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The effect of 1,10-phenanthroline on the chromosome damage and sister-chromatid exchanges induced by streptozotocin in mammalian and insect cells

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Abstract

The effect of the metal chelating agent 1,10-Phenanthroline (PNT) on the streptozotocin (STZ)-induced chromosomal aberrations (CAs) and sister-chromatid exchanges (SCEs) in Chinese hamster ovary (CHO) and mosquito (*Aedes albopictus*) cells was investigated. Treatment of CHO and mosquito cells with STZ produced a significant and dose-response increase in the yield of CAs as well as SCEs ($p < 0.05$). The addition of PNT prevented the induction of CAs by STZ in both types of cells, causing a significant decrease in the frequency of STZ-induced CAs (46.5–72.5%) ($p < 0.05$). This fact indicates that intracellular transition metals are implicated in STZ-induced CAs and that the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow OH^{\circ} + OH^{-} + Fe^{3+}$) is partly responsible for the production of CAs by this compound. On the other hand, the addition of PNT to CHO and mosquito cell cultures did not prevent the induction of SCEs by STZ. Therefore, it is valid to assume that the induction of CAs and SCEs by STZ occurs by different mechanisms.

Keywords: Streptozotocin; 1,10-Phenanthroline; Chromosomal aberration; Sister-chromatid exchange

1. Introduction

Streptozotocin (STZ) is an antibiotic isolated from *Streptomyces achromogenes* which exhibits marked antileukemic activity [1,2]. This compound has antibacterial, tumoricidal, carcinogenic and diabetogenic properties [1–4]. Chemically, STZ is an *N*-nitrosourea, and is related to agents such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a nitrogen mustard derivative that is commonly used in cancer therapy [1]. Because STZ is usually used to experimentally induce diabetes mellitus in laboratory animals and since it has been considered as a potential compound for the clinical treatment of malignant diseases, there is an intensive search to establish the exact mechanisms underlying cytotoxicity by STZ. It has been shown that STZ is a potent alkylating agent which directly methylates DNA [5,6] giving rise to chromosome and DNA damage [7–13]. At the chromosome level, STZ induces chromosomal aberrations (CAs) and sister-chromatid exchanges (SCEs) in mammalian and insect cells [8,9,11,14]. The precise mechanisms by which STZ induces CAs and

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SCEs remain unknown. Previous reports provided evidence that free radicals may be involved in the diabetogenic action of the drug [15–18]. We recently demonstrated that STZ-induced CAs in CHO and mosquito cells can be prevented to a great extent by the incorporation of antioxidant compounds into the cells [14]. This finding suggests that free radicals are involved in the clastogenesis by STZ. In order to gain further insights into a possible involvement of free radicals in the clastogenic action of STZ, in this work, we examined the effect of the iron chelator 1,10-phenanthroline (PNT) — which inhibits hydroxyl radical formation- on the ability of STZ to induce CAs and SCEs in mammalian and insect cells. Our results show that the treatment of cells with PNT markedly reduce the induction of CAs but does not prevent the induction of SCEs by STZ.

2. Materials and methods

2.1. Culture conditions and STZ treatments

CHO cells were grown in Ham's F10 medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C and 5% CO_2 atmosphere. A mosquito cell line (ATC-15) initiated from first-instar larvae of *Aedes albopictus* was grown at 28°C in Mitsuhashi and Marmorosch culture medium (Sigma) supplemented with 20% fetal calf serum and antibiotics. Both types of cells were cultured as monolayer in TC25 Corning flasks containing 3×10^5 cells/ml. During the log phase of growth the cells were treated for 30 min with STZ (Calbiochem-Novabiochem, La Jolla, CA, CAS No. 18883-66-4) at the doses indicated in Tables 1–4. Since STZ is rather unstable at pH 7 [1], it was prepared immediately before use by dissolving the drug in sodium citrate 0.02 M (pH 4.4). PNT (Sigma, CAS No. 5144-89-8) (9 μg/ml) was dissolved in double-destilled water and added to the cultures 15 min before the addition of STZ. At the end of the pulse treatment with STZ, PNT or STZ plus PNT, the cells were washed twice with Hanks, balanced salt solution and kept in culture with fresh culture medium until harvesting. For the determination of STZ-induced SCEs, BrdU (10 μg/ml, Sigma, CAS No. 59-14-3) was added to the culture medium.

2.2. Cell harvesting and cytogenetic analysis

For CAs analysis, cultures were harvested at 18 h (CHO) or 24 h (mosquito) after the end of treat-

Table 1
Chromosomal aberrations induced by STZ (30 min exposure) in CHO cells in the presence or absence of PNT (added 15 min before treatment)

STZ (mM)	PNT (9.0 μg/ml)	MI (%)	Aberrations (frequency per 100 cells)							Total aberrations	PCI[a]	% Cells with aberrations
			Chromatid-type			Chromosome-type						
			Breaks	Exch.	T	Dic.	Rings	Del.	T			
0.0	−	4.4	9	0	9	1	0	0	0	10		8
0.0	+	4.3	7	0	7	1	0	0	1	8		7
0.5	−	2.7	21	5	26	1	1	4	6	32[b]		25
0.5	+	5.5	8	0	8	0	1	2	3	11	72.5	9
1.0	−	2.1	32	6	38	3	4	3	10	48[b]		30
1.0	+	9.2	22	3	25	2	0	2	4	29	48.2	23
2.0	−	3.7	70	31	101	2	0	1	3	104[b]		51
2.0	+	5.3	27	10	37	0	0	1	1	38	66.0	27
4.0	−	4.7	81	32	113	6	0	1	7	120[b]		70
4.0	+	8.2	47	13	60	1	2	3	6	66	48.4	42

STZ, streptozotocin; PNT, phenanthroline; MI, mitotic index (1000 cells analysed): Exch, Exchanges: Dic, Dicentric chromosomes: Del, Deletions; T, Total aberrations.

[a] Percentage of clastogenesis inhibition (PCI, see Section 2). Differences between observed and expected values resulted statistically significant for all the combined treatments ($p < 0.05$) except for STZ 1.0 mM plus PNT (borderline significance).

[b] Statistically significant with respect to control value ($p < 0.05$).

Table 2
Chromosomal aberrations induced by STZ (30 min exposure) in mosquito cells in the presence or absence of PNT (added 15 min before treatment)

STZ (mM)	PNT (9.0 μg/ml)	MI (%)	Aberrations (frequency per 100 cells)							Total aberrations	PCI[a]	% Cells with aberrations
			Chromatid-type			Chromosome-type						
			Breaks	Exch.	T	Dic.	Rings	Del.	T			
0.0	−	9.6	6	0	6	0	2	0	2	8		7
0.0	+	11.3	13	1	14	0	0	0	0	14		11
0.5	−	10.7	26	0	26	0	0	0	0	26[b]		23
0.5	+	11.0	8	0	8	1	1	1	3	11	72.5	10
1.0	−	9.9	33	7	40	2	1	1	4	44[b]		34
1.0	+	12.3	27	2	29	0	1	1	2	31	46.5	19
2.0	−	11.8	38	8	46	1	0	1	2	48[b]		29
2.0	+	12.1	10	12	22	1	0	0	1	23	62.9	21
4.0	−	6.7	38	27	65	3	0	1	4	69[b]		45
4.0	+	13.3	15	16	31	0	1	0	1	32	61.4	26

STZ, streptozotocin; PNT, phenanthroline; MI, mitotic index (1000 cells analysed); Exch, Exchanges; Dic, Dicentric chromosomes; Del, Deletions; T, Total aberrations.

[a]Percentage of clastogenesis inhibition (PCI, see Section 2). Differences between observed and expected values resulted statistically significant for all the combined treatments ($p < 0.05$) except for STZ 1.0 mM plus PNT (borderline significance).

[b]Statistically significant with respect to control value ($p < 0.05$).

ments. For SCEs analysis, CHO cells were harvested at 24 h and mosquito cells were harvested at 48 h after the end of treatments. During the last 2.5 h (CHO) or 5 h (mosquito) of culture the cells were exposed to colchicine (0.1 μg/ml) (Sigma, CAS No. 64-86-8). Chromosome preparations were made according to the flame-drying method (i.e., slides were dried over a flame). To visualize SCEs, chromosome spreads were treated with 1 μg/ml of Hoechst 33258 (Sigma, CAS No. 23491-45-4) for 20 min in 0.1 M phosphate buffer (pH 6.8). Afterwards, slides were mounted in the same buffer and exposed for 1 h to the fluorescent light delivered by a battery of 5 Hitachi 10-W fluorescent tubes Following light ex-

Table 3
Induction of SCEs in CHO cells by treatment with STZ and the effect of PNT

STZ (mM)	PNT (9.0 μg/ml)	SCEs/cell (Mean ± S.E.)
0.0	−	8.68 ± 0.30
0.0	+	8.58 ± 0.28
0.1	−	16.88 ± 0.55[a]
0.1	+	17.88 ± 0.69[b]
0.5	−	30.30 ± 1.38[a]
0.5	+	No data[c]

STZ, streptozotocin; PNT, phenanthroline; 50-s mitosis per each treatment were scored.

[a]Statistically significant compared to control value ($p < 0.01$).

[b]Statistically non significant compared to the corresponding single treatment with STZ ($p > 0.05$).

[c]All metaphases observed in this dose and in the doses 1.0, 2.0 and 4.0 mM (with or without PNT) corresponded to first mitosis.

Table 4
Induction of SCEs in mosquito cells by treatment with STZ and the effect of PNT

STZ (mM)	PNT (9.0 μg/ml)	SCEs/cell (Mean ± S.E.)
0.0	−	3.12 ± 0.28
0.0	+	4.00 ± 0.35
0.1	−	4.64 ± 0.32[a]
0.1	+	3.94 ± 0.32[b]
0.5	−	5.60 ± 0.33[a]
0.5	+	5.36 ± 0.53[b]
1.0	−	7.02 ± 0.44[a]
1.0	+	6.40 ± 0.42[b]
2.0	−	8.52 ± 0.67[a]
2.0	+	8.98 ± 0.56[b]
4.0	−	10.92 ± 0.53[a]
4.0	+	11.48 ± 0.61[b]

STZ, streptozotocin; PNT, phenanthroline; 50-s mitosis per treatment were scored.

[a]Statistically significant compared to control value ($p < 0.01$).

[b]Statistically non significant compared to the corresponding single treatment with STZ ($p > 0.05$).

posure, slides were rinsed in deionized water, dried, and treated according to the procedure of Korenberg and Freedlender [19]. One hundred metaphases per treatment were scored for CAs and fifty second mitoses per dose endpoint were scored for SCEs.

The percentage of cells in mitosis for each endpoint (mitotic index, MI) was estimated over a total of 1000 cells randomly selected.

2.3. Statistical analysis

The percentage of clastogenesis inhibition (PCI) for each combined treatment of STZ and PNT was calculated applying the following formula:

$$\mathrm{PCI} = 100 - \frac{T \times 100}{E}$$

were T is the total number of aberrations produced by the combination of STZ plus PNT (i.e., the observed frequency of aberrations for the combined treatment) and E equals the sum of the aberrations induced by STZ only and by PNT only (i.e., expected frequency of aberrations for the combined treatment) (see Tables 1 and 2).

The significance of differences in aberration frequencies among different treatments was obtained by comparing Poisson distributions of observed and expected values with 95% confidence intervals [20]. The Student's t test was used for SCEs data analysis. The level of significance chosen was $p < 0.05$.

3. Results

3.1. Effect of PNT on STZ-induced CAs

The data on STZ-induced CAs in the presence or absence of PNT in CHO and mosquito cells are presented in Tables 1 and 2, respectively. A statistically significant increase in the frequency of CAs was observed in CHO and mosquito cells following treatment with increasing doses of STZ. ($p < 0.05$) (Tables 1 and 2). On the other hand, treatments with PNT alone did not produce any significant increase of CAs over control values ($p > 0.05$). The presence of PNT during the STZ treatments prevented the induction of CAs by 46.5% to 72.5% in both cell lines ($p < 0.05$, except for STZ 1.0 mM plus PNT, which exhibited borderline significance) (Tables 1 and 2). This protective effect was observed throughout the concentration range of STZ tested and was more evident at the lowest dose of STZ used (0.5 mM) (Tables 1 and 2).

On the other hand, the presence of PNT during STZ treatments in CHO cells produced a marked increase in the mitotic index (MI) in all the cell cultures examined with respect to the MI values observed in cultures treated with STZ only (Table 1). In mosquito cells, this effect was only evident at the highest dose of STZ used (4.0 mM) (Table 2).

3.2. Effect of PNT on STZ-induced SCEs

Induction of SCEs by STZ in CHO and mosquito cells is shown in Tables 3 and 4, respectively. The exposure to STZ significantly increased the frequencies of SCEs in CHO and mosquito cells as a linear function of drug concentration ($p < 0.01$). On the other hand, treatments with PNT alone or STZ plus PNT did not produce any significant alteration in the frequencies of SCEs in regard to control or STZ values, respectively ($p > 0.05$) (Tables 3 and 4). This indicates that PNT per se does not induce SCEs and that the addition of PNT does not prevent the STZ − induced SCEs. It must be noted that STZ produced an arrest of cell cycle progression in CHO but not in mosquito cells, as shown by the accumulation of cells in the first cycle and the absence of second mitosis in CHO cultures corresponding to treatments with STZ doses over 0.5 mM (Table 3). In addition, our results show that PNT affords no protection to CHO cells from the inhibition of cell-cycle progression caused by STZ (see Table 3).

4. Discussion

It has been shown that STZ induces CAs and SCEs in mammalian and insect cells[8,9,11,14]. Although the exact mechanisms by which STZ exerts its clastogenic activity remain to be fully elucidated, our previous observation that antioxidant compounds prevents to a great extent the induction of CAs in CHO and mosquito cells by STZ [14] suggests that

free radicals are involved in the production of CAs by this compound. In order to gain further insights into the mechanisms involved in the clastogenic action of STZ, in this report we investigated whether the addition of PNT into the culture medium can influence the yield of CAs and SCEs induced by STZ in CHO and mosquito cells. PNT is a compound that enters the cell and, by forming a complex with iron, prevents the Fenton reaction from occurring, thus blocking the production of the hydroxyl radical [21]. In a previous report, Eizirik et al. [22] showed that PNT does not protect against the diabetogenic effects of STZ, thus they ruled out the hypothesis that hydroxyl radicals generated via an iron-catalyzed reaction induces the STZ diabetogenic effects. Nevertheless, our present results show that the addition of PNT to CHO and mosquito cell cultures inhibits to a great extent the yield of STZ-induced CAs. This fact indicates that intracellular transition metals are implicated in the induction of CAs by STZ and that the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow OH^\circ + OH^- + Fe^{3+}$) is partly responsible for the production of CAs by this compound. Our previous finding that superoxide dismutase (which converts O_2^- into H_2O_2), catalase (a scavenger of H_2O_2) and mannitol (a scavenger of OH^-) inhibits the chromosome damage induced by STZ [14] and our present finding that PNT inhibits the production of CAs by STZ allow us to speculate that STZ generates O_2^-, which acts as a reducing agent of Fe^{3+}; $O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$, so that the sum of this and the Fenton reaction, i.e., the iron-catalyzed Haber–Weiss reaction, provides a good explanation for the induction of CAs by STZ: $H_2O_2 + O_2^- \rightarrow OH^\circ + OH^- + O_2$. According to this view, the OH radical thus produced is the agent which ultimately causes CAs by STZ.

On the other hand, our results showed that the exposure to STZ significantly increased the frequencies of SCEs in CHO and mosquito cells as a linear function of drug concentration and produced an arrest of cell cycle progression in CHO cells. Similar findings were made by Capucci et al. [9,10], in CHO cells exposed to lower doses of STZ than those employed in our study It has been reported that PNT avoids the induction of SCEs generated by H_2O_2 and O_2^- [23]. The fact that the addition of PNT did not affect the yield of STZ − induced SCEs in CHO and mosquito cells strongly suggests that neither metal ions nor free radicals are involved in the production of SCEs by this compound. Therefore, it is valid to assume that the induction of CAs and SCEs by STZ occurs by different mechanisms. The previous finding by Capucci et al. [9] that STZ induces SCEs but not CAs in V79 cells gives further support to this assumption. Most likely, SCEs induction by STZ results from DNA methylation by the drug [5,6,8,9,11]. Clearly, our results demonstrate that the mechanisms by which STZ induces chromosome damage are very complex. An additional evidence of this is the fact that PNT did not totally prevent the STZ-induced CAs. However, it is possible to speculate that STZ exerts its clastogenic effect partly by participating in a redox cycling process to generate free radicals, which in the presence of transition metal ions, can induce CAs, presumably by site specific generation of OH radicals through an iron-catalyzed Haber–Weiss reaction. Clearly, additional studies are needed to fully elucidate the exact mechanisms by which STZ induces chromosome damage. These studies are currently being performed in our laboratory.

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