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The mechanism of polyamine analog-induced enhancement of cisplatin cytotoxicity in the U-251 MG human malignant glioma cell line

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Abstract *Purpose*: During the last decade, several polyamine analogs have been developed as antineoplastic agents that replace intracellular polyamines but cannot mimic the biological functions of polyamines related to cell growth. It has been shown that pretreatment of several human brain tumor cell lines with some of these polyamine analogs increases the cytotoxicity of *cis*-diamminedichloroplatinum (CDDP). It has also been established that some of these polyamine analogs affect chromatin organization. In the study reported here we attempted to elucidate the mechanism by which polyamine analog-induced changes in DNA and chromatin structure may increase CDDP cytotoxicity. *Methods*: We studied the micrococcal nuclease sensitivity of the nuclei and measured the amount of platinum incorporated into the nucleosomal and linker regions of chromatin isolated from CDDP-treated U-251 MG human malignant brain tumor cells with or without pretreatment with two cytotoxic polyamine analogs 1,11-bis(ethylamino)-4,8-diazaundecane (BE-3-3-3) and 1,19-bis(ethylamino)-5,10,15-diazanonadecane (BE-4-4-4-4). *Results*: The pretreatment with the polyamine analogs decreased the MNase sensitivity and increased the incorporation of CDDP preferentially into the linker region of the chromatin. *Conclusions*: Pretreatment of cells with polyamine analogs probably alters the structure and/or the organization of the linker region such that more CDDP incorporates into the linker DNA. This is probably the reason for the observed enhancement of CDDP cytotoxicity in the polyamine analog-pretreated cells.

Key words Polyamine analog · *cis*-Diamminedichloroplatinum · Micrococcal nuclease · Chromatin structure · Atomic absorption spectroscopy

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Introduction

The polyamines spermidine and spermine and their precursor diamine putrescine have been detected in all mammalian cells [12]. In the last couple of decades, it has been demonstrated that the naturally occurring polyamines are absolutely required for the growth and proliferation of almost all living cells [8]. However, the specific biological function(s) of polyamines related to cell growth have only recently begun to be elucidated. Polyamines are polycations under normal physiological conditions. It is assumed that polyanions, such as DNA, will be the primary intracellular binding site(s) for polyamines. In vitro studies have demonstrated that the natural polyamines bind to DNA and induce various changes in DNA secondary structure including chromatin condensation (reviewed in reference 1). Based on these studies, we proposed that polyamines assist cell division by condensation and packaging of the newly synthesized DNA into chromatin. An inhibition of this function, therefore, should block the cell cycle and inhibit cell proliferation.

In recent years, several polyamine analogs have been designed and synthesized [1, 3, 10]. These analogs have been developed as anticancer agents with the idea that the analogs should prevent cell growth by poorly mimicking the growth related functions of their natural counterparts [3, 8, 10]. It has been demonstrated that some of these analogs arrest the growth of several human tumor cells both in tissue culture as well as in nude mouse xenografts [3, 6, 8, 10]. Two of the analogs, 1,11-bis(ethylamino)-4,8-diazaundecane (BE-3-3-3) and 1,19-bis(ethylamino)-5,10,15-diazanonadecane (BE-4-4-4-4), are either in or are being considered for clinical trials. In the last 5 years, several studies have shown that polyamine analog treatment opens up the structure of chromatin from a compact to a relaxed state (reviewed

in reference 1). Such relaxation of chromatin structure can be exploited therapeutically by combining polyamine analogs with DNA-binding anticancer agents that prefer binding to relaxed chromatin over condensed chromatin. *cis*-Diamminedichloroplatinum (II) (CDDP) is one such agent used widely in cancer chemotherapy. It has two chloride atoms separated by a distance of 3.3 Å [11]. This distance is perfectly suited for a nucleophilic attack by CDDP at two consecutive DNA bases which are 3.4 Å apart to form an intrastrand crosslink between two adjacent DNA bases. Hayes and Scovell [7] have demonstrated that nuclei isolated from CDDP-treated chicken erythrocytes are more resistant to MNase digestion than their untreated counterparts. It was concluded that CDDP preferentially binds to the linker region of DNA and inhibits MNase digestion. Therefore, CDDP cytotoxicity may be affected by changes in DNA and chromatin structure that may alter the structure and/or the organization of linker DNA.

We have reported that pretreatment with the polyamine analogs BE-3-3-3 and BE-4-4-4-4 increases the cytotoxicity of CDDP in cultured human brain tumor cell lines [4]. We were unable to show a clear correlation between these results and the abilities of the analogs to induce B- and Z-DNA structures in vitro. We have previously reported that some polyamine analogs affect chromatin condensation and probably change the structure and/or the organization of linker DNA [2]. Since CDDP preferentially binds to linker DNA [7], we studied the effects of pretreatment of U-251 MG human brain tumor cells with the polyamine analogs BE-3-3-3 and BE-4-4-4-4 on the incorporation of CDDP into DNA in chromatin by following the MNase sensitivity of isolated cell nuclei as well as by measuring the amount of platinum in nucleosomal and linker DNA by atomic absorption spectroscopy. Pretreatment with the polyamine analogs decreased MNase sensitivity of the nuclei and increased platinum incorporation into the linker DNA in CDDP-treated cells. The increased incorporation of CDDP in the linker region of the polyamine analog-treated cells was probably a consequence of changes in the structure of linker DNA in the analog-treated cells.

Materials and methods

Materials

BE-3-3-3 was a kind gift from Warner-Lambert-Parke-Davis (Rochester, N.Y.) and BE-4-4-4-4 was obtained from the drug synthesis and development section of the National Cancer Institute (Bethesda, Md.). Mycoplasma-free U-251 MG human malignant glioma cells were obtained from the tissue bank of the Brain Tumor Research Center of the University of California, San Francisco.

Cell culture

Cells were grown in monolayer cultures in complete Eagle's minimum medium containing nonessential amino acids, 10% fetal calf serum, and gentamicin (100 μg/ml) in 175-cm^2 tissue culture flasks following a previously described procedure [4]. Cultures were incubated at 37 °C in a humidified atmosphere of 95% air/5% CO_2. Control flasks were seeded with 2×10^6 cells and flasks for analog treatment were seeded with 2.4×10^6 cells. This compensated for the growth-inhibitory effects of the analogs and insured that the cell density was equal in all flasks at the time of CDDP treatment. Cells were treated 1 day after cell seeding with 10 μ*M* of the analogs. After another 48 h, the cells were treated for 1 h with 10 μ*M* CDDP. Cells were then trypsinized, harvested and counted using a hemacytometer.

Nucleus isolation

Nuclei were isolated following a procedure described elsewhere [9]. Briefly, the cells were collected in a precooled 50-ml plastic tube and centrifuged for 10 min at 4 °C to make a pellet. The pellet was then washed twice with 2.5 ml ice-cold phosphate-buffered saline. The pellets were transferred to sterile precooled plastic Sorvall tubes and were incubated in ice for 15–30 s with sterile lysis buffer (1 ml/5×10^6 cells) containing 0.25 *M* sucrose, 0.06 *M* KCl, 0.05 *M* NaCl, 0.01 *M* morpholino sulfonic acid (MES), 0.01 *M* $MgCl_2$, 0.001 *M* $CaCl_2$, 0.0001 *M* phenylmethanesulfonyl fluoride (PMSF) and 0.5% Triton X-100 (pH 6.5). Nuclei were pelleted by centrifugation at 5000 rpm for 10 minutes at 4 °C in a Beckman J2-21M refrigerated centrifuge using a JA-17 rotor. The pellet was washed twice with the same volume of lysis buffer and once with a sterile storage buffer containing 0.005 *M* $MgCl_2$, 0.01 *M* PIPES, 0.2 *M* NaCl and 0.01 *M* $CaCl_2$ (pH 6.8), resuspended in the minimum volume of the storage buffer [2], and stored at 4 °C. The purity of the nuclear preparation and the concentration of DNA in the nuclei were determined from its UV absorption spectrum in 0.1% sodium dodecyl sulfate.

MNase assay

A stock solution of MNase was prepared by dissolving a small amount of lyophilized powder in 200 μl digestion buffer. MNase assay was performed following a previously standardized procedure [2]. The enzyme activity was determined by following the digestion of calf thymus DNA at 37 °C in a Perkin-Elmer Lambda 4C UV-visible spectrophotometer connected to an electrical heating system. By definition, one unit of the enzyme digests 1.0 OD (A_{260}) of DNA in 30 min at 37 °C.

MNase digestion of isolated nuclei

Nuclei were digested with MNase following a previously described procedure [2]. Briefly, stock suspensions of nuclei were diluted with digestion buffer to a final concentration of 1 A_{260}/ml. Precalculated volumes of nuclear suspension were then distributed to 1.5-ml sterile siliconized plastic Eppendorf tubes so that the volume after enzyme addition would be 70 μl. The aliquots were incubated in ice for 15–30 s. Chilled enzyme solution (0.2 units) was then added and the tubes were incubated at 37 °C. We have previously shown that under these conditions MNase exclusively digests linker DNA [2]. After appropriate time intervals, the reaction was stopped by adding equal volumes of ice-cold 10 m*M* ethylene glycol tetraacetic acid (EGTA), and the mixture chilled in ice. In the zero-time tube, EGTA was added before the addition of the enzyme. Each sample was diluted with the storage buffer to 0.5 ml final volume and centrifuged at 10 000 rpm (300 *g*) for 10 min in a microfuge. The amount of digested DNA in the supernatant was measured from the A_{260} values determined using a Perkin-Elmer spectrophotometer. The absorption at 260 nm was fitted to the equation [2]:

$$A^t_{260} = P1\left(1 - e^{-P2 \cdot t}\right)$$

using a Marquadt-Levenberg algorithm in an IBM-PC personal computer, where A^t_{260} is the absorption at 260 nm at time "*t*" from

the start of the reaction, and P1 and P2 are the variable parameters. The initial velocity (v_0) of the enzyme reaction was calculated from the equation:

$$v_0 = (dv/dt)_{t \to 0} = P1 \times P2$$

Atomic absorption spectroscopy

Isolated nuclei were digested with 0.2 U MNase for 60 min following the procedure described above. Nuclei were precipitated by centrifugation at 10,000 rpm (300 *g*) for 10 min at 4 °C, washed three times with isotonic sodium phosphate buffer (pH 7.4), and both the supernatant + wash and the pellet were lyophilized in a Speed-vac centrifuge connected to a refrigeration unit. Samples were digested with concentrated nitric acid and the platinum contents of the DNA in both the MNase-digested supernatant (linker DNA) and the undigested pellet (nucleosomal DNA) fractions were determined by atomic absorption spectroscopy in a graphite furnace with a standardized temperature program and monitored at 265.9 nm with spectral bandpass of 0.2 nm in the microanalysis laboratory of the Chemistry Department of the University of California, Berkeley, and at the analytical laboratory of the University Wisconsin Cancer Center. Equal volumes of isotonic phosphate buffer containing 10 m*M* EGTA were similarly dehydrated and used as controls and the platinum contents of the controls were subtracted from the experimental values obtained during analysis.

Results

In the experiments reported here, we used 10 μ*M* CDDP in cells pretreated with 10 μ*M* polyamine analogs for 48 h. We have previously reported that under these conditions polyamine analogs deplete over 90% of intracellular polyamines and increase the cytotoxicity of CDDP [2, 4, 6]. The data presented here were representative of experiments that were repeated three times. In each set of experiments, the digestion kinetics were run twice in triplicate. The data points and the error bars shown in Figs. 1–3 are, respectively, the means and the standard deviations of the representative digestion experiment performed twice in triplicate.

The time course of MNase digestion of control and CDDP-treated U-251 MG cell nuclei is shown in Fig. 1. No appreciable difference was seen between the A_{260} values at the endpoints of digestion (limit digests) between control and CDDP-treated cells. A comparison between the A_{260} values of the digested nuclei with that of the limit digests showed that under our conditions less than 10% of the total nuclear DNA (initial A_{260}/ml = 1) was digested.

The time course of MNase digestion of the control and CDDP-treated U-251 MG cell nuclei pretreated with 10 μ*M* BE-4-4-4-4 is shown in Fig. 2. The 'limit digest' of control BE-4-4-4-4 cell nuclei was markedly greater than that of the nuclei from cells treated with both BE-4-4-4-4 and CDDP. The time course of MNase digestion of control and CDDP-treated U-251 MG cell nuclei pretreated with 10 μ*M* BE-3-3-3 is shown in Fig. 3. Similar to the findings in BE-4-4-4-4-treated cells, the limit digest of control BE-3-3-3-treated cells was markedly higher than that of the cells treated with both BE-3-3-3 and CDDP.

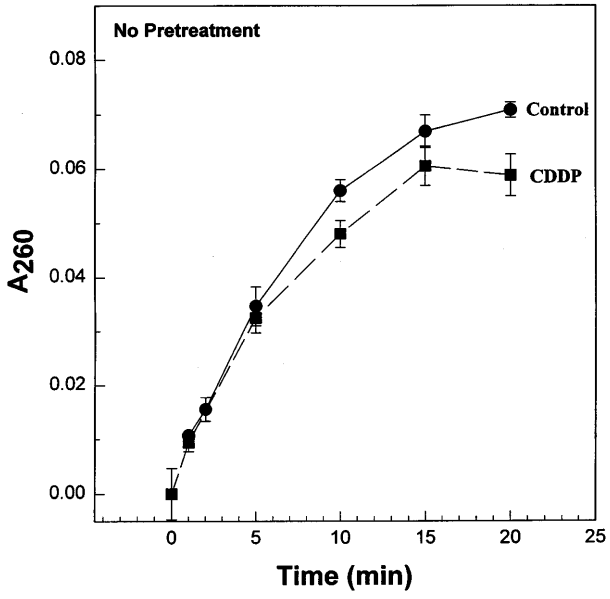

Fig. 1 Effect of CDDP treatment on the time course of MNase digestion of nuclei isolated from U-251 MG human brain tumor cells. Each data point and the associated error bars are, respectively, the mean and the standard deviations of data collected from three separate sets of experiments in which, in each set, the digestion was carried out twice in triplicate (● control untreated cells, ■ cells treated with 10 μ*M* CDDP)

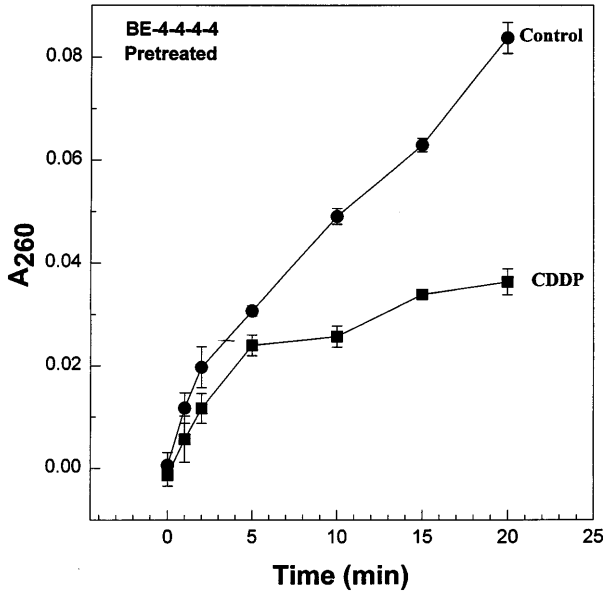

Fig. 2 Effect of CDDP treatment on the time course of MNase digestion of nuclei isolated from U-251 MG human brain tumor cells pretreated with 10 μ*M* BE-4-4-4-4 for 48 h. Each data point and the associated error bars are, respectively, the mean and the standard deviations of data collected from three separate sets of experiments in which, in each set, the digestion was carried out twice in triplicate (● control BE-4-4-4-4 cells, cells treated with BE-4-4-4-4 and 10 μ*M* CDDP)

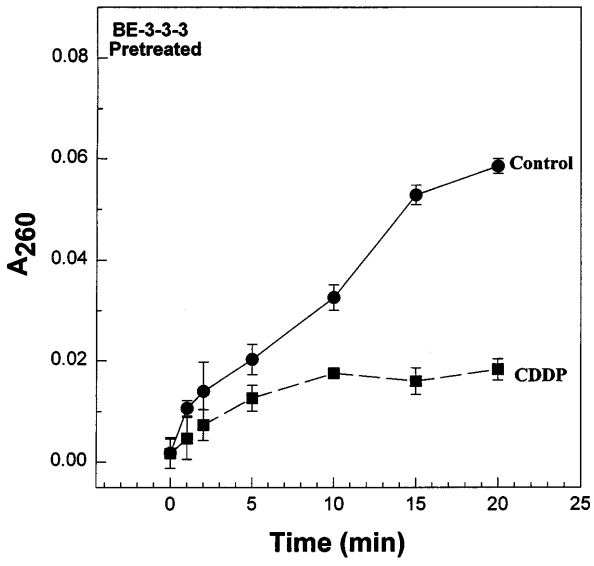

Fig. 3 Effect of CDDP treatment on the time course of MNase digestion of nuclei isolated from U-251 MG human brain tumor cells pretreated with 10 μ*M* BE-3-3-3 for 48 h. Each data point and the associated error bars are, respectively, the mean and the standard deviations of data collected from three separate sets of experiments in which, in each set, the digestion was carried out twice in triplicate (● control BE-3-3-3 cells, cells treated with BE-3-3-3 and 10 μ*M* CDDP)

The initial velocities (v_0) of the MNase digestion kinetics are shown in Table 1. Except for cells treated with both BE-3-3-3 and CDDP, all other cells had very similar v_0 values. In the case of BE-3-3-3 + CDDP-treated cells, the v_0 values were one order of magnitude lower than the values for all other cells.

In order to further confirm that the decrease in the MNase digestion rate was indeed a consequence of an increase in platinum incorporation in linker DNA, we studied the amount of platinum incorporated into linker and nucleosomal DNA by atomic absorption spectroscopy. Each experiment was repeated three times in triplicate. The mean values and standard deviations of a representative experiment are shown in Table 2. We have previously determined that under our digestion conditions over 90% of the nuclear DNA is digested to its mononucleosomal form [2]. The results in Table 2 show that there was only a small difference in the amount of platinum incorporated into the nucleosomal and the linker region of the analog-untreated cells. Also, there was only a marginal increase in the amount of platinum in the nucleosomal DNA in both BE-3-3-3- and BE-4-4-4-4-treated cells. In contrast, the amount of platinum incorporated into linker DNA was about twofold higher in the BE-4-4-4-4-pretreated cells and about threefold higher in the BE-3-3-3-pretreated cells as compared with the cells treated only with CDDP.

Table 1 Initial velocity (v_0) of MNase digestion of nuclei isolated from U-251 MG human brain tumor cells

Analog	v_0 (A_{260}/min)	
	Control untreated	CDDP-treated
–	0.009	0.008
BE-4-4-4-4	0.010	0.007
BE-3-3-3	0.007	0.0002

Table 2 Platinum incorporation in nucleosomal and linker DNA in polyamine analogue treated cells as measured by atomic absorption spectroscopy

Analogue	Component	Pt (μg)/DNA O.D.$_{260}$
Control	Nucleosomes	$7.1 \pm 1.1 \times 10^{-5}$
	Linker	$13.1 \pm 1.5 \times 10^{-5}$
BE-4-4-4-4 (10 μ*M*)	Nucleosomes	$18.1 \pm 0.6 \times 10^{-5}$
	Linker	$27.3 \pm 1.2 \times 10^{-5}$
BE-3-3-3 (10 μ*M*)	Nucleosomes	$11.2 \pm 0.9 \times 10^{-5}$
	Linker	$38.2 \pm 1.7 \times 10^{-5}$

It should be noted that no cellular polyamines, polyamine analogs, or unreacted CDDP were detected in the nuclear preparations (data not shown). Therefore, the changes in the enzyme kinetics reported here were not a consequence of the presence of polyamine, polyamine analogs or CDDP during the enzyme digestion kinetic study. The importance of this point is discussed below.

Discussion

The MNase digestion kinetics data corroborate our previous observation that less than 10% of the total nuclear DNA is in the linker region of the chromatin [2]. Both the initial rates (Table 1) and the limit digest (Fig. 1) in CDDP-treated cells decreased minimally as compared with the untreated cells. This observation differs from that previously reported by Hayes and Scovell [7]. However, in their studies, 50 μ*M* CDDP was used in HeLa cells. Considering that U-251 MG brain tumor cells are more resistant to CDDP than are HeLa cells and we used a lower CDDP concentration (10 μ*M*) than used in the previous studies, it is not surprising that the difference in the MNase digestion kinetics observed in our system was much less significant than that reported by Hayes and Scovell [7]. As mentioned above, the low CDDP concentration was used to follow the standardized conditions where polyamine analog treatment maximally affects the CDDP cytotoxicity in cultured brain tumor cells under physiologically relevant conditions [4].

Both BE-3-3-3 and BE-4-4-4-4 treatments caused a significant inhibition of MNase digestion (Figs. 2 and 3). Since CDDP treatment did not appreciably change the extent of limit digest in the analog-untreated cells (Fig. 1), this difference suggests a higher incorporation of platinum in the linker region of the analog-treated cells. It should also be noted that there was a small but reproducible increase in the amount of limit digest in BE-4-4-4-4-treated cells as compared with control

untreated and BE-3-3-3-treated cells (compare Fig. 2 with Figs. 1 and 3). This difference was probably because of an inhibition of nucleosome formation in BE-4-4-4-4-treated cells. We have also observed an inhibition of nucleosome formation in another human brain tumor cell line after a 96-h treatment with another polyamine analog related to BE-4-4-4-4 [2]. However, the difference in the magnitude of the limit digests after the 96-h analog treatment observed in that case is more pronounced than the difference observed after the 48-h treatment with BE-4-4-4-4 in the experiments reported here. Apart from the time of incubation, the difference could also be because of a difference between the analogs or the cell lines or a combination of all of these three factors. It should be noted that none of the polyamine analogs [2] nor unreacted CDDP was detected in the isolated nuclei that were used for the MNase digestion kinetic experiments (data not shown). Therefore, the observed changes in the MNase digestion kinetics were a result of a change in the chromatin structure because of analog and CDDP treatment and not a result of the presence of polyamines and/or unreacted CDDP in the enzyme reaction mixture.

Neither CDDP, nor any of the analogs alone, nor a combination of BE-4-4-4-4 and CDDP appreciably affects the initial MNase digestion rates (v_0). In contrast, a combination of BE-3-3-3 with CDDP markedly decreased v_0 (Table 1). It seems likely that the change in v_0 was a consequence of a change in the K_m values for the enzyme–DNA complex. Such a change indicates a change in the structure of linker DNA that changes the affinity of MNase for the substrate. However, this point can only be confirmed by detailed enzyme kinetic analysis at various substrate concentrations.

Our in vitro studies have shown that BE-4-4-4-4 inhibits nucleosome formation [5]. Such inhibition can cause an increase in the amount of linker DNA, thus causing an enhancement of CDDP cytotoxicity in BE-4-4-4-4-pretreated cells. However, our in vitro studies did not show any effect of BE-3-3-3 on nucleosome formation [5] and, therefore, cannot explain the increase in CDDP cytotoxicity in the BE-3-3-3-pretreated cells. The decrease in the v_0 values (Table 1) and the increase in platinum incorporation in linker DNA in BE-3-3-3-treated cells (Table 2) suggest that BE-3-3-3 treatment alters the structure of linker DNA [4] such that the DNA becomes more exposed to CDDP attack. Thus, the data reported here establish that both BE-3-3-3 and BE-4-4-4-4 increase CDDP cytotoxicity by affecting chromatin structure and exposing linker DNA to CDDP adduct formation, but the exact mechanism of BE-3-3-3-induced enhancement of CDDP cytotoxicity may be slightly different from that of BE-4-4-4-4-induced enhancement.

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