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Binding Characteristics of (−)-(R)-2-Aminomethylpyrrolidine(1,1-cyclobutanedicarboxylato)-2-platinum(II) to DNA, RNA and Protein Molecules in HeLa Cells and Its Lethal Effect: Comparison with *cis*- and *trans*-Diamminedichloroplatinums(II)

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HeLa S-3 cells were treated with ^{195m}Pt-radiolabeled (−)-(R)-2-aminomethylpyrrolidine(1,1-cyclobutanedicarboxylato)-2-platinum(II) (DWA2114R) under various conditions, and the relationship between the lethal effect of the agent and the number of platinum (Pt) atoms binding to DNA, RNA and proteins was examined. The values of mean lethal concentration for the cells treated with DWA2114 at 37°C for 1, 2 and 3 h were 137.3, 75.10 and 51.17 *μM*, respectively. Cells were treated identically and the numbers of Pt atoms combined with DNA, RNA and protein molecules were determined after fractionation of the cells. In this way, the D_0 values (D_0, dose that would give an average of one lethal event per member of the population), expressed as the drug concentration, were substituted for the number of Pt atoms combined with each fraction. The target volumes, the efficacy of Pt atom to kill cells expressed as the reciprocals of the D_0 values, were then calculated for each fraction. Our findings suggested that DNA was the primary target molecule for cell killing by DWA-2114R. The target volumes for DNA were 3.36×10^4, 4.00×10^4 and 4.10×10^4 nucleotides for 1-, 2- and 3-h treated cells, respectively. The cell-killing effects of DWA2114R were lower than those of *cis*-diamminedichloroplatinum(II) (CDDP) by factors of 1.54, 1.42 and 2.51 for 1-, 2- and 3-h treatments at 37°C, respectively, in terms of the target volume, while those in terms of the mean lethal dose (D_0) were 14.8, 11.2 and 16.0, respectively. The efficacy of DWA2114R in killing the cells was 2.6 times greater than that of CDDP in the 3-h treatment at 0°C.

Key words: DWA2114R — ^{195m}Pt-DWA2114R — HeLa cell — Mean lethal dose — Target volume

cis-Diamminedichloroplatinum(II) (CDDP) is in wide clinical use as an anticancer drug.[1–3] However, its toxic side effects, e.g., vomiting, neurotoxicity, ototoxicity, and most importantly, its nephrotoxicity, as well as its low solubility in water, limit the dose.[4–6] Therefore, in recent years, many analogs have been synthesized with improved properties as regards toxicity and solubility.[7] One of these is a newly developed antitumor agent, (−)-(R)-2-aminomethylpyrrolidine(1,1-cyclobutanedicarboxylato)platinum(II) (DWA2114R).[8] Both *in vivo* and *in vitro* pharmacokinetic studies of the distribution and metabolism of this compound are now being carried out. For these studies, the synthesis and utilization of radiolabeled DWA2114R are essential.

Since 1986, we have been engaged in improving the methods used for the synthesis of ^{195m}Pt-radiolabeled CDDP.[9] Recently, we described methods for the synthesis of ^{195m}Pt-CDDP[10] and -DWA2114R,[11] using HPLC. The use of ^{195m}Pt-CDDP allowed us to determine the number of Pt atoms binding to a DNA molecule in HeLa cells treated with CDDP at the mean lethal concentration. Hence the target volume (a measure of the efficiency of the Pt atom for killing the cells) could be determined at low doses of CDDP. This investigation suggested that the molecular target for cell killing by CDDP was DNA.[12] In the present study, we examined the rate of binding of Pt atoms to biologically important molecules in cultured HeLa cells treated with ^{195m}Pt-DWA2114R, and we determined the target volume. A comparison of the findings here with those in our previous studies[12, 13] should reveal the mechanism(s) underlying the antitumor activity of these Pt compounds.

MATERIALS AND METHODS

^{195m}Pt-DWA2114R Ten mg of 95%-enriched ^{194}Pt (purchased from Oak Ridge National Laboratory, USA) was irradiated in the hydraulic conveyor of the KUR (Kyoto University Reactor) at a thermal neutron flux of approximately 8.15×10^{13} $n\cdot cm^{-2}\cdot s^{-1}$ for 75 h. After a 3-day cooling period, to eliminate undesired radioactivity due mainly to ^{191}Pt and ^{197}Pt, the synthesis of ^{195m}Pt-DWA2114R was carried out according to a method described elsewhere[9] (specific activity, 6.7×10^6 Bq/mg-DWA2114R; chemical and radionuclidic purity, higher than 99.2%).

HeLa cells HeLa cells were cultured in a monolayer (petri dish, 90-mm diameter) in MEM supplemented with 10% calf serum and 1 m*M* L-glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Exponentially growing cells were trypsinized, collected, and resuspended in the same medium. One ml of cell suspension, containing 1.0×10^7 cells, was incubated with ^{195m}Pt-DWA2114R (5.0–200 μg/ml) for 1, 2 or 3 h at 37°C. After incubation, 10- and 990-μl aliquots of the cell suspensions were taken from the same sample, for the cell survival assay and the measurement of the fractional distribution of ^{195m}Pt, respectively.

Cell survival assay After appropriate dilution, the cells were subcultured in 6-cm petri dishes, at a density of 500–2000 cells/plate. After the addition of fresh medium, the cells were incubated for at least 10 days at 37°C in a CO_2-incubator. The cells were then stained with 1% crystal violet, and colony-forming capacity was determined. All survival experiments were performed in triplicate for each concentration.

Fractional distribution of ^{195m}Pt The distribution of ^{195m}Pt-radioactivity in the DNA, RNA and protein fractions of treated cells was measured by using the fractionation method of Schneider.[14]

Other experimental procedures All other experimental procedures were the same as those described in our previous paper.[12] In brief, we obtained the D_0 values (from the cell survival assay) as the concentration of DWA2114R with which the cells were treated, and these values were replaced by the numbers of Pt atoms bound to the cellular macromolecules (determined by the measurement of fractional distribution of ^{195m}Pt). We then divided the number of Pt atoms by the number of constituent units of each macromolecule per cell. The mean values of macromolecule content obtained from the 24 samples each of DNA, RNA and protein fractions was $1.90\pm0.04\times10^{-11}$, $2.72\pm0.06\times10^{-11}$, and $2.47\pm0.08\times10^{-9}$ g/cell, respectively. The number of constituent units was calculated assuming the molecular weight of the constituent units to be 350 for DNA and RNA, and 120 for proteins. Finally, we took the reciprocal of the quotient to be the target volume, i.e., the measure of the efficiency of the Pt atom for killing the cells.

RESULTS

Dose-survival curves of HeLa cells Fig. 1 shows the dose-survival curves of HeLa cells treated with ^{195m}Pt-DWA2114R for 1, 2, and 3 h at 37°C, and for 3 h at 0°C. In most cases, the semilogarithmic dose-survival curves consisted of straight lines. From the slopes of the lines, the D_0 values for the cells treated under various conditions were determined to be 137.3, 75.10, and 51.17 μM for 1-, 2- and 3-h incubation, respectively, at 37°C and 444.7 μM for 3-h incubation at 0°C. The D_0 for DWA2114R in each treatment was considerably higher than that obtained for CDDP treatment.[12] Namely, at 0°C for 3 h, the D_0 for DWA2114R was around 2-fold higher than that for CDDP (444.7/221), and these values for 37°C treatments for 1, 2, and 3 h, respectively, were 14.8-fold (137.3/9.3), 11.2-fold (75.10/6.7), and 16.0-fold (51.17/3.2) higher than those of CDDP (data for CDDP are from ref. 12). The efficacy of DWA2114R in killing the cells was 2.6 times greater than that of CDDP at 0°C in the 3-h treatment. Under these conditions the difference in the efficacy of the two drugs was greatest.

^{195m}Pt-distribution among various fractions Fig. 2 demonstrates the fractional distribution of ^{195m}Pt in the various fractions of HeLa cells treated with different concentrations of ^{195m}Pt-DWA2114R at 37°C for 1 h. From the semilogarithmic curves of the radioactivity vs. concentration plots, the relationships between incorporation into each fraction and the drug concentration were linear. There were clear differences in the incorporation behavior of DWA2114R and CDDP. The amounts of ^{195m}Pt incorporated into the three kinds of macromolecules were considerably higher with CDDP: around 9.3-fold for DNA, around 4.5-fold for protein, and only around 1.09-fold for the RNA fraction. The figures demonstrate

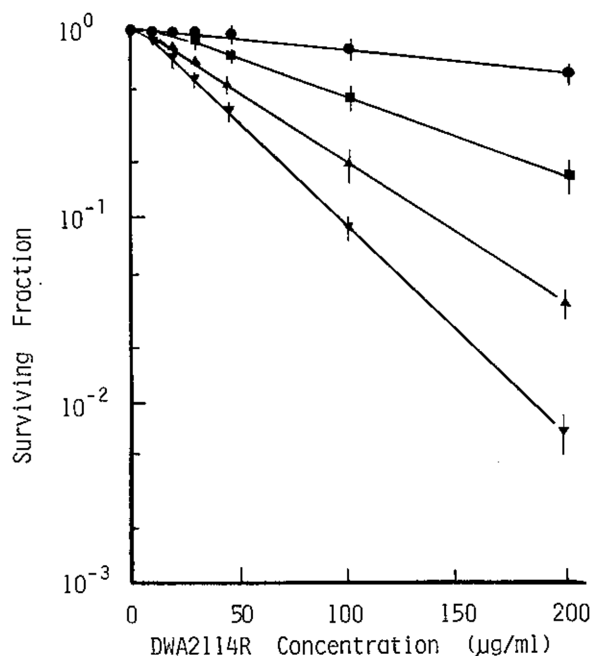

Fig. 1. Dose-survival curves of HeLa S-3 cells treated with ^{195m}Pt-DWA2114R under various conditions. Values given are means±SD from 3 separate experiments. Incubation conditions: ● 0°C-3 h, ■ 37°C-1 h, ▲ 37°C-2 h and ▼ 37°C-3 h.

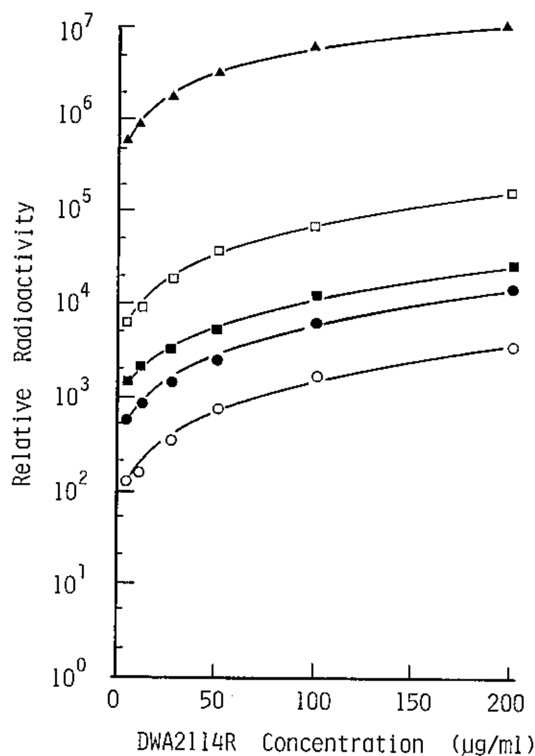

Fig. 2. ^{195m}Pt distribution in various fractions of HeLa cells treated with ^{195m}Pt-DWA2114R at 37°C for 1 h. Fractions: ○ DNA, ● RNA, ■ protein, □ TCA-soluble and ▲ medium.

that incorporation into the DNA fraction was of the order of 0.01% of the total radioactivity, and those into the RNA and protein fractions were about 0.01% and 1.0% respectively. Fig. 3a shows ^{195m}Pt distribution in the DNA fraction of cells treated under the different conditions. Incorporation in this fraction increased with incubation time; however, it did reach a plateau level. The incorporation was extremely high in the 3-h incubation at 0°C (it was less than one-tenth of, for example, the 1-h incubation at 37°C in the CDDP-treatment). Such marked incorporation was not observed with the two other fractions (Fig. 3 b and c). From the figures, we easily obtained the % Pt binding to each fraction at the mean lethal concentration. For DNA, these percentage were 0.0125%, 0.0192% and 0.0349% for treatment at 37°C for 1, 2, and 3 h, respectively, and 0.0058% for the 3-h treatment at 0°C. These values for RNA were 0.0820%, 0.134%, 0.169% and 0.0358%, while those for proteins were 0.203%, 0.361%, 0.556% and 0.069%, respectively. The % Pt binding to macromolecules at the mean lethal concentration did not vary significantly with drug concentration.

D_0, Pt-%, number of Pt-atoms binding to macromolecules and target volume The numbers of Pt atoms bound to DNA, RNA, and proteins under various incubation conditions at the mean lethal concentration were calculated on the basis of Figs. 1 (D_0) and 3 (% Pt), and the results are shown in Table I. The number of Pt atoms was then divided by the number of constituent units of each macromolecule per cell, and the reciprocal of the quotient, the target volume, was calculated. The target volume, i.e., the efficiency of Pt atom in killing the cells, increased with increasing incubation time, since the number of Pt atoms bound to macromolecules decreased with incubation time. The decrease of Pt atom binding to macromolecules is an immediate consequence of the increase of % Pt binding with incubation being overcome by a decrease in D_0.

DISCUSSION

In a previous study,[12] we determined the target volumes for three kinds of biologically important molecules in CDDP-treated cells, according to the single hit model. We concluded that, of the three molecules, only DNA was the target molecule for cell killing by CDDP since its molecular weight was high enough to include the calculated target volume. We also found that high-molecular-weight RNA (except t-RNA), under some limited conditions, could also be target molecules for cell killing by *trans*-diamminedichloroplatinum(II) (TDDP).[13] However, our findings here, outlined below, suggest that only the DNA molecule was the target material for cell killing by DWA2114R. Table I shows that the target volume of proteins was 7.35×10^5 amino acids for 1 h treatment at 37°C, for example. Proteins usually have a molecular weight of the order of 10^4–10^5, corresponding to 100–1000 amino acids. This means that about one out of 7.35×10^2–7.35×10^3 protein molecules combines with one Pt atom at the mean lethal concentration. It is very unlikely that the cell would be killed by the inactivation of only one out of 7.35×10^2–7.35×10^3 protein molecules, when almost all the protein molecules (99.86%–99.99%) would remain intact. This is also the case for RNA, since the target volume was 0.73×10^4 nucleotides, if RNA is assumed to be the target for cell death due to DWA2114R. The value is too large to allow a single hit on all RNA molecules in the cells at the mean lethal concentration. Thus, it is apparent that RNA and proteins are unlikely to be the targets of DWA2114R in causing cell death. On the other hand, the target volume calculated for DNA under these conditions, namely 3.36×10^4 nucleotides, is small enough to be contained within the DNA molecule. Assuming the molecular weight of DNA to be 3×10^8 (about 10^6 nucleotides), then 3.36×10^4 nucleotides, the calculated target volume of DNA for

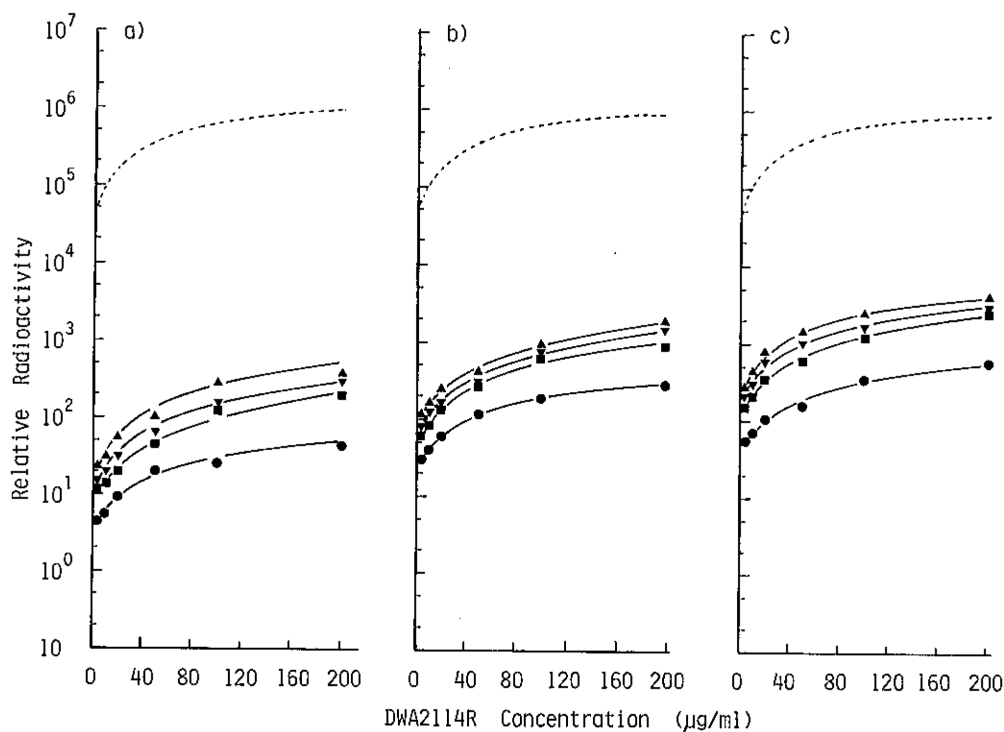

Fig. 3. ^{195m}Pt distribution in DNA (a), RNA (b) and protein (c) fractions of HeLa cells treated with ^{195m}Pt-DWA2114R under different incubation conditions. Dotted lines indicate medium level. Incubation conditions: ● 0°C-3 h, ■ 37°C-1 h, ▲ 37°C-2 h and ▼ 37°C-3 h.

Table I. D_0, Number of Pt-Atoms Binding to Three Kinds of Macromolecules and Target Volumes of HeLa Cells Treated with ^{195m}Pt-DWA2114R

Treatment	0°C-3 h	37°C-1 h	37°C-2 h	37°C-3 h
D_0 (μg/ml)	202.5	62.5	34.2	23.3
(μM)	444.7	137.3	75.10	51.17
DNA				
% Pt binding	0.0058	0.0125	0.0192	0.0275
No. of Pt atoms/cell (×10^5)	15.48	10.30	8.65	8.43
Target volume (×10^4 nucleotides)	2.24	3.36	4.00	4.10
RNA				
% Pt binding	0.0358	0.0820	0.134	0.169
No. of Pt atoms/cell (×10^5)	95.52	67.55	60.38	51.89
Target volume (×10^4 nucleotides)	0.51	0.73	0.81	0.95
Protein				
% Pt binding	0.0690	0.203	0.361	0.556
No. of Pt atoms/cell (×10^7)	1.841	1.672	1.627	1.707
Target volume (×10^5 amino acids)	6.68	7.35	7.56	7.21

1-h treatment at 37°C, means that binding of 30 Pt atoms per DNA molecule is needed to kill the cell. As the corresponding numbers for CDDP[12] and TDDP[13] were about 19 and 763 per DNA molecule, respectively, it can be concluded that, in terms of the efficiency of the Pt atom for killing cells, DWA2114R was 1.6 times less toxic than CDDP and 25.4 times more toxic than TDDP. It should be noted that, in terms of D_0, the cell-killing effect of TDDP was greater than that of DWA2114R by a factor of only 1.2. The relative efficiency of DWA2114R compared with that of TDDP varied depending on the treatment. Fig. 4 shows variations in D_0, in the percentage and number of Pt atoms binding to the DNA molecule, and variations in the target volume under different incubation conditions for DWA2114R treatment (from Table I), and for CDDP[12] and TDDP[13] treatments. The relative efficiency compared with TDDP decreased from 25.4 to 13.3 for 2 h incubation (4.00/0.301) and to 6.6 for 3 h incubation (4.10/0.623), while that compared with CDDP did not vary greatly, being 1.54 (5.17/3.36), 1.42 (5.71/4.00), and 2.51 (10.3/4.10) for 1-, 2- and 3-h treatments, respectively. It can therefore be concluded that prolonged incubation increased the efficiency of Pt in killing cells to a greater extent with DWA2114R and

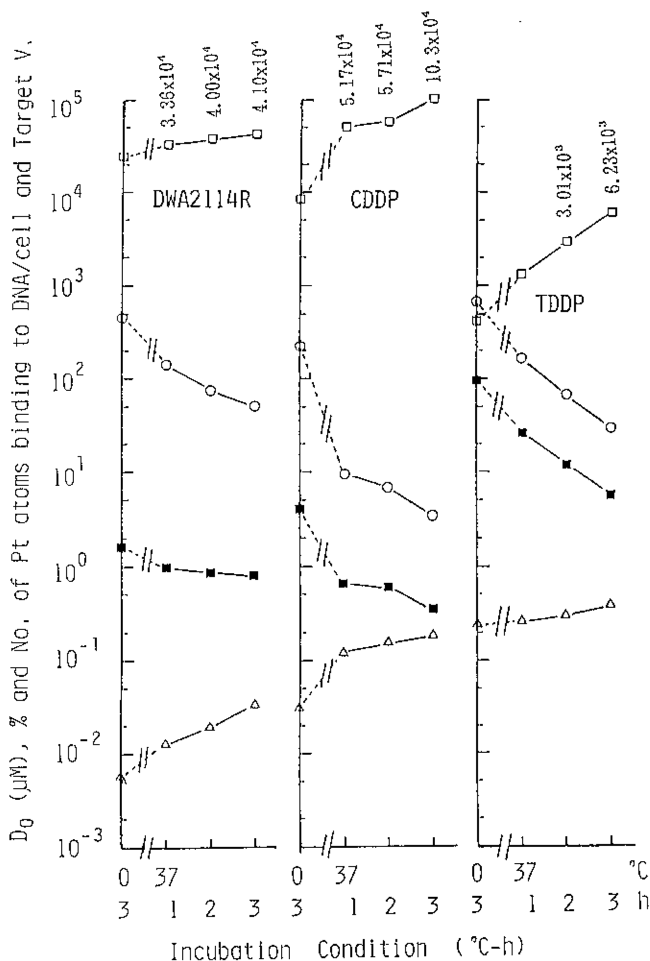

Fig. 4. Comparison of D_0 (μM), percentage and number ($\times 10^6$) of Pt atoms bound to DNA molecules (/cell), and the target volume (nucleotides), for the Pt compounds DWA-2114R, CDDP, and TDDP. ○ D_0, △ %-Pt, ■ number of Pt atoms and □ target volume.

CDDP than with TDDP. The most notable feature in this respect is that the target volume, that is, the cell-killing efficiency of Pt, for 3-h incubation at 0°C was 2.6 times greater with DWA2114R (2.24×10^4 nucleotides) than with CDDP (0.87×10^4 nucleotides), although D_0 was twice as high (less toxic) for DWA2114R (444.7 μM) than for CDDP (221 μM). This suggests that the binding of DWA2114R with the DNA molecule is much more lethal than that of CDDP at low temperature. There is much evidence to demonstrate that bifunctional bonds provide much higher cytotoxicity[15-18] than mono-functional bonds, and that the proportion of bifunctional bonds in treated cells varies[19, 20] depending on the incubation conditions and on the type of drug. Thus, our present findings suggest that the proportion of bifunctional DNA-DNA cross-links is higher with DWA2114R than with CDDP at a low temperature. In any case, our results here indicate that it is important to consider not only the number of Pt atoms combined with DNA but also the chemical structure of the binding and the chemical behavior of the compounds, in the analysis of the mechanism responsible for the antitumor action of these Pt compounds.

In conclusion, this study suggests that target volume analysis provides much more precise information on the mechanism underlying the lethal action of these compounds than does D_0 analysis. We believe the use of ^{195m}Pt-compounds and the subsequent analytical technique employed here to be valuable for examining the number of Pt atoms, and hence, the target volumes of cells treated with various Pt compounds.

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