Inorganica Chimica Acta, 159 (1989)

The Effect of *cis*-Platinum on Nucleotide Metabolism*

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(Received August 26, 1988)

Abstract

Deoxyguanosine 5'-triphosphate (dGMP) and hypoxanthine, a catabolic intermediate, have been reacted with *cis*- and *trans*-diamminedichloroplatinum(II) under defined conditions and their reaction products characterized. Their potential as substrate in the catabolism after platination was diminished or abolished with regard to alkaline phosphatase, nucleoside phosphotransferase or xanthine oxidase as degrading enzymes. The effect on the phosphatase reaction was to severely lower the maximum catalytic rate (V_{max}) while not affecting the Michaelis–Menten constant (K_M). Xanthine oxidase could no longer bind platinated hypoxanthine and was thus unable to oxidize it to uric acid. On that basis platinated hypoxanthine is likely to accumulate during chemotherapeutic treatment of cancer with *cis*-diamminedichloroplatinum(II).

Introduction

cis-Diamminedichloroplatinum(II) (*cis*-platinum) and related platinum(II) compounds are potent chemotherapeutica in the treatment of several forms of cancer [1]. A mechanistically important target is DNA; but a pleiotypic action of platinum(II) seems likely on chemical and biological grounds. A general feature of platinum(II) action is the impairment of normal metabolic substrate function of biomolecules and in particularly of guanine moieties that became fixed to platinum(II) in their N7-position [2, 3]. For instance, synthesis and repair of DNA are inhibited at positions of platinated purines [4–6]. Where such lesions are bypassed, fidelity of DNA copying is lost, and mutations are manifested [7]. Small molecules like adenosine(5')tetraphospho(5')adenosine (Ap_4A), a supposed pleiotypic cell growth effector [8], can chelate with platinum-(II) and subsequently become resistant against hydrolytic degradation *in vitro* [9]. If nucleotides and in particularly second messengers of that kind (cAMP, cGMP) and other small biomolecules or their catabolites become resistant after platination against degradation they are expected to accumulate. Enhanced intracellular levels of such molecules, however, may contribute to the antitumoral action of *cis*-platinum. We report here the reaction of dGMP and of hypoxanthine with *cis*-/*trans*-diamminedichloroplatinum(II) and their partial and respectively total resistance against catabolic degradation after platination.

Experimental

Alkaline phosphatase (EC 3.1.3.1) (1500 unit/mg) and xanthine oxidase (EC 1.1.3.22) (0.1 unit/mg) were purchased from Boehringer/Mannheim and from Sigma, respectively. Nucleoside phosphotransferase from *Lupinus luteus* (EC 2.7.1.77) (0.02 unit/mg) was a gift of Dr Guranowski, Poznan, Poland.

cis-Platinum and *trans*-diamminedichloroplatinum-(II) (*trans*-platinum) were gifts of Degussa, Frankfurt. Stock solutions (1–2 mM) were prepared in aqueous 10 mM KNO_3 by incubation for 24 h at 25 °C allowing the dichloroplatinum(II) complexes to form the chloroaquaplatinum(II) complexes.

Hypoxanthine, dGMP, guanosine and other nucleotides/nucleosides were purchased from Sigma and all other chemicals from Merck, Darmstadt.

The assay mixture for alkaline phosphatase (100 μl) contained 50 mM Tris–HCl (pH 7.6), 2 mM (5–100 μM for the measurement of kinetic parameters) dGMP or dGMP–platinum(II) adduct and 10^{-4}–10^{-1} unit/ml enzyme. The reaction was stopped after 3 min (initial rates) or 15 min (reaction completion) at 37 °C by the addition of 0.9 ml phosphate assay mixture. This contained 100 μl of the molybdato/sulfuric acid stock solution (2.5 g/100 ml $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 3 M H_2SO_4), 10 μl of a 1% (*v*/*v*) Triton X100 solution and water. After 30 min at room temperature the absorbance

*Abbreviations used in this paper: *cis*-platinum = *cis*-diamminedichloroplatinum(II), *trans*-platinum = *trans*-diamminedichloroplatinum(II).

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0020-1693/89/$3.50

at 660 nm wavelength was measured [10]. The amount of phosphate was determined with reference to 2–20 μM phosphate solutions as standards. The assay for nucleoside phosphotransferase was the same as for alkaline phosphatase.

The assay mixture (1 ml) for xanthine oxidase contained 20 μM hypoxanthine or hypoxanthine platinum(II) adduct, 2 mM potassium phosphate (pH 7.8), 200 mM NaCl, 0.1 mM EDTA and 0.01–0.2 unit enzyme. The production of uric acid was followed by difference spectroscopy (Zeiss DMR10, thermostated 1 cm quartz cuvettes) at 293 nm wavelength and 37 °C [11]. Initial velocities were obtained from the slopes of the progress curves.

Kinetics of platination were followed at 37 °C by difference spectroscopy (300 nm wavelength for dGMP and 276 or 248 nm for hypoxanthine) of a mixture containing 10 mM KNO_3 (pH 5.5) without enzymes. Stoichiometry measurements were conducted by the same method and using the tangent method of ref. 12. Preparative amounts of adducts were synthesized from mixtures of stoichiometric quantities of platinum complex and ligand by 24 h incubation at room temperature in the dark (10 mM KNO_3, pH 5.5). Large amounts of adducts of hypoxanthine were separated from the starting material by fractionation on Dowex 50 WX 8, 200–400 mesh (H-form) in the presence of a gradient 0–1 M sodium sulfate.

Reaction products of dGMP were analysed by HPLC on LKB-Ultropak TSK ODS 120 T 5 μm (reversed phase) by gradient elution of 0–30% (by vol.) methanol/water in solution of 0.1 M ammonium phosphate (pH 5.8).

Results and Discussion

Alkaline Phosphatase and Nucleoside Phosphotransferase

The reaction of dGMP with *cis*-platinum or *trans*-platinum lead to progressive inhibition of alkaline phosphatase with time (Fig. 1). Several effects were observed: (i) initial rates of the phosphatase reaction decreased, (ii) enzymatic kinetics of the phosphatase reaction changed from monophasic in the absence to biphasic in the presence of *cis*-platinum, (iii) the level of inorganic phosphate that was liberated during the first phase (approximately 10 min) followed platination kinetics with a second order rate constant of 10 M^{-1} min^{-1}. This value is in accord with 6 M^{-1} min^{-1} for the reaction of dGMP and *cis*-platinum monitored by the change in absorbance at 300 nm wavelength (not shown). An incubation of *cis*-platinum with the enzyme during catalysis did not cause measurable inhibition and supported the conclusion that the inhibition in Fig. 1 was due to coordination of dGMP with the

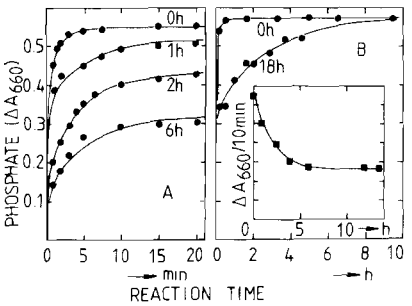

Fig. 1. Enzymatic dephosphorylation of dGMP in the presence of alkaline phosphatase. Inhibition after reaction of dGMP with *cis*-platinum. Panel A, dephosphorylation kinetics for a mixture of dGMP and *cis*-platinum after various times of platination. Panel B, the same reaction as for Panel A at an extended time scale. Figure inset, the release of phosphate as a function of platination time. The concentrations of the platination mixture were 1 mM dGMP, 1 mM *cis*-platinum. Samples in the inset were incubated with alkaline phosphatase for 10 min. Concentrations of 20 μM dGMP or adduct were employed during the enzymatic assay. The concentration of released phosphate is given in absorbance units at 660 nm wavelength of the assay [10].

metal complex and not due to inactivation of the enzyme. Comparable results were obtained for nucleoside phosphotransferase.

Michaelis–Menten Parameters

The effect of the coordination of dGMP with *cis*-platinum on the substrate properties were established in terms of K_M and V_{max} according to [13] from initial rate measurements (not shown). Values were $K_M = 120 \pm 15$ μM for both free and platinated dGMP (either *cis*-platinum or *trans*-platinum), whereas V_{max} was lower by a factor 4.2 for the *cis*-platinum adduct than for free dGMP with alkaline phosphatase. Similarly the K_M was 400 ± 50 μM for free and platinated dGMP and V_{max} lower by a factor of 77 for the adduct than for free dGMP with nucleoside phosphotransferase. The effects of *trans*-platinum were similar to those of the *cis* isomer with a 4-fold decrease in V_{max} for alkaline phosphatase and a 37-fold decrease for nucleoside phosphotransferase.

Stoichiometry of cis-dGMP–Platinum

The reaction of *cis*-platinum with dGMP gave a coordination product (80–90% pure) that consisted of 2 molecules dGMP per molecule of metal ion as seen by HPLC. This was in agreement with the stoichiometric value measured spectrophotometrically at 300 nm wavelength (0.5 mM dGMP and 0–1 mM *cis*-platinum, 22 h at 25 °C). The same stoichiometry was reflected by the initial rates of inorganic phosphate release in the presence of alkaline phosphatase and nucleoside phosphotransferase,

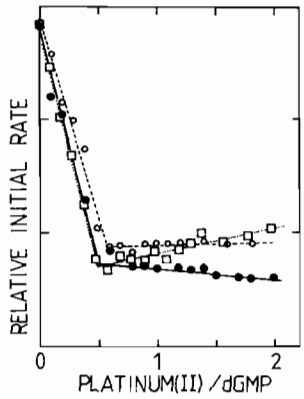

Fig. 2. The stoichiometry of the dGMP/*cis*-platinum complex expressed through the inhibition of phosphate release catalysed by alkaline phosphatase (circles) or nucleoside phosphotransferase (squares). Initial rates were measured. Symbols refer to *cis*-platinum (–○–, –■–) or to *trans*-platinum (–●–).

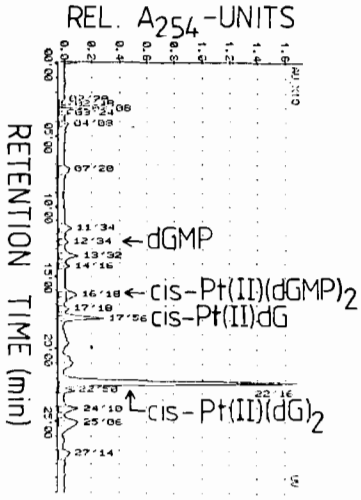

Fig. 3. Analysis of the *cis*-platinum adduct of dGMP. The enzymatic reaction mixture (1 ml) contained 50 μM adduct, 0.06 unit enzyme and 50 mM Tris–HCl (pH 7.6). After 2 h incubation at 37 °C the mixture was loaded directly onto the HPLC column. Elution peaks were assigned according to the results of Eastman [14].

respectively (Fig. 2). We conclude that the bisadduct *cis*-[Pt-$(NH_3)_2$(dGMP)$_2$] and the monoadduct have impaired substrate properties for the enzymatic removal of phosphate. The same result holds for *trans*-platinum.

Nonequivalence of Phosphates

The biphasic kinetics in Fig. 1 indicated that approximately half of the phosphate in the bisadduct *cis*-[Pt$(NH_3)_2$(dGMP)$_2$] was released during the first 10 min and the rest during a much longer time period. We interpreted this as evidence that the two phosphates in the adduct were probably released in sequence, and that the observed effect on V_{max} was concerned with the first phosphate. The release of phosphates was finally complete as demonstrated by an analysis of the products by HPLC (Fig. 3).

Xanthine Oxidase

The reaction of hypoxanthine, a substrate of xanthine oxidase, with *cis*-platinum was followed by UV difference spectroscopy (absorbance minimum at 248 nm, maximum at 270 nm, isosbestic points at 240 and 259 nm wavelength). The reaction kinetics were bimolecular and followed a rate constant of 8 ± 3 M^{-1} min^{-1} (pH 5.5, 10 mM KNO_3, 37 °C). The photometrically measured stoichiometry of the platination reaction was 1.0 ± 0.1 mol *cis*-platinum per mole hypoxanthine. The same kinetic and stoichiometry values were found when the progress of the platination was followed in the enzymatic assay for the oxidation to uric acid in the presence of added xanthine oxidase (Fig. 4). Progress of platination was indicated as a complete loss in substrate activity during formation of the monoadduct (Fig. 4, inset). The enzyme itself was not inhibited as indicated in control experiments with fresh hypoxanthine in the same mixture that contained the adduct.

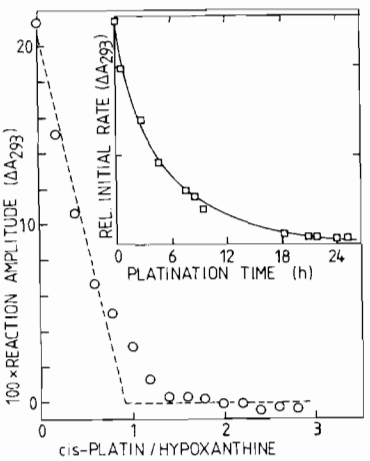

Fig. 4. The loss of substrate activity of hypoxanthine after adduct formation with *cis*-platinum. The oxidation of hypoxanthine was catalysed by xanthine oxidase. The absorbance difference refers to the total amount of uric acid that was formed during the enzymatic reaction (1–48 h) and the abscissa to the molar fraction of reactants in the platination mixture. Platination time was 24 h. Inset, the initial rate of uric acid synthesis from hypoxanthine (an equivalent of 10 μM in the enzymatic assay) as a function of the incubation time in the platination mixture (1 mM hypoxanthine, 1 mM *cis*-platinum, 10 mM KNO_3, pH 5.5, 37 °C).

Loss of Binding Affinity

cis-Platinum was separated from unreacted hypoxanthine by ion exchange chromatography on Dowex 50. Pure adduct was inactive as substrate of xanthine oxidase over the concentration range 5–50 μM tested in the standard assay. In the competition assay at concentrations as high as 100 μM adduct (10–20 μM hypoxanthine in the standard assay, close to K_M) the adduct was not inhibitory. We conclude that after the reaction with *cis*-platinum, hypoxanthine has lost its substrate binding properties.

Conclusions

The catabolism of platinated dGMP in the complex *cis*-$[Pt(NH_3)_2(dGMP)_2]$ is inhibited at the level of enzymatic removal of the phosphates and blocked for oxidation to uric acid at a later point. On that basis a temporary accumulation of platinum(II) adducts of dGMP and a long lasting accumulation of platinated hypoxanthine is expected during cancer chemotherapy with *cis*-platinum and related metal complexes. Whether or not platinum(II)–purine adducts are permissive for other catabolic reactions remains to be investigated. Accumulated platinated catabolites could be obstructive for cell survival and thus could contribute to the cytotoxicity of platinum(II) compounds.

Acknowledgement

The financial support by the Deutsche Forschungsgemeinschaft grant SFB 234 C7 is gratefully acknowledged.

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