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ENHANCED LEVELS OF CYCLIC AMP, ADENOSINE(5′)TETRAPHOSPHO(5′)ADENOSINE AND NUCLEOSIDE 5′-TRIPHOSPHATES IN MOUSE LEUKEMIA P388/D_1 AFTER TREATMENT WITH *cis*-DIAMMINEDICHLOROPLATINUM(II)*

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Abstract—As part of the exploration of the mechanism of platinum(II) complex-induced growth inhibition and/or cytotoxicity, we studied the intracellular levels of several nucleotides during treatment of mouse leukemia P388/D_1 at selected concentrations of 1 μM *cis*-diamminedichloroplatinum(II) (*cis*-DDP) and 20 μM of its *trans*-isomer (*trans*-DDP). The effects and their time-dependences are correlated with those on cell growth parameters previously published (Just G and Holler E, *Cancer Res* **49**: 7072–7078, 1989). The effects of *cis*-DDP are strong and irreversible, whereas those of *trans*-DDP are marginal and reversible, in parallel with similar effects on cell growth parameters. Concentrations of nucleoside 5′-di- and 5′-triphosphates increase in parallel with cellular DNA and protein content by three- to four-fold after 60 hr of treatment. The nucleoside monophosphates dAMP, dGMP and dTMP reveal concentration maxima during exponential cell growth that are two- to six-fold higher than in the control cultures. Levels of cyclic AMP, adenosine(5′)tetraphospho(5′)adenosine (Ap_4A) and CDP rise three- to five-fold above those in the control cultures within a few hours of the start of treatment. The level of coenzyme NAD^+ falls below that of the control, concomitantly with an arrest of cells in the G2 phase of the cell cycle and with the appearance of giant cells. Due to the high reactivity of *cis*-DDP and the continuous concentration increase during the treatment, purine nucleoside 5′-triphosphates provide a possibility for the acquisition of resistance to *cis*-DDP. The correlation of responses of metabolically and regulatory active nucleotides with biological effects suggests their function in antitumorigenesis.

Several human tumors are successfully treated with *cis*-diamminedichloroplatinum(II) (*cis*-DDP‡) [1]. DNA damage in tumor cells is caused by intrastrand crosslinks of *cis*-DDP with purine N7 at d(GpG) and d(ApG) sequences. This is thought to represent the main route leading to inhibition of tumor cell proliferation (for a recent review see Ref. 2). One of the reasons for this assumption is that *trans*-diamminedichloroplatinum(II) (*trans*-DDP), which is therapeutically ineffective, cannot form these types of adducts.

Because *cis*-DDP is chemically very reactive, it appears that other biochemical targets besides DNA exist and can contribute to cell growth inhibition and cytotoxicity. Several observations seem to support this hypothesis. (i) Minimal fractions of the overall incorporated platinum are detected in coordination with DNA [3]. (ii) Because of repair, inhibition of DNA synthesis should be transient and thus may not be as critical for *cis*-DDP-induced cytotoxicity as hitherto assumed [4]. Attempts to correlate the amount of *cis*-DDP required for inhibition of DNA synthesis *in vitro* with the concentration needed for inhibition of cell growth are indeed unsuccessful [5]. (iii) Amino acid transport is impaired by *cis*-DDP. Certain *cis*-DDP resistant cell lines display alterations in their plasma membrane transport systems [6–9]. Other evidence for an involvement of membrane proteins in the mode of action of platinum(II) complexes is provided by the fact that mechanisms of alkylating drugs have features in common with those of *cis*-DDP [10]. Their targets are, besides DNA, several distinct cytoplasm membrane proteins involved in transport or hormone signal transduction [10, 11]. (iv) Cells treated with *cis*-DDP are arrested in the G2 phase of the cell cycle, suggesting an involvement of pathways that regulate cell division [12, 13]. (v) In a previous investigation employing the P388/D_1 cell line, we found that 1 μM *cis*-DDP inhibits cell division, while synthesis of DNA and protein continues at almost unperturbed and balanced rates, leading to the appearance of giant cells [13]. (vi) Diadenosine(5′)tetraphospho(5′)-adenosine (Ap_4A), a proposed pleiotypic growth factor or alarmone [14], reacts easily with *cis*-DDP to yield a bidentate platinum(II) complex [15]. The *cis*-DDP and *trans*-DDP adducts of Ap_4A cannot be hydrolysed nor can they bind to other biomolecules [16, 17]. (vii) Purine nucleoside 5′-triphosphates react with *cis*-DDP in cell-free systems at rates as fast as observed for DNA (unpublished results). (viii) Platinum(II) adducts of dGMP are only slowly

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‡ Abbreviations: *cis*-DDP, *cis*-diamminedichloroplatinum(II); *trans*-DDP, *trans*-diamminedichloroplatinum(II); Ap_4A, adenosine(5′)tetraphospho(5′)adenosine.

dephosphorylated and are resistant against oxidation at the level of the hypoxanthine adducts of *cis*- or *trans*-DDP [18]. By forming the adducts, various nucleotides seem to cease to be available for biochemical reactions and are thus removed from active cellular pools. (ix) Reactions of *cis*-DDP with several cellular components may be implicated in drug resistance, and such components have been summarized recently [19].

The biochemical events that accompany *cis*-DDP-induced growth inhibition and/or cytotoxicity, besides the binding of the drug to nucleic acids, are largely unknown. In order to detect changes in the biochemistry of cells during the drug treatment, we examined the nucleotide pool of mouse leukemia P388/D_1 with regard to the biological effects seen during treatment with platinum(II) complexes [13]. The nucleotide pool is a suitable compartment for studying biochemical effects of such a treatment for the following reasons. (i) Nucleotides can disappear in the form of platinum(II) adducts. (ii) The relative concentrations of nucleotides reflect the cell energy charge. (iii) Nucleotide biosynthetic activities such as those of nucleoside kinases and dihydrofolate reductase can decrease [20], or increase as for cyclic AMP [21]. (iv) Catabolic activities can increase as for NAD^+ due to its function as a substrate of nuclear ADP-ribosyltransferase in DNA-repair [22]. (v) The concentration of Ap_4A may increase because of its role as an alarmone [23]. The geometrical isomer *trans*-DDP serves as a control for effects that were not related to the typical antitumor activity of *cis*-DDP [24].

MATERIALS AND METHODS

Mouse leukemia P388/D_1 cultures were obtained from Dr Reubl (Munich, Germany). RPMI/glutamine (without carbonate) was purchased from Biochrom (Berlin, Germany) and Gibco (Glasgow, U.K.), horse serum from Boehringer (Mannheim, Germany), [^{3}H]thymidine and other radioactively labelled nucleotides from Amersham–Buchler (Braunschweig, Germany), ethidium bromide, calf thymus DNA, pronase, bovine serum albumin and ribonuclease A from Sigma (Munich, Germany), and all other chemicals were of the highest available grade from Merck (Darmstadt, Germany). *cis*-DDP and *trans*-DDP were gifts of Degussa (Frankfurt, Germany).

Phosphate buffered saline (PBS), pH 7.0, contained 2.63 mM KCl, 1.46 mM KH_2PO_4, 136 mM NaCl, 7 mM Na_2HPO_4 and 1 mM $NaH_2PO_4 \cdot H_2O$ in distilled water.

Cell culture. Mouse leukemia P388/D_1 cultures were seeded at (8–10) $\times 10^4$ cells/mL and grown in 200 mL suspension cultures without shaking in the presence of 5% CO_2 at 37°. Growth medium was RPMI containing 10% heat inactivated horse serum (30 min at 56°), $NaHCO_3$ (10 mM), hepes buffer (10 mM, pH 7.4) and glutamine (2 mM). The doubling time of cells was 12–14 hr [25]. Freshly prepared solutions of *cis*-DDP or *trans*-DDP in dimethylformamide (0.01% by vol.) were added at the end of the lag phase (9 hr after seeding). The control culture (platinum absent) contained the same amount of dimethylformamide.

Protein. Amounts of (1–4) $\times 10^5$ cells for each time point were washed twice with PBS, pelleted at 1000 *g* in silane treated vials, resuspended in 0.1 volume PBS (with regard to the volume of the growth medium that had contained these cells) and homogenized by ultrasonification (Branson Sonifier B15). After centrifugation at 5000 *g*, the protein in the supernatant was measured according to the method of Bradford [26]. Cell numbers were determined with a coulter counter Model ZM.

Cell extraction and HPLC-analysis. Amounts of (1–10) $\times 10^6$ cells for each time point were washed twice with PBS and centrifuged at 1000 *g* in silane treated vials. Immediately after addition of 20 μL of a mixture of ^{3}H-marker nucleotides (dCTP, dGTP and dTTP, 30–50 Ci/mmol, each 20 nM in PBS) the cell pellet was extracted according to a modified method of Shrylock *et al.* [27]. Added to the pellet per 1 $\times 10^7$ cells, during vigorous stirring on a whirl mix were 5 mL of an 80% (by vol.) hot (75°) methanol/water mixture containing 0.5 mM EDTA. After 2 min, the extract was cooled on ice for 15–30 min and then centrifuged at 5000 *g*. The supernatant was immediately lyophilized and stored at −60° until HPLC analysis.

HPLC analysis was performed with low pressure mixing equipment from LKB (Bromma, Sweden). Radioactive marker nucleotides were detected by a flow monitor from Raytest (Straubenhart, Germany) in series with the UV-detector (254 nm wavelength, 10 μL cell, 1 cm light path). Signals from both detectors were recorded, synchronized, stored and evaluated on an IBM-XT compatible computer with chromatography software from Nuclear Interface (Münster, Germany).

Quantitative analysis of intracellular nucleotides was carried out in two successive chromatographic separations of the cell extract. After removing particles by centrifugation, lyophilisates (50–200 μL) in PBS were chromatographed on a semi-preparative TSK DEAE 5PW weak anion exchange column (75 × 7.5 mm) from Bio Rad (Munich, Germany) with a 60 min linear gradient from 0.02 to 0.7 M ammonium acetate at pH 9.0. [^{3}H]Nucleotides added prior to extraction served as quantitative markers for yield calculations. Typically, yields were 63 ± 7%, for the extraction and ion-exchange chromatography together. The fractionated eluates were separated into six pools, which had been selected on the basis of the elution positions of unlabelled nucleotide markers. These markers (free nucleotides and platinum(II) adducts) were also employed to identify the extracted nucleotides of interest. Pools were lyophilized, resuspended in 50–200 μL PBS and analysed by reversed phase chromatography on a μ-bondapak C_{18} column from Waters (Milford, MA, U.S.A.). Elution was carried out in a series of methanol gradients in 0.1 M ammonium phosphate buffer (pH 5.4) according to the following schedule: 5 min, 0.3%; 10 min, 0.3–3%; 15 min, 3–15%; 3 min, 15%; 3 min, 15–0.3%, 6 min, 0.3% methanol (by vol.) [27, 28]. Typical yields were 90 ± 10%. Figure 1 gives an example for both the ion exchange and the reversed phase chromatography.

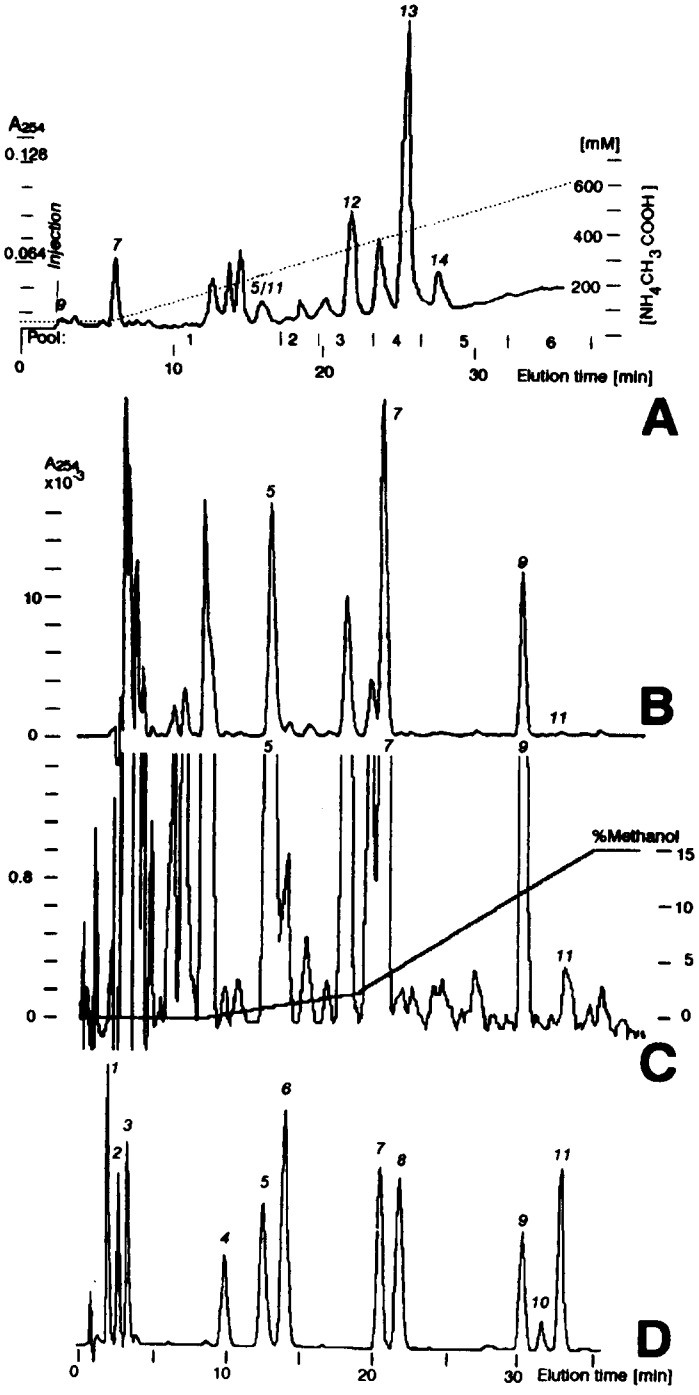

Fig. 1. Representative analytical HPLC chromatograms. An example of nucleotide analysis is given using an extract of mouse leukemia P388/D_1 cells, after a 15 hr treatment with 1 μM *cis*-DDP. Results are shown for the primary separation of all nucleotides by ion exchange HPLC on DEAE 5PW, the separation of this effluent into six pools, and the subsequent separation for one arbitrarily chosen pool by reversed phase HPLC on μ-bondapak C18. Elution gradients are indicated by broken lines, the concentrations of the eluents is displayed on the right side of the figures. Nucleotides are represented by numbers: (1) CMP, (2) UMP, (3) dCMP, (4) TMP, (5) AMP, (6) $NADP^+$, (7) NAD^+, (8) dAMP, (9) adenosine, (10) *cis*-DDP-Ap_4A adduct, (11) cAMP, (12) ADP, (13) ATP and (14) GTP. For details see Materials and Methods. Panel A: primary HPLC on DEAE 5PW. Panel B: secondary HPLC on μ-bondapak C18 of pool 1 in panel A. Panel C: same as panel B but with higher resolution of the absorbance scale. Panel D: elution profile under conditions of panel B for a mixture of standard nucleotides.

Quantities were compared to the cell number and protein content of the same samples. The values were reproducible within ±10% error for chromatography except for dAMP, dTMP and cAMP which were within ±20% error, and within ±30% error for different cell extracts from the same cell crops.

Binding of platinum(II) complexes to nucleotides. The kinetics of the reactions between the platinum(II) complexes and either ATP or GTP were followed photometrically at 280–285 nm wavelength in a Zeiss DMR 10 spectrophotometer. Stock solutions of 2 mM platinum(II) complexes containing 3 mM KCl were equilibrated over night at 37°. Reaction solutions at 37° contained 0.1–1.0 mM nucleotide, 3 mM KCl and 10 mM Mops buffer (pH 7.0). The chloride ion concentration was the same as in the cytoplasm. Reactions were started by the addition of stock solutions containing 2 mM platinum complexes to give final concentrations of one tenth of the ATP or GTP concentration. Bimolecular reactions followed pseudo-first order kinetics under these conditions. Although *cis*-DDP bound to ATP in a 1:1 (mol:mol) stoichiometry [29], reaction kinetics were biphasic following an increase in absorbance. Time-dependences were treated as a superposition of a slow and a fast exponential, as described previously [30].

RESULTS

Effects on intracellular concentrations of nucleotides

After separation of nucleotides by anion exchange and reversed phase HPLC, more than 250 different compounds were distinguished in the eluate. The concentrations of eluting material varied by orders of magnitudes (Fig. 1B and C). Although in general resolution was high, the degree of separation of the small amounts of dGTP from the large amounts of ATP was not sufficient to allow a quantitative measurement of dGTP.

Because of the variation in cell size, it was meaningful to relate nucleotide levels to those of protein. The effect of this choice is demonstrated for AMP in Fig. 4: the effect of the treatment with *cis*-DDP appears small when related to the protein content and large when related to the cell number.

Many of the eluting bands increased in size in response to the treatment of the cells with the platinum(II) compounds. The effects of *cis*-DDP were often pronounced and irreversible in contrast to those of *trans*-DDP which were marginal and always reversible. Including the control, maximum levels of nucleotides were often observed 20–50 hr after seeding when the cultures were in the exponential growth phase. Four main groups of nucleotides have been distinguished on the basis of their concentration and of their time–response to the cell treatment with *cis*-DDP.

Representatives of the first group were the nucleoside 5′-triphosphates ATP, CTP, GTP and dTTP and the nucleoside 5′-diphosphates ADP and GDP (Fig. 3, Table 1); CDP was an exception (Fig. 4). Typically, nucleotide levels began to rise above those of the control 10–15 hr after addition of *cis*-DDP to the culture and continued to increase throughout the 70 hr observation period. At the end of the observation period, concentrations were three to four times higher than those of the control.

The second group consisted of the deoxyribonucleoside 5′-monophosphates dAMP, dGMP, dTMP and the ribonucleotide 5′-monophosphates AMP, CMP and GMP (Fig. 2, Table 1). The increase in nucleotide level relative to the control was marginal for the ribonucleotide in comparison with that for the deoxyribonucleotides. In contrast to the first group, the effects were reversible. Nucleotide levels were normal after 70 hr. Maximum concentrations varied between two- and five-fold depending on the kind of nucleobase.

The nucleotides of the third group were cyclic AMP, Ap_4A and CDP (Fig. 4). Exposure of cells to *cis*-DDP typically provoked an immediate rise in nucleotide concentration relative to the control (probably within less than 6 hr). The concentration of Ap_4A approached its maximum after 30 hr (10-fold higher than in the control). All nucleotide concentrations remained high for the rest of the observation period. In contrast, treatment with *trans*-DDP had only marginal effects.

Figure 4 also shows the time-dependence for the concentration of NAD^+. Its level was not affected during the first 15 hr of treatment but then fell below the level of the control where it remained until the end of the observation period. For *trans*-DDP, the level of NAD^+ decreased insignificantly.

Table 1 summarizes the various nucleotides investigated. The maximum concentrations and corresponding time periods of the treatment with the platinum(II) complexes are given. The concentrations were calculated on the basis of the previously measured values of cell volumes [13]. The nucleotide levels in Figs 2–4 compare well with those of the absolute concentrations in Table 1, except in the case of long periods of treatment with *cis*-DDP when cell size became highly variable so that the assumption of an average cell volume seemed no longer meaningful [13].

The value of the cell energy charge was calculated as a function of growth time [31]. By comparison with the control it was found that the treatment of the cells with the platinum(II) complexes did not affect this parameter beyond the experimental error (not shown).

Kinetics of nucleotide adduct formation

The high intracellular concentrations of purine nucleotides, especially of the nucleoside 5′-triphosphates, suggests the possibility of a reaction between these nucleotides and the platinum(II) complexes. The reaction will render platinum(II) complexes inactive for a subsequent attack on DNA (unpublished results).

The reactions of ATP or GTP were investigated under conditions of 3 mM chloride ions, pH 7.0 and 37°, which were as close as possible to intracellular conditions. An example is shown in Fig. 5. Kinetics were biphasic. Under conditions of excess nucleotide over platinum(II) complex, the fast phase followed a concentration-dependent pseudo-first order reaction. Second order rate constants as calculated from such dependences were $(22 \pm 10)\ M^{-1}min^{-1}$ for

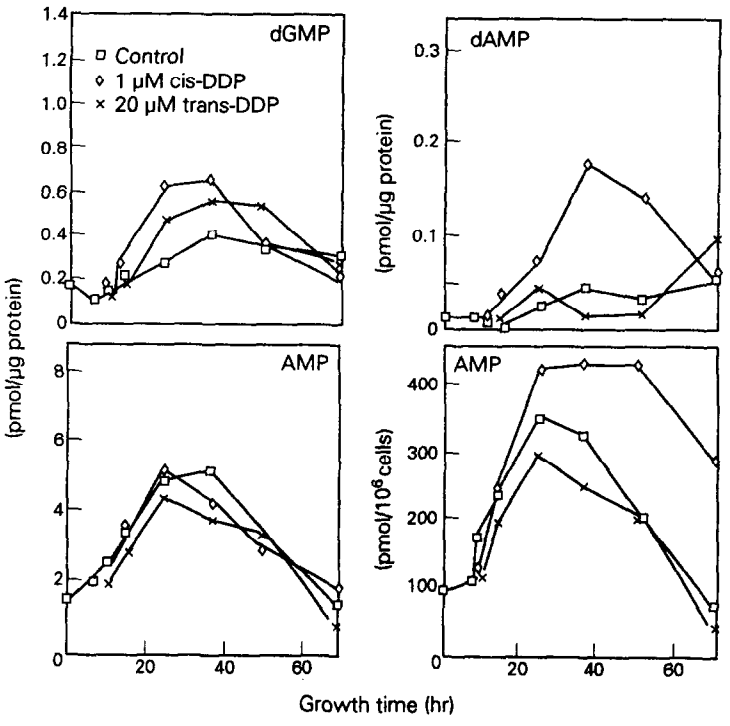

Fig. 2. Levels of AMP, dAMP and dGMP, respectively, in mouse leukemia P388/D_1 cells during treatment with platinum(II) complexes. As an example, levels of AMP are related to either protein content or cell number.

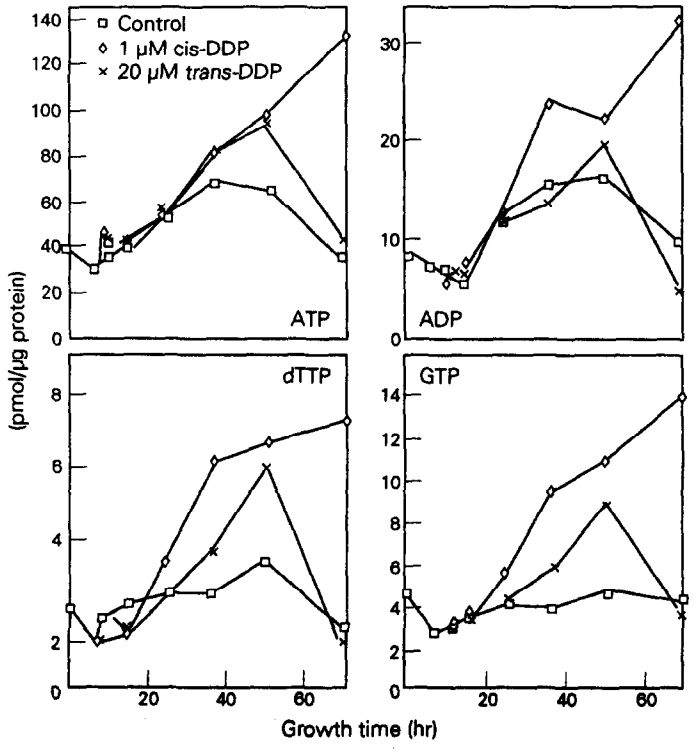

Fig. 3. Levels of ATP, ADP, dTTP and GTP, respectively, in mouse leukemia P388/D_1 cells during treatment with platinum(II) complexes.

Table 1. Maximum concentrations of nucleotides in extracts of platinum(II)-treated mouse leukemia P388/D_1 cells

	Maximum concentration* of nucleotides during treatment with				
	cis-DDP†		*trans*-DDP‡		Control§
Nucleotide	t_{max}‖ (hr)	(mM)	t_{max}¶ (hr)	(mM)	(mM)
Group 1					
ATP	60††	28		3	4
		16††	40	17	13
ADP	60	7		0.5	1
		4.8	40	3.8	3.6
CTP	60	1.5		0.3	0.1
		1.1	40	1.1	0.2
GTP	60	3.4		0.5	0.6
		2.5	40	2.1	0.8
GDP	60	1.2		0.3	0.15
		1.2	40	1.6	0.35
dTTP	60	0.15		0.01	0.01
		0.15	40	0.15	0.08
Group 2					
AMP	20	0.6		0.45	0.55
		0.6	20	0.45	0.55
CMP	20	0.14		0.14	0.14
		0.14	20	0.14	0.14
UMP	30	0.26		0.3	0.18
		0.22	40	0.32	0.16
dAMP	30	0.026		0.002	0.004
		0.006	60	0.008	0.005
dGMP	30	0.12		0.1	0.08
		0.12	40	0.1	0.08
dTMP	40	0.01		0.02	0.005
		0.005	50	0.025	0.005
Group 3					
Ap_4A	30	0.01		0.003	0.001
		0.01	40	0.004	0.001
cAMP	6	0.009		0.003	0.002
		0.009	6	0.003	0.002
CDP	6	1.1		0.2	0.3
		1.1	6	0.2	0.3
Group 4**					
NAD^+	40	0.8		1.3	1.2
		0.8	20	0.8	1.2
Others					
NADH	40	0.02		0.02	0.03
		0.02	50	0.025	0.03

* Calculated on the basis of an average volume per cell determined by flow cytometry [13].
† One μM *cis*-DDP.
‡ Twenty μM *trans*-DDP.
§ In the absence of platinum(II) complexes.
‖ Time at maximum concentration of nucleotide during treatment with *cis*-DDP.
¶ Time at maximum concentration of nucleotide during treatment with *trans*-DDP.
** Concentration minimum relative to control.
†† For each nucleotide, two sets of data are presented, the upper line refers to the time t_{max} of maximum concentration of the nucleotide during treatment with *cis*-DDP and the lower line to the time t_{max} of maximum concentration of the nucleotide during treatment with *trans*-DDP.

ATP/*cis*-DDP, (35 ± 15) $M^{-1}min^{-1}$ for GTP/*cis*-DDP, (33 ± 15) $M^{-1}min^{-1}$ for ATP/*trans*-DDP and (80 ± 40) $M^{-1}min^{-1}$ for GTP/*trans*-DDP. Rate constants for the slow reactions were concentration-independent and had the averaged value $(3 \pm 1) \times 10^{-3}$ min^{-1} for both platinum(II) complexes. The relative amplitudes of the fast reactions were $(35 \pm 15)\%$ for *cis*-DDP and $(12 \pm 10\%)$ for *trans*-DDP. The values did not depend on the kind and concentration of nucleotides.

The fast reaction reflected the attack of the nucleotides by the chloroaqua form of the platinum(II) complexes. The slow reaction was attributed to the dissociation reaction of the first chloro ligand from diamminedichloroplatinum(II) to yield the chloroaqua form. Our stock solutions containing 2 mM *cis*-DDP or 2 mM *trans*-DDP and 3 mM chloride ions consisted of a mixture of dichloro/

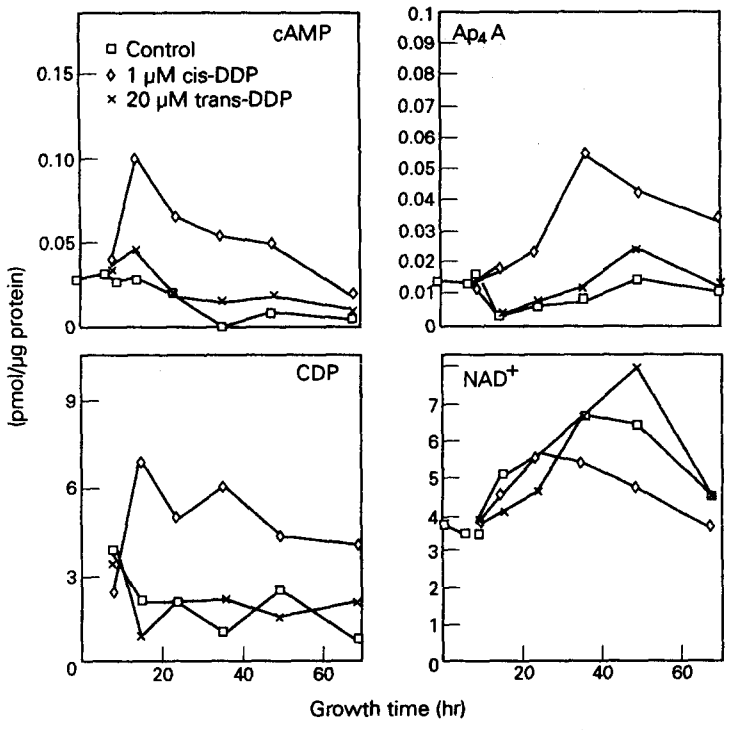

Fig. 4. Levels of cAMP, Ap_4A, CDP, and NAD^+ in mouse leukemia P388/D_1 cells during treatment with platinum(II) complexes.

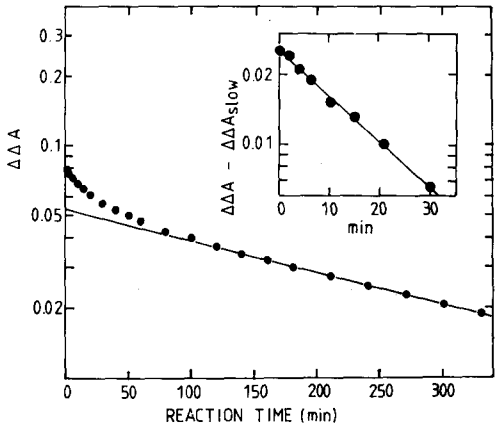

Fig. 5. Reaction of *cis*-diamminechloroaquaplatinum(II) with GTP, measured by the increase in absorbance at 290 nm wavelength and 37°. The concentrations were 0.5 mM GTP, 0.05 mM *cis*-diamminechloroaquaplatinum(II), 3 mM KCl and 10 mM Mops buffer (pH 7.0). The main figure is a semilogarithmic plot of the absorbance change as a function of reaction time. Data are treated as a superposition of two exponential time-dependences. The inset shows the semilogarithmic plot of the fast exponential obtained from the superposition by subtraction of the slow exponential (line in the main figure). Symbols refer to the change in absorbance $\Delta\Delta A = \Delta A_\infty - \Delta A_t$, where ΔA_∞ is the absorbance change at infinite time (obtained by extrapolation) and ΔA_t is the absorbance change at any time. $\Delta\Delta A_{slow}$ denotes the absorbance change indicated by a line in the main figure.

chloroaqua forms of the platinum(II) complexes. This can be shown by mass law calculations, employing the equilibrium constants of 3.3 mM (*cis*-DDP) and 0.32 mM (*trans*-DDP), for the dissociation of the first chloro ligand from diamminedichloroplatinum(II) [32, 33]. The calculated fractions of monochloro forms were 43% for *cis*-DDP and 9% for *trans*-DDP, corresponding with the above reaction amplitudes. The rate constant for the dissociation of the first chloride ion is 0.003 min^{-1} for *cis*-DDP and 0.005 min^{-1} for *trans*-DDP [32, 33]. These values compare excellently with the value for the slow reaction phase (above).

DISCUSSION

The nucleotide pool may be regarded, with some restrictions, as an indicator of the metabolic and regulatory state of the cell. We have recently investigated the effects of selected concentrations of 1 μM *cis*-DDP and 20 μM *trans*-DDP on the growth of mouse leukemia P388/D_1 cells [13]. These concentrations were chosen because they both inhibited the incorporation of [^{3}H]thymidine by 90%. In the present study, we measured the effects of the platinum(II) complexes on the size of nucleotide levels at the same drug concentrations. Our findings are discussed within the context of the previous findings.

Treatment with *cis*-DDP markedly and irreversibly increases the concentrations of most nucleotides in contrast to *trans*-DDP, which induces small and

reversible effects. This correlates with the marginal and transient effects of *trans*-DDP on growth, cell size and cell cycle distribution in spite of its 20-fold higher concentration in the culture medium [13]. The effects of *cis*-DDP are discussed in more detail below.

The levels of most nucleotides never decreased as a consequence of the drug treatment, indicating that pool depletion by adduct formation with *cis*-DDP was not substantial. The concentration increase observed for nucleoside 5′-diphosphates and 5′-triphosphates (Fig. 3) follows a time-dependence, which is similar to the one for the cellular DNA and protein content [13]. These dependences parallel the increase in cell size and the number of cells arrested in the G2 phase of the cell cycle. The rise in ATP concentration, the increasing number of cells not undergoing mitosis and yet the relatively unperturbed rate of ongoing, balanced DNA and protein synthesis [13], together with a relatively constant cell energy charge may suggest that drug treatment inhibits the energy-dependent synthesis and function of the spindle apparatus and/or other energy requiring processes of mitosis and cell separation. This explanation is in agreement with the recent observation that polyploid nuclei are formed in *Physarum polycephalum* during treatment with low doses of platinum(II) complexes [34].

Of particular interest are the responses by cyclic AMP and Ap_4A (Fig. 4), suggesting an effect of *cis*-DDP on the levels of regulatory nucleotides. The rise in the concentration of cyclic AMP is an early effect (within 6 hr after the addition of the drug) and precedes the arrest of cells in the G2 phase of the cell cycle (6–12 hr) [13]. Although a similar rise in cyclic AMP level has been recently reported by Tisdale [35] for other cell lines, a relation to growth parameters has not been established. The chemotherapeutically inefficient drug *trans*-DDP induces a marginal concentration increase only (Fig. 4). The net synthesis of cyclic AMP by isolated membrane fragments from $P388/D_1$ cells is stimulated by treatment with *cis*-DDP and is due to both an activation of adenylate cyclase and an inhibition of a low K_m-form phosphodiesterase [36, 37]. Exposure of human cancer cells to site-selective cyclic AMP analogues effects growth inhibition, probably by suppression of the action of TGFα via the protein kinase A regulatory subunits [38]. Our results confirm the previous conclusion, suggesting that an increase of cyclic AMP levels may be an integral part of the mechanism of antitumor drugs [21, 39].

The rapid rise in Ap_4A concentration following the addition of the drug (Fig. 4) is in agreement with the suggested function of Ap_4A as an alarmone [23]. An increase in the Ap_4A level also correlates with DNA repair [40, 41]. We believe that Ap_4A is a new and important candidate for involvement in the mechanism of *cis*-DDP induced cell growth inhibition and/or cytotoxicity.

Levels of deoxyribonucleoside 5′-monophosphates, in contrast to those of ribonucleoside 5′-monophosphates, increase transiently during treatment with either *cis*-DDP or *trans*-DDP (Fig. 2, Table 1). Because the increase is also observed for dTMP, which is not a target of platinum(II) complexes [24], these results are in agreement with an enhancement of DNA repair that removes DNA segments containing the platinum(II) lesions [42]. In contrast with the transient behaviour of the monophosphates, the level of NAD^+ responds at a later time during the *cis*-DDP treatment (Fig. 4). NAD^+ is the substrate of nuclear ADP-ribosyltransferase active during some form of eucaryotic DNA repair. This enzymatic reaction is also involved in other processes such as cellular differentiation, transformation and gene rearrangements [22 and references therein]. The difference in timing of the observed effects on NAD^+ and deoxyribonucleoside 5′-monophosphate levels may reflect different phases of repair.

The observation of an increase in the levels of the purine nucleoside 5′-triphosphates, particularly, has not been reported before. The increase is at variance with a reduction in activities of nucleoside kinases and dihydrofolate reductase reported by Hall *et al.* [20]. It remains to be seen, whether the discrepancy refers to the particular ligands of *cis*-DDP and *cis*-malonatodiammineplatinum(II) used in the experiments. Our result is in agreement with a recent report of an enhancement of these enzyme activities for 3.2-fold *cis*-DDP resistant A2780 human carcinoma cells [43].

The effects on the nucleotide concentrations and their time-dependences suggest that *cis*-DDP may function as a "pleiotypic" effector. Several of the nucleotides seem to have defined roles in antitumorigenesis. Some of the roles, like that of CDP, are not yet understood. The function of DNA as the sole target seems unlikely in face of the rapid responses of the cyclic AMP and Ap_4A. The cell membrane should be considered as another important target [39]. Cells with elevated cyclic AMP content show increased sensitivity to *cis*-DDP [19 and references therein] suggesting that pleiotypic effects could, at least, sensitize cells for treatment with platinum(II) complexes.

Platinum(II) scavenging by nucleoside 5′-triphosphates is possibly due to their high intracellular concentrations. This has not hitherto been recognized. Platinum(II) complexes on their way to nuclear DNA convert to their monochloromonoaqua forms [44]. In this form they react easily with adenine and guanine nucleoside 5′-triphosphates. Nucleoside 5′-triphosphates are particularly reactive because of their high negative charge. The concentration of ATP increases from 4 mM in control cells to 28 mM during treatment with *cis*-DDP (Table 1), corresponding to a decrease in the half-life from 7.8 to 1.1 min for *cis*-DDP, and similarly for *trans*-DDP. In comparison, sulfur-containing molecules like glutathione, cysteine and methionine capture *cis*-DDP with half-lives of 3 hr (5 mM glutathione) to 4 days (50 μM cysteine or methionine) [45]. The nucleoside 5′-triphosphate-dependent increase in scavenger capacity during drug treatment is likely to be a source of acquired *cis*-DDP resistance.

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