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Authors
Seki, S
Hongo, A
Zhang, B
et al.

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Inhibition of Cisplatin-mediated DNA Damage *in vitro* by Ribonucleotides

Shuji Seki,[1,3] Atsushi Hongo,[2] Bo Zhang,[1,4] Kosuke Akiyama,[1] Altaf H. Sarker[1] and Takafumi Kudo[2]

[1]*Department of Molecular Biology, Institute of Cellular and Molecular Biology,* [2]*Department of Obstetrics and Gynecology, Okayama University Medical School, 2-5-1, Shikata-cho, Okayama 700*

Effects of ribonucleotides on *cis*-diamminedichloroplatinum(II) (cisplatin)-mediated DNA damage were studied by incubating pUC18 DNA with cisplatin in the presence of nucleotide, and by monitoring conformational change and sensitivity change to restriction enzyme *Hpa*II of the DNA due to the platinum-DNA adduct formation. The cisplatin-mediated DNA damage was inhibited in a dose-dependent fashion by ATP or GTP, substantially at their physiological intracellular concentrations, and almost completely by 5 m*M* ATP or 2 m*M* GTP. The inhibitory effect of nucleotide on the platination of DNA was in the order of GTP > ATP≫CTP > UTP, and of nucleoside triphosphate > nucleoside diphosphate > nucleoside monophosphate. Nucleoside did not show any significant effect on platination of DNA. To elucidate the mechanism of the nucleotide effects on platination of DNA, interaction between ATP or GTP and cisplatin was analyzed by high-performance liquid chromatography. The results suggested that ATP inhibits cisplatin-mediated DNA damage both by forming a platinum-ATP adduct and by non-covalent ionic interaction with cisplatin, while GTP acts largely by forming platinum-GTP adducts.

Key words: Cisplatin — Ribonucleotide — DNA damage — Platination of nucleotide

cis-Diamminedichloroplatinum(II) (cisplatin) is an important cancer chemotherapeutic agent used for treatment of several malignancies.[1,2] The antineoplastic activity is thought to be based on its interaction with cellular DNA resulting in the formation of various types of adducts.[2-4] The major lesions induced by treatment of isolated DNA with cisplatin have been shown to be intrastrand cross-links between bases of guanine and guanine (GG) (60–65% of total platinum adducts) and adenine and guanine (AG) (20–25%).[2-7] Rarer lesions are 1,3 intrastrand cross-links between G residues separated by one base residue in GNG sequences (5–6%), DNA interstrand crosslinks, DNA-protein crosslinks and platinum-DNA monoadducts.[2-7]

Although cisplatin is a potent antineoplastic drug, the development of drug resistance in tumors and its nephrotoxicity restrict its therapeutic applications.[2,8] Many different cellular mechanisms appear to contribute to the resistance, including increased adduct repair and increased levels of glutathione and metallothionein, which can potentially inactivate the drug.[8-12]

In the present paper, the possibility that ribonucleotide concentrations in the vicinity of DNA-cisplatin interaction sites can influence the degree of platinum-DNA adduct formation is studied.

[3] To whom correspondence should be addressed.

[4] Present address: Department of Pathology, Beijing Medical University, 100083 Beijing, China.

MATERIALS AND METHODS

Cisplatin was kindly provided by Nippon Kayaku Co., Ltd., Tokyo. Other reagents were purchased from the following sources: ribonucleoside triphosphates (NTPs), adenosine, guanosine, adenosine-5′-monophosphate (AMP), guanosine-5′-monophosphate (GMP), adenosine-5′-diphosphate (ADP) and guanosine-5′-diphosphate (GDP) from Seikagaku Kogyo Co., Ltd., Tokyo, agarose from Nippon Gene Co., Ltd., Toyama, ethidium bromide from Nacalai Tesque Inc., Kyoto, and *Hpa*II restriction endonuclease from Toyobo Co., Ltd., Osaka. Stock solutions of nucleotides were adjusted to pH 7.0 with 1 *N* NaOH.

Preparation of pUC18 DNA Growth of HB101 strain cells of *Escherichia coli* transformed with pUC18 plasmid, amplification of the plasmid, harvesting and alkaline lysis of the bacteria, and purification of the plasmid DNA were conducted by the reported large-scale isolation procedure.[13] The prepared DNA was dissolved in TE buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0) and stored at −80°C until use.

Assay for nucleotide effect on cisplatin-mediated DNA damage Cisplatin-mediated DNA damage was measured essentially as described previously.[14] Briefly, the reaction mixture (20 μl) containing 1 μg (0.56 pmol) of pUC18 DNA (concentration: 28 n*M* DNA; 150 μM DNA nucleotide), an appropriate concentration of nucleotide, 15 μM cisplatin and TE buffer was incubated at 37°C for 16 h in the dark. Finally, the DNA was analyzed by agarose

gel electrophoresis.[14-16] When the amount of cisplatin added to the reaction mixture was varied, it was expressed as the molar ratio of cisplatin molecules to the nucleotides (or phosphate moieties) of pUC18 DNA in the mixture.

Digestion with restriction endonuclease The cisplatin-treated DNA was digested with *Hpa*II in a reaction mixture (20 μl) consisting of 0.5 μg of cisplatin-treated DNA, 1 unit of *Hpa*II and 2 μl of 10-fold-concentrated, appropriate ionic strength buffer for the enzyme.[13, 14] The digestion was conducted at 37°C for 2 h.

Agarose gel electrophoresis Agarose gels were prepared at 1% or 2% in TBE buffer (0.089 *M* Tris-borate, 0.089 *M* boric acid and 0.002 *M* EDTA, pH 8.0) supplemented with or without ethidium bromide (EtdBr) at 0.5 μg/ml.[13] The electrophoresis buffer was TBE buffer with or without 0.5 μg/ml EtdBr depending on the gel used. A portion (0.25 μg DNA) of each DNA sample was loaded into a slot of a submerged agarose gel, and electrophoresed at 50 V for 100 min in a mini gel electrophoresis system.[14] After electrophoresis, the EtdBr-free gel was stained for 60 min with 0.5 μg/ml of EtdBr in TBE buffer.[15, 16]

Reaction of cisplatin with nucleotide and HPLC analysis of platinated nucleotide Nucleotide (ATP or GTP) was incubated at 37°C for 16 h with varying concentrations of cisplatin in 10-fold-diluted TE buffer. After the incubation, the mixture was chilled to 0°C, and adjusted to pH 5.5 by adding a large volume of 100 m*M* $(NH_4)_2HPO_4$ buffer (pH 5.5). High-performance liquid chromatography (HPLC) was conducted with a Waters 600E system equipped with a 486 tunable absorbance detector and a 741 data module. Separation of nucleotides and adducts was performed at room temperature on a Waters RPC_{18} column by eluting with a 0–15% (v/v) methanol gradient in 100 m*M* $(NH_4)_2HPO_4$ buffer (pH 5.5).[17] Ultraviolet absorbance was recorded at 254 nm wavelength. HPLC-eluted materials were identified on the basis of retention times by comparison with known materials.[17]

RESULTS

Conformational analysis of cisplatin-damaged DNA When DNA is exposed at 37°C for more than several hours to cisplatin, intrastrand cross links between adjacent bases in GG and AG sequences and, as a rarer lesion, between G residues separated by one base residue in GNG sequences are known to occur.[2-7] Such cross links produced on closed circular, superhelical DNA (the S band in figures) cause unwinding of the negative superhelix, which can be detected in agarose gel electrophoresis by the reduced mobility (Fig. 1).[2, 14, 18, 19] In contrast, the mobility of nicked, open circular DNA increased cisplatin-dose-dependently, probably because the nicked, open circular DNA (the O band in figures) formed a more compact structure after platination (Fig. 1).[14, 18, 19] Under the present experimental conditions (incubation at 37°C for 16 h at one-tenth molar ratio of cisplatin per DNA nucleotide), the mobility of the cisplatin-damaged superhelical pUC18 DNA was reduced significantly and became almost equal to that of cisplatin-damaged open circular DNA (Fig. 1, lane 11).

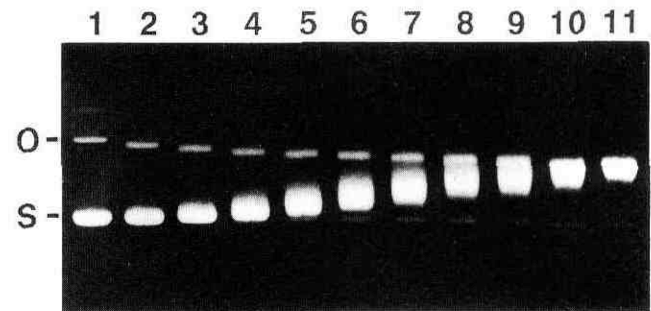

Fig. 1. Effects of cisplatin on conformation of pUC18 plasmid DNA. The reaction mixture (20 μl final volume) for cisplatin treatment contained 1 μg of pUC18 DNA (150 μM DNA nucleotide), varying amounts of cisplatin and TE buffer. The mixture was incubated at 37°C for 16 h in the dark. A part (5 μl; 0.25 μg DNA) of the incubated sample was electrophoresed at 50 V for 100 min on 1% agarose gel. The gel was stained for 60 min with ethidium bromide at 0.5 μg/ml. Lanes (1–11) correspond to DNA samples incubated at molar ratios of cisplatin and pUC18 DNA nucleotide of 0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07, 0.08, 0.09 and 0.1. Abbreviations used: S, closed circular, superhelical DNA; O, nicked, open circular DNA. The same abbreviations are also used in Figs. 2, 4 and 5.

Analysis of cisplatin-damaged DNA by restriction endonuclease The platinated sites of DNA are known to be resistant to the restriction enzymes which recognize and cleave the initial, non-platinated DNA sequence.[2, 19] To detect and analyze the platination of DNA by restriction enzymes, *Hpa*II (recognition sequence, CCGG; 12 cutting sites in pUC18) was used in the present experiments because of the high incidence of intrastrand cross-links between G residues.[2-7, 14] pUC18 DNA treated with cisplatin under the present conditions was highly resistant to *Hpa*II, and cleaved only a single site (shown later in Figs. 3 and 5B, lane 2).[14]

Effects of ribonucleoside triphosphate on cisplatin-mediated DNA damage The cisplatin-mediated DNA damage detected in terms of conformational change of pUC18 DNA and the decrease of the *Hpa*II sensitivity was inhibited dose-dependently by ATP and GTP (Figs. 2A, 2B and 3). The assay in terms of conformational change showed that apparent complete inhibition of the

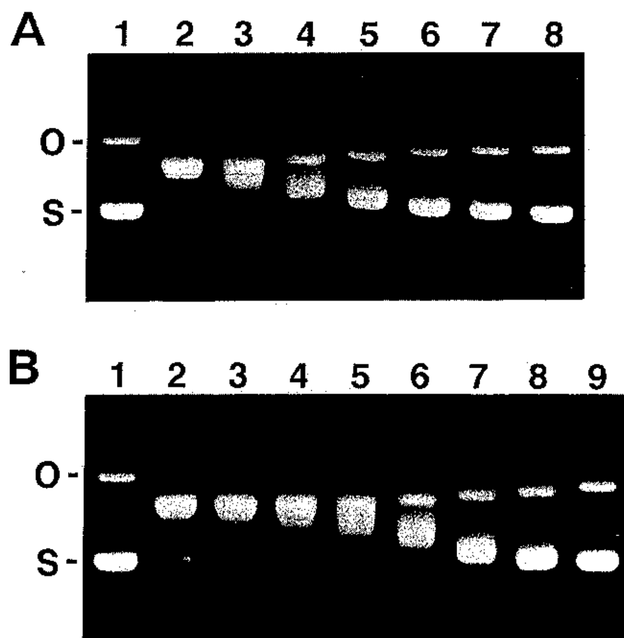

Fig. 2. Effects of ribonucleoside triphosphate on cisplatin-mediated DNA damage. The incubation mixture (20 μl final volume) consisted of 1 μg of pUC18 DNA, TE buffer, ATP or GTP at the indicated concentration and 15 μM cisplatin. The incubation, gel electrophoresis and staining were performed as described in the legend to Fig. 1. A. Effect of ATP. Lane 1, control pUC18 DNA; lane 2, cisplatin and no ATP; lane 3, 0.2 m*M* ATP; lane 4, 0.5 m*M*; lane 5, 1 m*M*; lane 6, 2 m*M*; lane 7, 3 m*M*; lane 8, 5 m*M*. B. Effect of GTP. Lane 1, control pUC18 DNA; lane 2, cisplatin and no GTP; lane 3, 0.01 m*M* GTP; lane 4, 0.05 m*M*; lane 5, 0.1 m*M*; lane 6, 0.2 m*M*; lane 7, 0.5 m*M*; lane 8, 1 m*M*; lane 9, 2 m*M*.

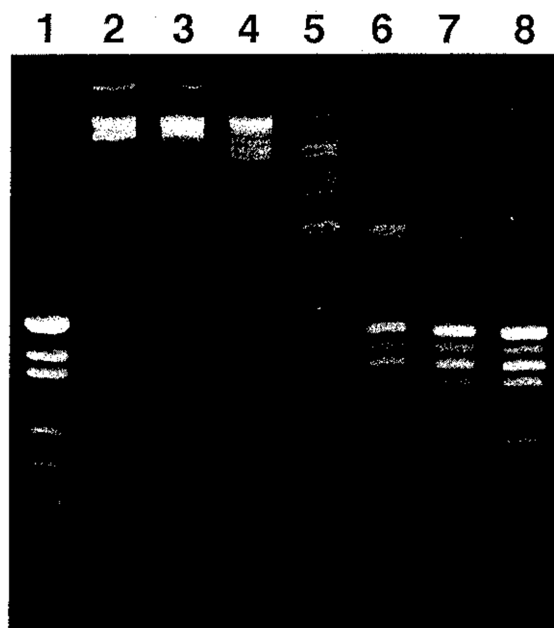

Fig. 3. Effects of ATP on cisplatin-mediated DNA damage analyzed in terms of sensitivity to *Hpa*II. The incubation mixture and conditions for the cisplatin treatment of pUC18 DNA were the same as described in the legend to Fig. 2. After the cisplatin treatment, the DNA was digested at 37°C for 2 h with 1 unit of *Hpa*II.[13, 14] A part (0.25 μg) of the sample was electrophoresed on a 2% agarose gel in the presence of EtdBr at 0.5 μg/ml as described in "Materials and Methods." Lane 1, control pUC18 DNA; lane 2, cisplatin and no ATP; lane 3, 0.2 m*M* ATP; lane 4, 0.5 m*M*; lane 5, 1 m*M*; lane 6, 2 m*M*; lane 7, 3 m*M*; lane 8, 5 m*M*.

cisplatin-mediated DNA damage occurred in the presence of 5 m*M* ATP or 2 m*M* GTP. ATP was required at about 2–3 times higher concentration than GTP to give the same degree of inhibition. The assay using *Hpa*II digestability was more sensitive than the conformational assay, as reported previously.[14] The *Hpa*II digestability assay confirmed the inhibitory effect of ATP or GTP (data not shown) on cisplatin-mediated DNA damage (Fig. 3).

The cisplatin-induced DNA damage was also inhibited by CTP and UTP. The inhibitory effects of nucleoside triphosphates on platination of DNA were in the order of GTP > ATP ≫ CTP > UTP (Fig. 4). A combined addition of ATP and GTP to the cisplatin-DNA reaction mixture showed an additive inhibitory effect on DNA damage by cisplatin (data not shown).

Effects of nucleoside diphosphate, nucleoside monophosphate and base on cisplatin-mediated DNA damage The cisplatin-mediated DNA damage was also inhibited

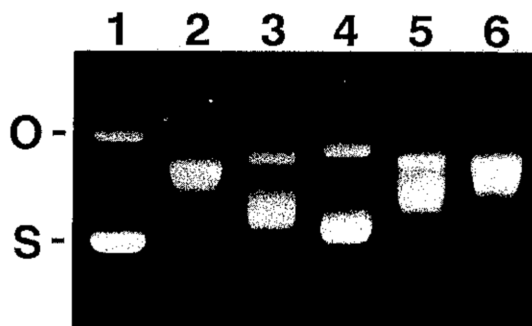

Fig. 4. Effects of various ribonucleoside triphosphates on cisplatin-mediated DNA damage. The incubation mixture and conditions for the cisplatin treatment of pUC18 were the same as described in the legend to Fig. 2. The conformational analysis of DNA was performed as described in "Materials and Methods." Lane 1, control pUC18 DNA; lane 2, cisplatin and no ribonucleotide; lane 3, 1 m*M* ATP; lane 4, 1 m*M* GTP; lane 5, 1 m*M* CTP; lane 6, 1 m*M* UTP.

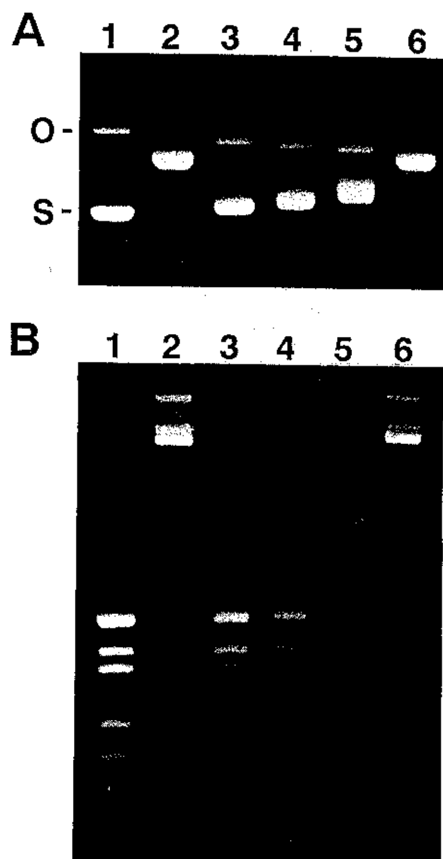

Fig. 5. Effects of ATP, ADP, AMP and adenosine on cisplatin-mediated DNA damage. The conformational analysis and *Hpa*II sensitivity test of pUC18 DNA incubated with cisplatin and adenosine nucleotide or adenosine were conducted as described in the legends to Figs. 2 and 3. A. Conformational analysis. Lane 1, control pUC18 DNA; lane 2, cisplatin and no nucleotide or base; lane 3, 3 m*M* ATP; lane 4, 3 m*M* ADP; lane 5, 3 m*M* AMP; lane 6, 3 m*M* adenosine. B. *Hpa*II sensitivity test. Lane 1, control pUC18 DNA; lane 2, cisplatin and no nucleotide or base; lane 3, 5 m*M* ATP; lane 4, 5 m*M* ADP; lane 5, 5 m*M* AMP; lane 6, 5 m*M* adenosine.

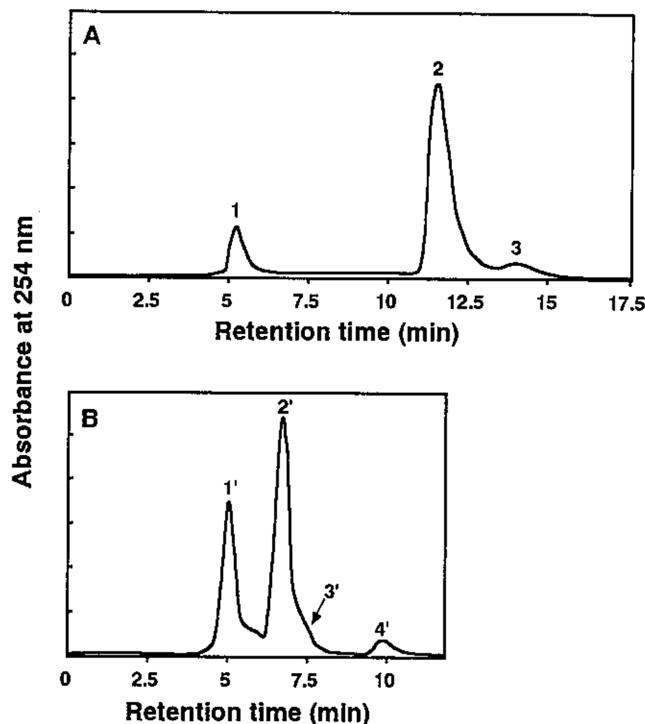

Fig. 6. HPLC elution profile of cisplatin-treated nucleotide. The reaction mixture (20 μl final volume), containing 15 μM cisplatin, 800 μM ATP (A) or GTP (B), was incubated at 37°C for 16 h in the dark. After the incubation, the mixture was chilled to 0°C, and diluted to an appropriate concentration with 100 m*M* $(NH_4)_2HPO_4$ buffer (pH 5.5). Separation of nucleotides and adducts was performed at room temperature on a Waters RPC_{18} column by eluting with a 0–15% (v/v) methanol gradient in 100 m*M* $(NH_4)_2HPO_4$ buffer (pH 5.5). Ultraviolet absorbance was recorded at 254 nm. A. 1, ATP-Pt adduct; 2, ATP; 3, ADP formed from ATP by dephosphorylation. B. 1′, GTP-Pt adduct; 2′, GTP; 3′, GDP formed from GTP; 4′, GTP-Pt-GTP adduct.

by nucleoside diphosphate (NDP) and nucleoside monophosphate (NMP). The inhibition was in the order of ATP>ADP>AMP, as shown in Fig. 5. Similar results were obtained with GTP, GDP and GMP. Adenosine showed no detectable effect on DNA damage by cisplatin (Fig. 5).

Platination of free nucleotide Monofunctionally platinated ATP or GTP was produced by incubating ATP or GTP at 37°C for 16 h with cisplatin (Fig. 6). The amount of monofunctionally platinated ATP or GTP formed was proportional to the cisplatin concentration added to the incubation mixture (data not shown). As the cisplatin concentration was increased, bifunctional adducts of GTP (GTP-Pt-GTP) appeared gradually (Fig. 6). Platinated nucleotides increased in proportion to the increase of nucleotide concentration (Fig. 7). The concentrations of cisplatin involved in platinum-nucleotide adduct formation were about 7 μM (47% of the initial concentration) in the presence of 5 m*M* ATP and about 14 μM (11 μM for the monoadduct and 3 μM for the diadduct; 93%) in the presence of 2 m*M* GTP.

DISCUSSION

A large body of evidence indicates that cisplatin manifests its cytotoxic activity by binding to DNA and inhibiting DNA replication and transcription.[2, 20] Concerning cisplatin binding to DNA, bifunctional platina-

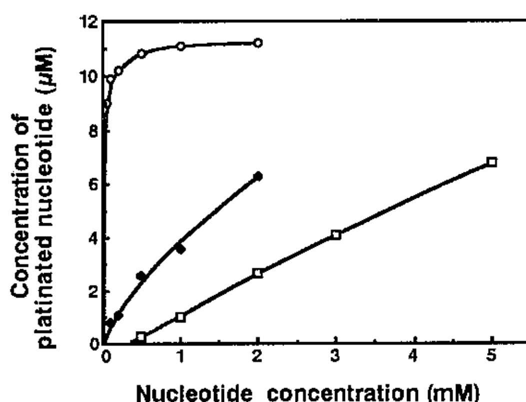

Fig. 7. Effect of varying nucleotide concentrations on platinated nucleotide formation. Nucleotide at the indicated concentration was incubated at 37°C for 16 h with 15 μM cisplatin. Platinated nucleotides were analyzed by HPLC as described in Fig. 6. (○), monofunctional GTP-platinum adducts; (□), monofunctional ATP-platinum adducts; (●), bifunctional GTP-platinum adducts.

tion is known to occur with guanosine $N(7)$ to guanosine $N(7)$, guanosine $N(7)$ to adenosine $N(1)$ or $N(7)$ and adenosine $N(7)$ or $N(1)$ to adenosine $N(7)$, and monofunctional platination at $N(7)$ of guanosine, $N(1)$ or $N(7)$ of adenosine and $N(3)$ of cytidine.[4]

The cisplatination of DNA is known to be inhibited reversibly or irreversibly by various chemicals such as chloride ion and thiol compounds.[2, 4, 14, 21] In plasma, where the chloride ion concentration is high (approximately 100 m*M*), cisplatin is predominantly in the inactive, dichloro form.[2] Inside the cell, where the chloride ion concentration is about 4 m*M*, cisplatin is converted to active, aquated forms (in which the chloride ligand is replaced by water) and is able to react with DNA.[2, 21] Thiol compounds are suggested to inhibit cisplatin-mediated DNA damage by combining with cisplatin.[22] Free nucleotides are also thought to inhibit cisplatin-mediated DNA damage because of their structural similarities with the target residues, but, so far as we know, no precise study on the subject has been reported.

It is known that each cell contains fairly large amounts of free ribonucleotides, especially ATP, and the nucleotide concentrations vary widely from tissue to tissue. For example, previous reports suggested that there are about 2–4 m*M* ATP and 0.3 m*M* GTP in rat liver, rat muscle and human brain cells, and about 1 m*M* ATP and 0.2–0.3 m*M* GTP in rat hepatoma, rat sarcoma and human glioblastoma cells.[23-26] Cellular concentration of dATP or dGTP is generally lower than one hundredth of that of the corresponding ribonucleotide.[23-26]

If free nucleotides interact with cisplatin, the platinum DNA adduct formation in cells is expected to be affected by the presence of free nucleotides, and as a result the cytotoxic effect of cisplatin may suffer a change depending on the nucleotide concentration in the vicinity of cisplatin-DNA interaction sites. To examine this notion, the effects of nucleotides on cisplatin-mediated DNA damage were studied *in vitro* in the present experiments. Free nucleotides inhibited cisplatin-mediated DNA damage in a dose-dependent fashion, possibly competing with DNA nucleotides for interaction with cisplatin. The inhibition by ribonucleoside triphosphate occurred in the order of GTP$>$ATP$\gg$CTP$>$UTP. The order was the same as that for adduct formation with platinum on DNA. Interestingly, the degree of the inhibition of platinum-DNA adduct formation depended on the degree of phosphorylation of ribonucleosides. The inhibitory effect was in the order of ATP$>$ADP$>$AMP or GTP$>$GDP$>$GMP. Ribonucleosides showed no inhibitory effect on platinum-DNA adduct formation. The results indicate that negative charges of the phosphate residues are important for the interaction between free nucleotides and cisplatin, as for the interaction between DNA nucleotides and cisplatin.[2, 4, 5, 27] As described in the "Results" section, the GTP-inhibition of platinum-DNA adduct formation is thought to be due to platination of GTP itself, but the degree of the ATP-inhibition is not directly proportional to the degree of ATP platination. In the latter case, non-covalent, ionic interaction between negative charge of ATP and positive charge of cisplatin may be involved in part in the inhibitory effect of ATP on platinum-DNA adduct formation by keeping the cisplatin away (shielding) from its target, the DNA.

Considering that ribonucleotides (ATP, GTP and others) are present in cells at concentrations possibly affecting the platinum-DNA adduct formation and that cellular nucleotide levels vary from cell to cell, nucleotides may have a significant influence on tumor sensitivity or resistance to cisplatin and cisplatin toxicity to host cells.

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