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Sequence-dependent Termination of Mammalian DNA Polymerase Reaction by a New Platinum Compound, (−)-(*R*)-2-Aminomethylpyrrolidine(1,1-cyclobutane-dicarboxylato)-2-platinum(II) Monohydrate

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We examined the mechanisms of the inhibition of DNA synthesis by a new platinum compound, (−)-(*R*)-2-aminomethylpyrrolidine(1,1-cyclobutane-dicarboxylato)-2-platinum(II) monohydrate (DWA-2114R), a derivative of the antitumor drug *cis*-diamminedichloroplatinum(II) (CDDP), using prokaryotic and eukaryotic DNA polymerases. Preincubating activated DNA with CDDP or DWA-2114R reduced its template activity for prokaryotic and eukaryotic DNA polymerases in a dose-dependent manner. DWA2114R required six times greater drug concentration and two times longer incubation time to show the same decrease of the template activity compared to CDDP. Treatment of primed pUC118 ssDNA templates with the two drugs followed by second-strand synthesis by prokaryotic and eukaryotic DNA polymerases revealed that DWA2114R bound to DNA in a similar manner to CDDP and these adducts blocked DNA elongation by DNA polymerases of eukaryotes as well as of prokaryotes. With these two drugs, the elongations by *E. coli* DNA polymerase I (Klenow fragment), T7 DNA polymerase and calf thymus DNA polymerase α were strongly arrested at guanine-guanine sequences (GG). Stop bands were also observed at adenine-guanine sequences (AG) guanine-adenine-guanine sequences (GAG) and mono-guanine sequence (G). Calf testis DNA polymerase β was also arrested efficiently at AG, GAG and G, but much more weakly at GG. This pattern was common to DWA2114R and CDDP.

Key words: Cisplatin — New platinum analogue — Replication inhibition — Chain elongation arrest — Mammalian DNA polymerase

Although *cis*-diamminedichloroplatinum(II) (CDDP)[4] is the most effective antitumor drug currently available for ovarian, testicular and pulmonary cancer,[1] its nephrotoxicity sometimes limits the dose which can be employed.[2,3] The other problem is that the tumor may become resistant to CDDP.[4] To overcome these problems, second-generation CDDP analogues with reduced toxicity and reduced cross resistance to CDDP have been developed and some are reaching the stage of clinical testing.[4] Recently, (−)-(*R*)-2-aminomethylpyrrolidine (1,1-cyclobutane-dicarboxylato)-2-platinum(II) monohydrate (DWA2114R), having the unsymmetrical diamine as a carrier ligand, has been developed.[5] This new analog has almost the same antitumor effect, but its nephrotoxicity is much weaker compared with CDDP.[2] It was also reported to show lower cross resistance with CDDP-resistant P388 murine leukemia cell line than other CDDP analogs (K. Kamisango and T. Matsumoto, unpublished data). Inhibition of replication due to inactivation of DNA as a template has been proposed to be responsible for the cytotoxity of CDDP.[6–8] A consensus exists that the platinum-DNA lesions formed by CDDP inhibit DNA synthesis *in vitro*.[6–8] Chromatographic analyses of CDDP-DNA adducts and immunochemical studies on CDDP-DNA adducts show that the CDDP-DNA adducts are mainly formed at GG and AG sequences, and adducts are also formed less efficiently at G and GNG (N is any intervening nucleotide).[7,9] *In vitro* replication of single-stranded M13mp10 DNA by the large fragment of *E. coli* DNA polymerase I is arrested by CDDP at specific sites which may involve bifunctional adducts at GG and AG.[5] In this study , we have examined the mechanism of the inhibition of DNA synthesis by the CDDP analog, DWA2114R, using prokaryotic and eukaryotic DNA polymerases in comparison with that by the original compound, CDDP. The results showed that DWA2114R gave similar arrest patterns to those of CDDP with all DNA polymerases used, at approximately 10 times higher concentration than CDDP.

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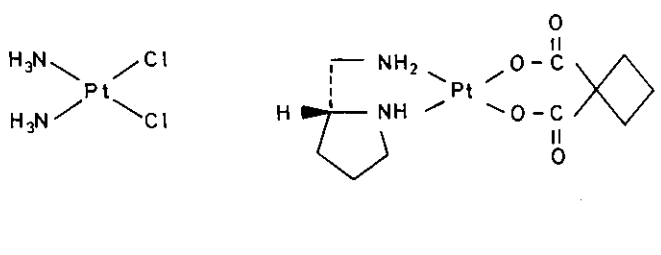
[4] The abbreviations used are: CDDP, *cis*-diamminedichloroplatinum(II); DWA2114R, (−)-(*R*)-2-aminomethylpyrrolidine-(1,1-cyclobutane-dicarboxylato)-2-platinum(II) monohydrate; D/N ratio, molar ratio of drug to nucleotide; bound D/N ratio, molar ratio of DNA-bound drug to nucleotide; ssDNA, single-stranded DNA.

MATERIALS AND METHODS

Chemicals [methyl-^{3}H]dTTP (20 Ci/mmol) and [α-^{32}P]dATP (800 Ci/mmol) were purchased from ICN Pharmaceuticals. Unlabeled deoxyribonucleoside triphosphates were obtained from Boehringer/Mannheim, Federal Republic of Germany. *cis*-Dichlorodiammine platinum (II) was purchased from Aldrich Chemical Company, Milwaukee, WI. Unlabeled and ^{14}C-labeled DWA2114R (17.2 mCi/mmol) were generous gifts from Chugai Chemical Co., Japan (Fig. 1).

Enzymes and DNAs DNA polymerase I Klenow fragment of *E. coli* was obtained from P-L Biochemicals, Inc., Milwaukee, WI. T7 DNA polymerase was obtained from Pharmacia-LKB Biotechnology Inc. DNA polymerase α from calf thymus was immunoaffinity-purified in our laboratory.[10] DNA polymerase β from bovine testis was purified by the method of Yoshida *et al.*[11] with some modifications. Specific activities of DNA polymerase α and DNA polymerase β were 200,000 units/mg protein and 100,000 units/mg protein, respectively, measured with activated DNA and poly(dA)/oligo(dT) as described previously.[10, 11] It was confirmed that these purified enzyme fractions showed no endonuclease activity as tested by examining the conversion of form I to form II of palasmid pBR322 DNA. pUC118 ssDNA was isolated from the phage obtained by precipitation with polyethylene glycol, followed by phenol extraction. This ssDNA was annealed to universal primer (15-mer, P-L Biochemicals) by heating the viral DNA with the primer at 60°C for 20 min and cooling slowly to room temperature.

Adduct formation Activated DNA (10 μg) was incubated at 37°C with CDDP at a drug concentration of 0.1 m*M* to 1.0 m*M* in 1 m*M* sodium phosphate/3 m*M* NaCl (pH 7.3), for 48 h. Reaction of DWA2114R with activated DNA was performed under similar conditions except that the incubation period was 96 h and the drug concentration was 0.5 m*M* to 5.0 m*M*. Primed pUC118 ssDNA (1 μg) was incubated at 37°C with CDDP and DWA2114R at a D/N ratio of 0.3 to 1.5 in the same buffer for 3 h (CDDP) and 12 h (DWA2114R). To quench the platinum reactions, concentrated NaCl (5 *M*) was added to raise the chloride concentration to 0.5 *M* and the samples were placed on ice. The samples were precipitated with ethanol and the precipitate was washed twice with ethanol to remove unbound platinum. The amount of platinum bound to the DNA was determined by flameless atomic absorption spectroscopy, or by using ^{14}C-labeled DWA2114R as described below.



CDDP DWA2114R

Fig. 1. The chemical structures of DWA2114R and CDDP.

Determination of bound D/N ratio using ^{14}C-labeled DWA2114R Primed pUC118 ssDNA (5 μg) was incubated at 37°C with [^{14}C]DWA2114R at a D/N ratio of 0.3 or 0.6 in 1 m*M* sodium phosphate/3 m*M* NaCl, pH 7.3, for 12 h. DNA in the reaction mixture was absorbed on DEAE paper (Whatman DE82). To remove the unbound DWA2114R, DEAE paper was washed at least twice for 5 min with 50 ml each of 0.1 *M* NaCl, water, and ethanol. The amount of DWA2114R bound to DNA was measured in terms of the radioactivity bound to the DEAE paper.

Reaction with T7 DNA polymerase and DNA polymerase I When activated DNA was used as a template, the reaction mixture (25 μl) contained 80 m*M* potassium phosphate (pH 7.2), 2 μg of either platinated or unplatinated activated calf thymus DNA, 80 μM each of dATP, dGTP, dCTP, 40 μM [^{3}H]dTTP (400 cpm/pmol), 2 m*M* 2-mercaptoethanol, 4 m*M* $MgCl_2$, and 1 unit of T7 DNA polymerase or DNA polymerase I . The reaction mixture for product analysis of the DNA elongation reaction (10 μl) contained 40 m*M* Tris-HCl, pH 7.5, 4 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol, 80 μM of dCTP, dGTP and dTTP, 5 μCi [α-^{32}P]dATP (8 μM), 1 μg of either platinated or unplatinated primed pUC118 ssDNA and 1 unit of DNA polymerase I or T7 DNA polymerase. The reaction mixture was incubated at 37°C for 30 min and then chased with 5 μl of 200 μM dATP for an additional 15 min at 37°C. At the end of the reaction, samples were placed on ice and then loaded on sequencing gels.

Reaction with DNA polymerase α The reaction mixture for activated DNA was the same as that in the case of DNA polymerase I. The reaction mixture for the second-strand DNA elongation reaction (25 μl) contained 1 μg of either platinated or unplatinated primed pUC118 ssDNA, 40 m*M* Tris-HCl pH 7.5, 4 m*M* $MgCl_2$, 2 m*M* dithiothreitol, 1 μg of single-strand-binding protein of *E. coli* (Sigma Biochemicals, Inc., USA), 80 μM each of dGTP, dCTP, dTTP, 5 μCi [α-^{32}P]dATP (8 μM) and 2 units of immunoaffinity-purified DNA polymerase α. Incubation was carried out at 37°C for 30 min followed by chase with 5 μl of 200 μM dATP for an additional 30 min at 37°C. At the end of the reaction, samples were concentrated 5-fold by ethanol precipitation. The DNA product was then electrophoresed on a sequencing gel.

Reaction with DNA polymerase β The reaction mixture for DNA polymerase β with activated DNA was essentially the same as that of DNA polymerase α except that 100 m*M* NaCl was added. The reaction mixture for product analysis (24 μl) contained 100 m*M* Tris-HCl pH 8.8, 8 m*M* $MgCl_2$, 100 m*M* NaCl, 50 μM each of dTTP, dCTP and dGTP, 5 μCi [α-^{32}P]dATP (8 μM), and 2 units of DNA polymerase β. Incubation was carried out as described for DNA polymerase α.

Electrophoretic analysis of synthesized DNA After the incubation, one volume of deionized formamide containing 0.3% xylene cyanol, 0.3% bromphenol blue, and 0.3% EDTA was added to the reaction mixture. Samples were boiled for 5 min and then subjected to electrophoresis at 1500 V on an 8% polyacrylamide in 7 *M* urea (Sanger *et al.*, 1977), and the gel was exposed overnight to Fuji RX film.

RESULTS

Effects of CDDP and DWA2114R on activities of DNA polymerases Neither CDDP nor DWA2114R inhibited the reactions of prokaryotic or eukaryotic DNA polymerases, when added directly to the reaction mixture up to 1 m*M* (data not shown). It is not likely, therefore, that these compounds directly inhibit the DNA polymerases by interacting with the enzyme proteins. Mixtures of activated DNA and platinum compounds of various concentrations were preincubated for 48 h (CDDP) and 96 h (DWA2114R) and their template activities were measured with prokaryotic DNA polymerases (T7, *E. coli*) and eukaryotic DNA polymerases α and β (Fig. 2). The activities were suppressed in a dose-dependent manner. With CDDP, the extent of inhibition was essentially the same among all DNA polymerases tested (Fig. 2A), while, with DWA2114R, the inhibition differed somewhat among the enzymes; DNA polymerases α and β showed higher activity than prokaryotic enzymes with the analogue-treated templates (Fig. 2B). This difference was not analyzed further. It is remarkable that DWA2114R requires 6 times greater drug concentration and 2 times longer incubation time to attain the same extent of inhibition. These results agree in part with those of Harder *et al.*[8]

DNA synthesis on the CDDP- and DWA2114R-modified pUC118 ssDNA For the further analysis of the inhibition, pUC118 ssDNA was treated with these drugs, hybridized with synthetic DNA primer, and used as a template for the DNA polymerase reaction. The reaction products were electrophoresed on a sequencing polyacrylamide gel. The exact positions of elongation arrest were determined by comparing the stop bands with lanes of dideoxynucleotide sequencing reactions using untreated DNA according to the method of Sanger *et al.*[12]

In Fig. 3, the reaction products formed by T7 DNA polymerase are shown. Sequence analysis of the termination sites produced by CDDP reveals a strong sequence specificity for guanine base. There were strong stop bands at the GG sequence and stop bands were also observed at AG, GAG and G sequences. Within the sequence of 15 bases (no. 86–101) where no G existed, no stop band was seen. When the drug concentration was

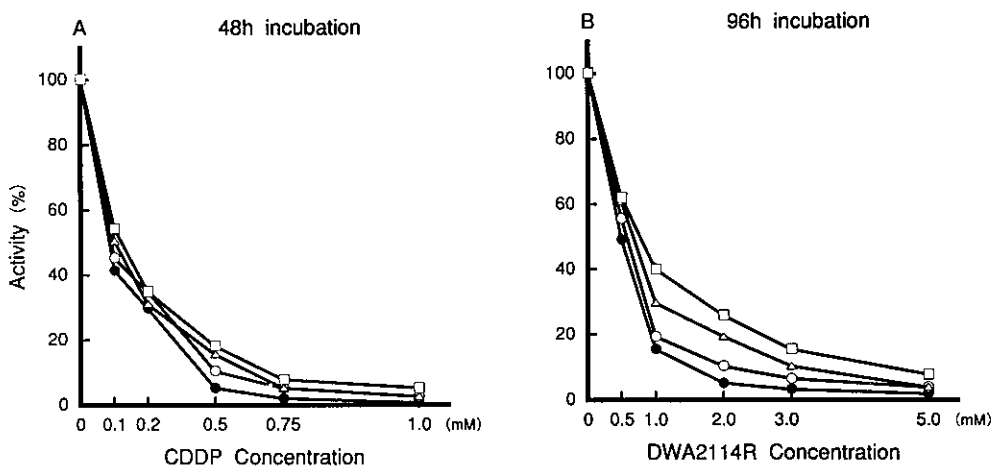


Fig. 2. Decrease of the template activity of the activated DNA by the treatment with CDDP (A) and DWA2114R (B). Activated DNA (10 μg) was incubated at 37°C at a drug concentration of 0.1 m*M* to 1 m*M* for 48 h (CDDP) or 0.5 m*M* to 5 m*M* for 96 h (DWA2114R). Activities of T7 DNA polymerase (●), *E. coli* polymerase I (○), calf thymus DNA polymerase α (□), calf testis DNA polymerase β (△) were measured as described in "Materials and Methods."

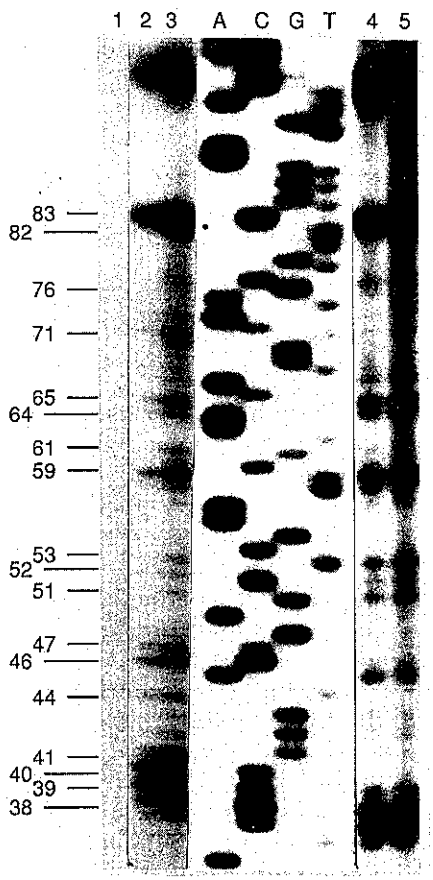


Fig. 3. Analysis of reaction products by T7 DNA polymerase with platinated template. Reactions were performed with 1.0 unit of T7 DNA polymerase using CDDP- and DWA2114R-modified pUC118 ssDNA as a template and reaction products were electrophoresed as described in "Materials and Methods." Lane 1, no modification control; lane 2, CDDP-modified DNA (bound D/N ratio=0.007); lane 3, CDDP-modified DNA (bound D/N ratio=0.014); lane 4, DWA2114-R-modified DNA (bound D/N ratio=0.007); lane 5, DWA-2114R-modified DNA (bound D/N ratio=0.014). Lanes A, C, G and T show sequence analysis using T7 DNA polymerase with dideoxy chain-terminating nucleotides on an unplatinated template. On the left side of the lanes positions of stop bands are shown. The numbers indicate the nucleotides added to the 3′-OH end of the primer.

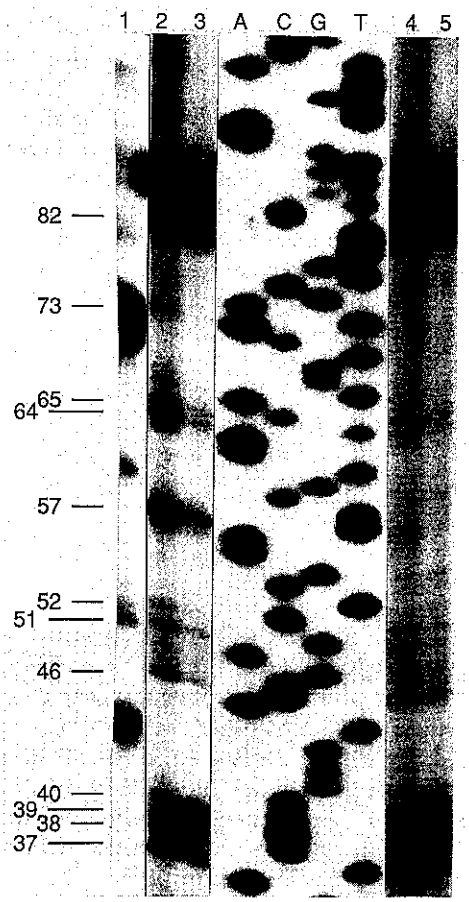


Fig. 4. Analysis of reaction products by calf thymus DNA polymerase α with platinated template. Reactions were performed with 2.0 units of immunopurified DNA polymerase α from calf thymus using CDDP- and DWA2114R-modified pUC118 ssDNA as a template and reaction products were electrophoresed as described in "Materials and Methods." Lane 1, no modification (control); lane 2, CDDP-modified DNA (bound D/N ratio=0.007); lane 3, CDDP-modified DNA (bound D/N ratio=0.014); lane 4, DWA2114R-modified DNA (bound D/N ratio=0.007); lane 5, DWA2114R-modified DNA (bound D/N ratio=0.014). Sequencing by Sanger's method is also shown. On the left side of the lanes, positions of stop bands are shown. The numbers indicate the nucleotides added to the 3′-OH end of the primer.

increased, the amount of shorter fragments increased. Analysis of the reaction products formed by *E. coli* DNA polymerase I (Klenow fragment) showed very similar patterns (data not shown). These results are consistent with those of Pinto and Lippard.[6] Treatment with DWA2114R gave a very similar pattern of arrest bands, although 10 times more concentration was required than in the case of CDDP. Control lanes show the products with the non-treated template. In addition to the bands indicated, bands at positions 42, 43, 66, 67 and 73 were seen (Fig. 3). However, these bands were not reproducible in several experiments, so we tentatively concluded that these were not arrest bands created by adduct formation.

In Fig. 4, reaction products formed by eukaryotic DNA polymerase α are shown. Lower processibility in the elongation reaction on templates compared with prokaryotic DNA polymerase resulted in several natural

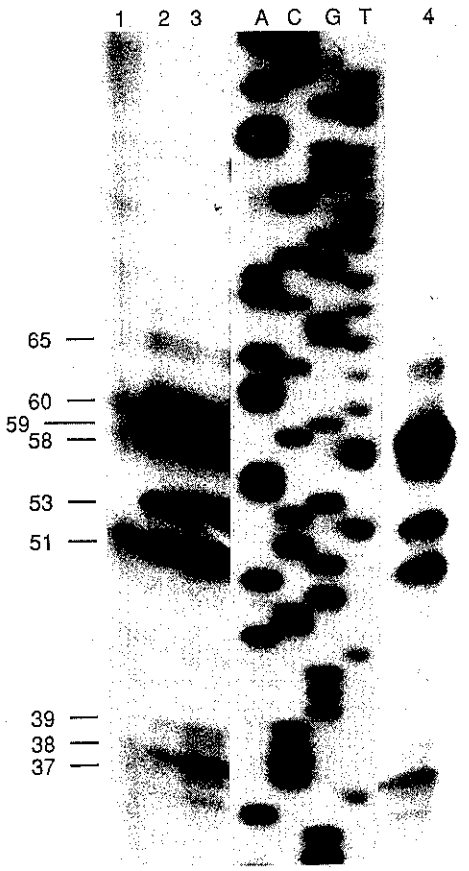


Fig. 5. Analysis of reaction products formed by calf testis DNA polymerase β with platinatinated template. Reactions for DNA polymerase β were performed as described in "Materials and Methods," using 2.0 units of DNA polymerase β from calf testis. Lane 1, no modification (control); lane 2, CDDP-modified DNA (bound D/N ratio=0.007); lane 3, CDDP-modified DNA (bound D/N ratio=0.014); lane 4, DWA2114-R-modified DNA (bound D/N ratio=0.014). Sequencing by Sanger's method is also shown. On the left side of the lanes, positions of stop bands are shown. The numbers indicate the nucleotides added to the 3'-OH end of the primer.

stop bands, which may be due to specific arrest of DNA polymerase α at "arrest sequences" such as the guanine cluster[13] at 84 and 85 nucleotides from the universal primer (no. 84–85) (Fig. 3). Within these limitations, analysis of the reaction products by DNA polymerase α revealed that the pattern of elongation arrest was essentially the same as that observed with prokaryotic polymerases. The arrest patterns of CDDP and DWA2114R were also exactly the same. It should be noted that some natural stop bands with DNA polymerase α disappeared on treatment with platinum compounds, possibly due to conformational changes of DNA arising from adduct formation.

In Fig. 5 the reaction products formed by eukaryotic DNA polymerase β are shown. This enzyme did not show strong stop bands with control DNA. With platinated DNAs, its pattern of arrests differed remarkably from those by other DNA polymerases. Much weaker stop bands at the guanine cluster (no. 38–41) and stronger stop bands at AG (no. 52–53, no. 59–60) and GAG (no. 52–54) appeared. At sequence GG (no. 47–48) no stop band was observed.

Comparison of patterns of elongation arrests The patterns of elongation arrests are summarized in Fig. 6. With either CDDP or DWA2114R, elongation by *E. coli* DNA polymerase I, T7 DNA polymerase, and mammalian DNA polymerase α was strongly arrested at GG sequence. Stop bands were also observed at AG, GAG and G sequences. There were three stop modes of DNA elongation at putative platinated DNA lesions on the template: stop bands appeared mostly at one base prior to the lesion, but sometimes at positions opposite to the lesion, and even one base beyond the lesion. The arrest patterns by DNA polymerase β differed from those of prokaryotic DNA polymerases and eukaryotic DNA polymerase α. With DNA polymerase β, arrests at G-clusters were less efficient than those in the cases of the other three enzymes, but arrests were highly efficient at AG, GAG and G sequences. These differences may reflect the difference in base-recognition mechanism by these enzymes. However, the stop modes by CDDP and DWA2114R were strikingly similar, indicating that these two drugs form similar adducts with DNA, and that these adducts are recognized by DNA polymerases in the same manner.

DISCUSSION

The main purpose of this study was to determine the inhibitory effects of a new platinum compound, DWA-2114R, on *in vitro* DNA synthesis in comparison with CDDP. Experiments showed that DWA2114R required approximately 10-times-higher drug concentration to show the same extent of decrease in its template activity as CDDP. This result agrees with the fact that the effective dose in tumor therapy of DWA2114R is 10 times higher than that of CDDP.[5] In order to determine whether the action mechanism of the new analogue, DWA2114R, is the same as that of CDDP or not, a single-stranded plasmid DNA of known sequence was modified with the two drugs and used as a template for DNA synthesis by four kinds of DNA polymerases purified from prokaryotes and eukaryotes. The analysis of reaction products on sequencing gel revealed that the arrest sites produced by DWA2114R were quite similar

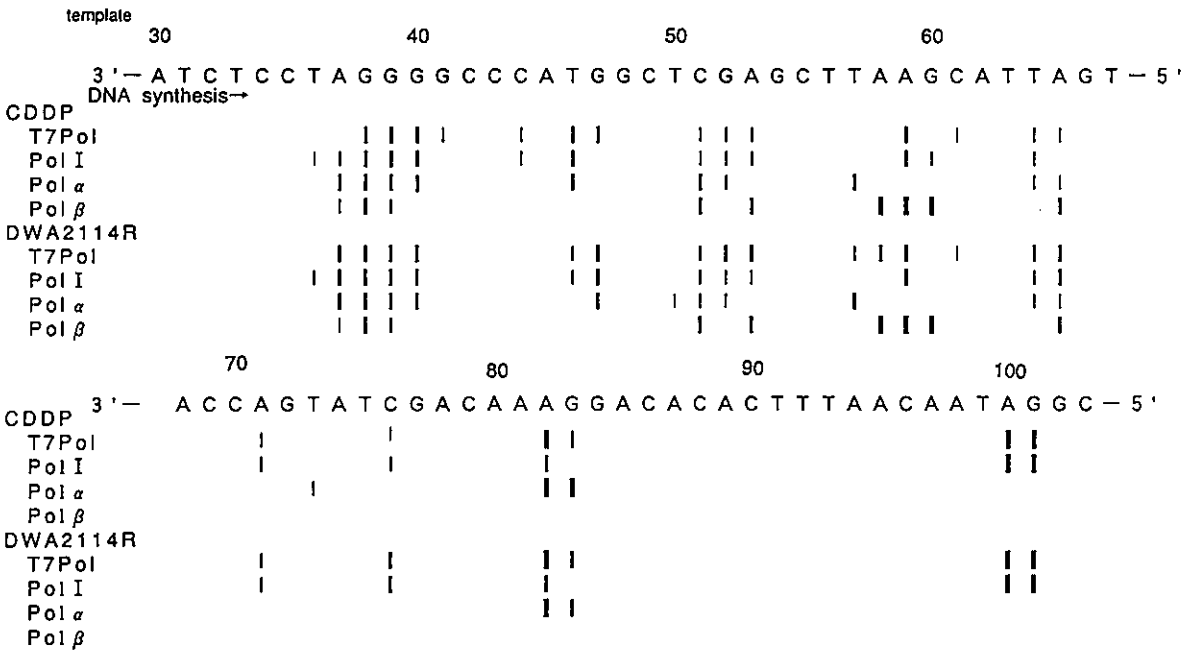


Fig. 6. Schematic presentation of the arrest sites of DNA polymerases on CDDP- and DWA2114R-modified DNA. The drugs used for modification and the enzymes are shown on the left side of the figure. Arrest bands from Figs. 3 to 5 are summarized schematically. The width of the bar represents the intensity of each band. The arrest sites of *E. coli* DNA polymerase I (Klenow fragment) (data not shown) are also shown. The numbers of deoxynucleotides added to the 3′-OH end of the primer are shown above the template sequence.

to those obtained by CDDP treatment, with all DNA polymerases tested. Therefore, it is likely that DWA-2114R forms adducts on DNA in a similar way to CDDP and the adducts block chain elongation by DNA polymerase. It was suggested that the cyclobutane dicardoxylate group (Fig. 1) of DWA2114R could be nonenzymatically cleaved to yield the active form, while the aminomethylpyrrolidine group at the other side of the compound remained attached to platinum to form bulkier adducts than those in the case of CDDP. The present results showing similar arrest patterns with CDDP and DWA2114R may indicate that the crosslinking of two adjacent purines including guanine is primarily important. The elongation arrest might be analogous to that caused by pyrimidine dimers in ultraviolet light-irradiated DNA.[14] Although we confirmed that the pyrrolidine group existed at DNA lesions using ^{14}C-labeled compounds, the significance of this group in the interference with DNA synthesis remains unclear. It was striking that DNA polymerase β showed arrest patterns different from those with the other three polymerases. The stop bands with DNA polymerase β were more prominent at AG, GAG and G than GG, while the other three enzymes stopped more efficiently at GG than at AG, GAG and G sequences (Fig. 6). Similar results were obtained with both CDDP and DWA2114R. In the case of a carcinogen, 4-hydroxyquinoline N-oxide, it was observed that DNA polymerase β was arrested at positions exactly one base prior to G where the carcinogen was attached.[15] It is conceivable that, in the case of platinum compounds, DNA polymerase β could bypass the crosslinkings at GG more easily than at AG, GAG and G but a definite conclusion must wait further study.

DWA2114R shows lower nephrotoxicity and other side effects compared with CDDP.[5] However, at the level of DNA replication, i.e., the ultimate target of drug action, these two drugs behaved in the same way. Therefore, the difference in side effects might be due to other factors, e.g., different pharmacokinetics or permeability.

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