmonella typhimurium* TA98

First treatment		Secondary treatment		His$^+$ revertants/ 10^7 survivors
Tetrandrine (187.5 μg/ml)	Diesel (0.5 mg/ml)	Tetrandrine (187.5 μg/ml)	Diesel (0.5 mg/ml)	
–	–	–	–	0.9
–	–	–	+	135.2
–	–	+	+	603.7 [a] (4.5) [d]
+	–	–	–	1.2
+	–	–	+	171.5 [b] (1.3) [d]
–	+	–	–	151.1
–	+	+	–	722.7 [c] (4.8) [d]

[a] Diesel mutagenicity after concurrent treatment with tetrandrine.
[b] Diesel mutagenicity after pre-treatment with tetrandrine.
[c] Diesel mutagenicity after post-treatment with tetrandrine.
[d] Ratio of diesel mutagenicity between with and without tetrandrine.

to insufficient concentrations of tetrandrine tested.

Tetrandrine potentiated the mutagenicity of both direct-acting mutagens (DEP, AP and TNF) and promutagens (2AA, AFB_1, BaP, FB and CSC), including various chemical classes, e.g., aromatics, mycotoxins, and nitro compounds. Without S9 activation, tetrandrine was not mutagenic. However, a high tetrandrine-enhanced mutagenicity of the direct-acting mutagens was observed. With metabolic activation, tetrandrine was also shown to have an enhanced effect at non-mutagenic doses. The enhancement of mutagenicity by tetrandrine in the His reversion assay was substantiated with the SOS/Umu assay system, where an obvious potentiation of SOS function by tetrandrine was demonstrated with a promutagen (2AA) and a direct-acting mutagen (TNF). The phenomenon of genotoxicity enhancement by other chemicals such as tumor-promoting agents (e.g., 12-*O*-tetradecanoylphorbol 13-acetate) and non-mutagenic carcinogens (e.g., diethylstilbestrol) has been reported by other investigators (Dewdney and Soper, 1984; Allaben et al., 1979).

The study on the tetrandrine-induced mutagenesis enhancement showed a higher enhancement at the lower doses of the mutagens. Since the plate incorporation assay does not compensate for toxicity and since a decrease in background growth was found under microscopic observation, the lower mutagenicity enhancement by tetrandrine with higher doses of mutagens probably resulted from more killing of cells when treated with the

TABLE 5

ENHANCING EFFECT OF TETRANDRINE ON THE MUTAGENIC ACTIVITY OF DIESEL EMISSION IN *Salmonella typhimurium* TA98 AND TA1538 [a]

Conc. of tetrandrine (μg/plate)	His$^+$ revertants/plate [b]	
	TA98	TA1538
0	494	76
93.7	768 (1.6) [c]	85 (1.1)
187.5	965 (2.0)	98 (1.3)
375	1465 (3.0)	91 (1.2)
750	2072 (4.3)	73 (1.0)

[a] In each plate, 0.3 mg diesel emission was added.
[b] Spontaneous revertants: TA98 = 21; TA1538 = 12.
[c] Enhancement ratio = induced revertants obtained from tetrandrine plus diesel/induced revertants obtained from diesel only.

TABLE 6

SOS INDUCTION IN *umu*C STRAIN TREATED WITH TETRANDRINE

Tetrandrine (μg/treatment)	S9	% Survival	β-Galactosidase unit [a]	SOS induction ratio [b]
0	–	100	54.5	
6.25	–	95.2	52.5	1.0
12.5	–	87.3	50.3	0.9
25	–	66.6	51.3	0.9
50	–	26.2	82.4	1.5
Positive control [c]	–	36.5	830.9	15.2
0	+	100	46.3	
25	+	94.3	46.2	1.0
50	+	77.7	46.5	1.0
100	+	27.3	61.6	1.3
150	+	6.7	60.0	1.3
Positive control [d]	–	43.1	713.0	15.4

[a] β-Galactosidase unit $= 1000 \times (OD_{420} - 1.75 \times OD_{550})/(t \times v \times OD_{600})$; where t = min of reaction and v = ml of cells.
[b] Induction ratio = experimental galactosidase unit/negative control galactosidase unit.
[c] 0.5 μg TNF/treatment.
[d] 2.5 μg 2AA/treatment.

TABLE 7

ENHANCING EFFECT OF TETRANDRINE ON THE SOS RESPONSE INDUCED BY MUTAGENS

Conc. of tetrandrine (μg)	Conc. of mutagen (μg)	S9 activation	β-Galactosidase unit [a]	SOS induction ratio [b]	% Enzymatic activity increased [c]
0	0	−	55.1	1	−
2.5	0	−	54.8	1	−
5.0	0	−	49.6	0.9	−
10.0	0	−	59.4	1.1	−
15.0	0	−	51.2	0.9	−
0	0.1 [d]	−	136.0	2.5	−
2.5	0.1	−	156.1	2.8	24.8
5.0	0.1	−	246.3	4.8	136.3
10.0	0.1	−	327.4	5.9	231.3
15.0	0.1	−	476.0	8.6	420.3
0	0	+	78.5	1	−
6.25	0	+	95.8	1.2	−
12.5	0	+	93.7	1.2	−
25.0	0	+	91.8	1.2	−
50.0	0	+	99.1	1.3	−
0	0.75 [e]	+	311.4	4.0	−
6.25	0.75	+	393.6	5.0	27.9
12.5	0.75	+	473.4	6.0	63.0
25.0	0.75	+	722.0	9.2	170.6
50.0	0.75	+	1051.2	13.4	309.2

[a] β-Galactosidase unit $= 1000 \times (OD_{420} - 1.75 \times OD_{550})/(t \times v \times OD_{600})$; where t = min of reaction and v = ml of cells.

[b] Induction ratio = experimental galactosidase unit/negative control galactosidase unit.

[c] % enzymatic activity increased = [(galactosidase unit induced by tetrandrine plus mutagen) − (galactosidase unit induced by mutagen)]/galactosidase unit induced by mutagen. Spontaneous enzyme units were subtracted.

[d] Trinitrofluorenone.

[e] 2-Aminoanthracene.

higher doses of mutagens in the presence of tetrandrine.

The enhancing effect of tetrandrine was found in the concurrent treatment of tetrandrine and mutagens and the post-treatment with tetrandrine, but not in the pre-treatment with tetrandrine. This finding indicated that the mutagenicity enhancement by tetrandrine occurred after the mutagen entered the bacterial cell. Therefore, tetrandrine may act intracellularly by influencing DNA repair pathways and/or by increasing mutation expression. Error-prone DNA repair has been suggested to be partially responsible for mutation induction (Perry and Walker, 1982). In the Ames Salmonella assay, TA98 contains plasmid pKM101, whereas TA1538 does not. Results from a comparative study between TA98 and TA1538 show that the tetradrine-induced enhancement of diesel mutagenicity observed in TA98 was abolished in TA1538 (Table 5). Hence, the mutagenicity enhancement by tetrandrine may be, at least in part, due to the potentiation of error-prone DNA repair. As no mutagenic activity was found for tetrandrine without metabolic activation, tetrandrine might enhance error-prone repair for induced, but not spontaneous, mutations. This assumption may further be supported by the results from the SOS function studies (Table 6), which showed no tetrandrine-induced SOS repair, but demonstrated enhancement of mutagen-induced SOS response by tetrandrine.

Tetrandrine has been demonstrated to be considerably effective for the treatment of silicosis. Our data show that this drug was not only a

mutagen but also a potent genotoxic enhancer. The impact of these findings on the health of tetrandrine-treated subjects is uncertain. Further studies with both in vitro and in vivo genotoxicity assessments need to be performed.

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