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Relationship between Cell-killing Efficiency and Number of Platinum Atoms Binding to DNA, RNA, and Protein Molecules in HeLa Cells Treated with *cis*-Diamine(glycolato)platinum(II)

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HeLa S-3 cells were treated with ^{195m}Pt-radiolabeled *cis*-diamine(glycolato)platinum(II) (254-S) under various conditions, and the relationship between the lethal effect and the numbers of Pt atoms binding to DNA, RNA, and proteins was examined. The mean lethal concentrations for the cells treated with 254-S at 37°C for 0.5, 1, 2, and 3 h were 67.1, 47.0, 26.8 and 8.1 μM, respectively. Using identically treated cells, we determined the numbers of Pt atoms combined with DNA, RNA, and protein molecules after fractionation of the cells. In this way, the D_0 values (D_0, the dose that causes an average of one lethal event per member of the population), expressed as the drug concentration, could be related to the number of Pt atoms combined with each fraction. The efficiency of the Pt atom in killing the cells, expressed as the reciprocal of the D_0 values, was then calculated for each fraction. The results suggested that DNA was the primary target for cell killing by 254-S. The target volumes for DNA were 3.96, 4.97, and 11.77 $\times 10^4$ nucleotides for 1-, 2-, and 3-h treated cells, respectively. In terms of the target volume, the cell-killing effects of 254-S were comparable to those of *cis*-diamine-dichloroplatinum(II) (CDDP), for which the target volumes under identical conditions were determined to be 5.17, 5.71, and 10.3 $\times 10^4$ nucleotides, respectively, while in terms of the mean lethal dose (D_0), the cell-killing effects of 254-S were lower than those of CDDP by a factor of 5.1 (47.0/9.3), 4.0 (26.8/6.7), or 2.5 (8.1/3.2) for 1-, 2-, or 3-h treatment, respectively.

Key words: *cis*-diamine(glycolato)platinum(II) — ^{195m}Pt-254-S — HeLa cell — Mean lethal dose — Target volume

Although *cis*-diaminedichloroplatinum(II) (CDDP) is now one of the clinically most important anticancer drugs,[1–3] the development of acquired resistance, as well as the toxic side effects of the drug, often limits its use.[4–6] One way to circumvent the clinical resistance to CDDP is the development of new platinum analogues that are not cross-resistant to CDDP. One of these analogues is the newly developed antitumor agent *cis*-diamine(glycolato)platinum(II), 254-S, a coordination complex with a novel ring structure in which glycolato is bound to the platinum ion as a bidentate ligand.[7] Pharmacokinetic studies, both *in vivo* and *in vitro*, of the distribution, metabolism, and cell-killing action of compound 254-S are now required. For these studies, the synthesis and utilization of radiolabeled 254-S is essential. Recently, we succeeded in synthesizing ^{195m}Pt-radiolabeled 254-S.[8] A comparison of the characteristics of this compound with those of other such compounds[9–12] should provide useful information to clarify the mechanism(s) of the antitumor activities of these Pt compounds. Here we report an investigation of the binding characteristics of ^{195m}Pt-254-S to DNA, RNA, and protein molecules in cultured HeLa cells. The reciprocal of the number of Pt atoms bound to these molecules at the mean lethal concentration should be a useful parameter of the cell-killing efficiency of Pt atoms.

MATERIALS AND METHODS

^{195m}Pt-254-S preparation Platinum-195m was produced by neutron bombardment of 95%-enriched ^{194}Pt (10 mg; purchased from Oak Ridge National Laboratory, U.S.A.) in the hydraulic conveyor of the Kyoto University Reactor (KUR) at a thermal neutron flux of approximately 8.15×10^{13} n·cm^{-2}·s^{-1} for 75 h. After cooling for about 3 days to eliminate undesired radioactivity, due mainly to ^{191}Pt and ^{197}Pt, the synthesis of ^{195m}Pt-radiolabeled 254-S (specific activity: 7.2×10^6 Bq/mg-254-S, chemical and radionuclidic purity: higher than 98.4%) was carried out, according to a method described elsewhere.[8]

HeLa cells HeLa S-3 cells were maintained as monolayers in Petri dishes (90 mm in diameter) in MEM supplemented with 10% calf serum and 1 mM 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 fresh medium. One ml of cell suspension, containing 1.0×10^7 cells was incubated with ^{195m}Pt-254-S (2.5–10 μg/ml) for 1, 2 or 3 h at 37°C. After incubation, cell suspensions were taken from each flask for cell survival assays (10 μl) and for the measurement of fractional distribution of ^{195m}Pt (990 μl).

Cell survival assay After appropriate dilution, the cells

were subcultured in 6-cm Petri dishes, at a density of 500–5000 cells/plate, depending on the treatment. After the addition of fresh medium, the cells were incubated for colony formation under standard conditions for at least 10 days, and the colonies were washed with normal saline, stained with 1% crystal violet, and scored. 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 after isolation of these fractions, using the method of Schneider[13] with a minor modification. For the measurement of ^{195m}Pt-radioactivity in each fraction, an NaI(Tl)-scintillation counter (Aloka, Tokyo, RLC-551) was employed. The DNA, RNA, and protein contents of each fraction were determined by the diphenylamine, phloroglucinol, and biuret methods, respectively.

Other experimental procedures All other experimental procedures were the same as those described in our previous paper.[9] In brief, we obtained the D_0 values (from the cell survival assay) as the concentration of 254-S with which the cells were treated, and these values were converted to the number of Pt-atoms bound to each macromolecule (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), assuming the molecular weight of the constituent unit to be 350 for DNA and RNA, and 120 for proteins. The values of mean macromolecule content obtained from 24 samples each of DNA, RNA, and protein fractions were $1.90 \pm 0.04 \times 10^{-11}$, $2.72 \pm 0.06 \times 10^{-11}$, and $2.47 \pm 0.08 \times 10^{-9}$ g/cell, respectively. Finally, we regarded the reciprocal of the quotient as being a measure of the cell-killing efficiency of the Pt atom. Thus, the efficiency was expressed as the number of constituent units to which the binding of 1 Pt atom was needed, in order to kill the cell.

RESULTS

Dose-survival curves of HeLa cells Fig. 1 illustrates the dependence of cell survival on the drug concentration when the cells were treated with ^{195m}Pt-254-S for 0.5, 1, 2, and 3 h at 37°C and for 3 h at 0°C. The semilogarithmic survival plots are straight lines with a slight shoulder. From the linear part of the plots, the D_0 values (dose resulting in a decrease in survival to 37% of the initial value) for the cells treated under various conditions were calculated to be 67.1, 47.0, 26.8, and 8.05 μM at 37°C for 0.5-, 1-, 2-, and 3-h treatments, respectively, and 206.6 μM at 0°C for 3-h incubation. Comparing this result with that for CDDP,[9] we found that the D_0 for 254-S in every treatment was considerably higher than that obtained for

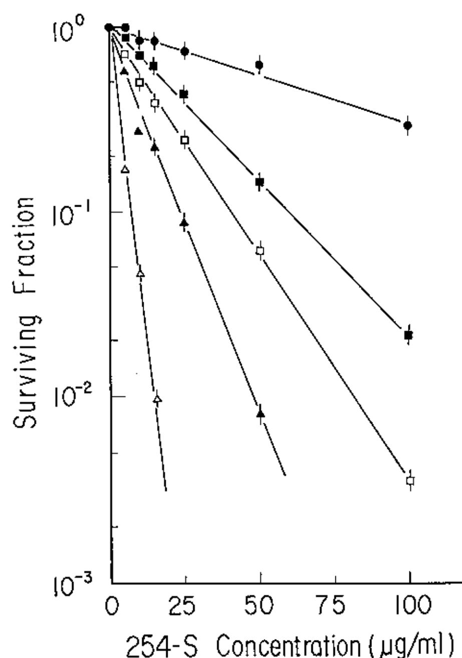

Fig. 1. Dose-survival curves of HeLa S-3 cells treated with ^{195m}Pt-254-S under various conditions. Values given are means ±SD of 3 separate experiments. Incubation conditions: ● 0°C-3 h, ■ 37°C-0.5 h, □ 37°C-1 h, ▲ 37°C-2 h and △ 37°C-3 h.

CDDP, except for treatment at 0°C. Namely, at 0°C for 3 h, the D_0 for 254-S was approximately identical (206.6 μM) with that for CDDP (221 μM), while the values for 37°C treatments were 5.1 (47.0/9.3), 4.0 (26.8/6.7), and 2.5 (8.1/3.2) times those for CDDP with 1-, 2-, and 3-h treatments, respectively. Relative to CDDP, the lethal effect of 254-S was the highest at 0°C for 3-h treatment.

^{195m}Pt-distribution among various fractions Fig. 2 shows the fractional distribution of ^{195m}Pt among the various fractions of HeLa cells treated with different concentrations of ^{195m}Pt-254-S under various conditions. An almost linear relationship can be seen between incorporation and drug concentration in all the fractions. Clear differences were observed between the incorporation of 254-S and CDDP,[9] in that the amounts of ^{195m}Pt incorporated into the DNA (Fig. 2a) and protein (Fig. 2c) fractions were considerably lower than was the case with CDDP, while no difference was observed in incorporation in the RNA fraction (Fig. 2b). At 37°C for 1-h incubation, incorporation of ^{195m}Pt into the DNA fraction was of the order of 0.03% of the total radioactivity (corresponding value for CDDP, 0.1%[9]), and incorporation into the RNA and protein fractions was about 0.10% and 0.23% (corresponding values for CDDP were 0.10% and 1.0%, respectively[9]). It can be seen from Fig. 2 that ^{195m}Pt

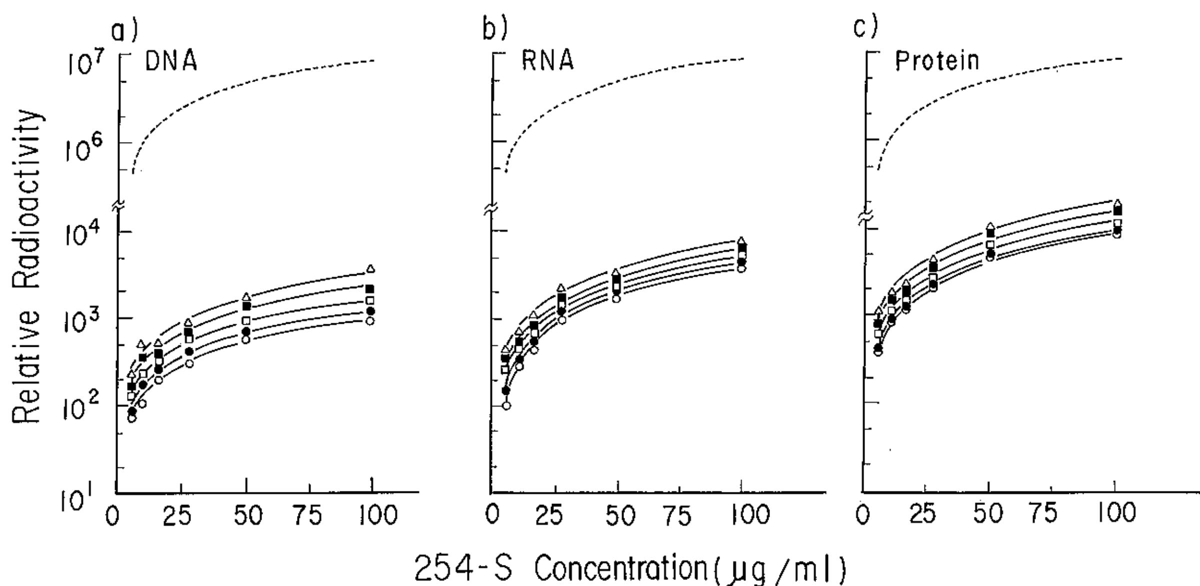

Fig. 2. ^{195m}Pt-distribution in DNA (a), RNA (b) and protein (c) fractions of HeLa cells treated with ^{195m}Pt-254-S under various incubation conditions. Dotted lines represent "medium level." Incubation conditions: ○ 0°C-3 h, ● 37°C-0.5 h, □ 37°C-1 h, ■ 37°C-2 h and △ 37 °C-3 h.

Table I. D_0, Number of Pt-atoms Bound to Three Kinds of Macromolecules (DNA, RNA, and Protein) and the Target Volumes of HeLa Cells Treated with ^{195m}Pt-254-S

	Treatment	0°C-3 h	37°C-0.5 h	37°C-1 h	37°C-2 h	37°C-3 h
	D_0(μg/ml)	61.67	20.21	14.03	8.01	2.40
	(μM)[a]	206.6±18.7	67.07±8.8	46.99±5.9	26.83±4.1	8.05±1.4
DNA	%-Pt binding	0.0232	0.0310	0.0432	0.0620	0.0640
	No. of Pt-cell (×10^5)	7.94	10.06	8.74	6.96	2.98
	Target volume (×10^4 nucleotides)	1.022	3.44	3.96	4.97	11.77
RNA	%-Pt binding	0.0508	0.0704	0.0968	0.128	0.145
	No. of Pt-cell (×10^5)	62.97	28.33	27.29	20.67	7.01
	Target volume (×10^4 nucleotides)	0.779	1.733	1.799	2.375	7.004
Protein	%-Pt binding	0.152	0.191	0.232	0.282	0.352
	No. of Pt-cell (×10^6)	18.87	7.68	6.54	4.54	1.700
	Target volume (×10^6 nucleotides)	0.652	1.60	1.88	2.71	7.24

a) Values are means±SD, while others are means of samples from 3 separate experiments.

incorporation into the DNA fraction increased with increasing 254-S concentration under all experimental conditions. It should be emphasized that the incorporation was extremely high with 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), while no such marked difference was observed between the two compounds in ^{195m}Pt-incorporation into the RNA and cold TCA-soluble fractions. From the figures, we easily obtained the %-Pt binding to each fraction at the mean lethal concentration. In the case of DNA, the values were 0.0250%, 0.0310%, 0.0432%, and 0.0620% for 0.5-, 1-, 2-, and 3-h treat-

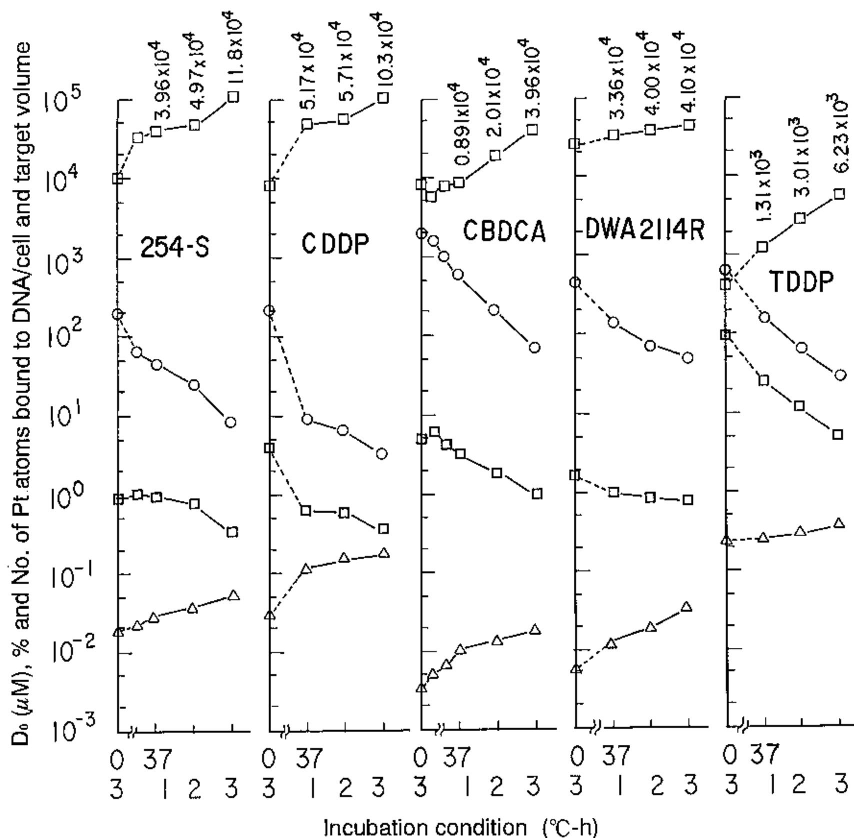

Fig. 3. Comparison of the D_0, the percentage and number of Pt-atoms combined to DNA molecules (/cell), and the target volume for several Pt-compounds. ○ D_0, △ %-Pt, ■ number of Pt-atoms and □ target volume. The values cited for other Pt-compounds are from ref. 9 (CDDP), ref. 10 (TDDP), ref. 11 (DWA2114R), and ref. 12 (carboplatin).

ments, respectively, at 37°C, and 0.0064% for the 3-h treatment at 0°C. The values for RNA were 0.0704%, 0.0968%, 0.1284%, 0.1452%, and 0.0508%, while the values for proteins were 0.191%, 0.232%, 0.282%, 0.352%, and 0.152%, 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 the findings shown in Figs. 1 (D_0) and 2 (Pt distribution), 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 reciprocals of the quotients, i.e., the efficiency of Pt atoms in killing the cells, were calculated. The efficiency of the 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 atoms bound to macromolecules is an immediate consequence of the fact that the increase of the %-Pt binding with incubation was more than compensated by the decrease in D_0.

DISCUSSION

In our previous study, we determined the target volumes for DNA, RNA, and protein molecules in CDDP-treated cells, assuming that the cells would be killed by inactivation of some type of constituent molecules through the binding of Pt atoms,[9] and that the Pt-compounds would react equally with different species of each of these categories of biologically important molecules.[14] 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. This was also the case for DWA-2114R[11] and carboplatin,[12] while in the case of *trans*-diaminedichloroplatinum(II) (TDDP)[10], $PtCl_4$, and $PtCl_6$,[15] not only DNA, but also high-molecular-weight RNA (except for t-RNA), under some limited conditions could also stoichiometrically be the target molecule for cell killing. The present results demonstrated that the target volumes for the DNA, RNA, and protein molecules of HeLa cells treated with 254-S for 1 h at 37°C were 3.96×10^4 and 1.80×10^4 nucleotides, and 1.88×10^6 amino acids, respectively. Of these values, only that of DNA is small enough to be contained within a

cellular DNA molecule, that is, only the DNA molecule can be stoichiometrically the target material. On the other hand, the target volumes calculated for RNA and protein molecules, i.e., 1.80×10^4 nucleotides and 1.88×10^6 amino acids, are too large to be included within usual RNA or protein molecules. In other words, large numbers of these molecules remain intact even after treatment with the D_0 concentration of 254-S. Thus, it is apparent that RNA and proteins are unlikely to be the target of 254-S in causing cell death. Assuming the molecular weight of DNA to be 10^7 (about 3×10^4 nucleotides),[16] the calculated target volume of DNA for 1-h treatment at 37°C, 3.96×10^4 nucleotides, implies that about 0.8 Pt-atoms per DNA molecule is needed to kill the cell. The corresponding numbers of Pt atoms per DNA molecule were about 0.6, 1.0, 3.7, and 25 in the cases of CDDP,[9] DWA2114R,[11] carboplatin,[12] and TDDP.[10] (Previously in refs. 9–12, we mistakely regarded the molecular weight of mammalian DNA as 3×10^8; these values have been recalculated on the basis of the correct value, namely 10^7). It can therefore be concluded that, in terms of the efficiency of the Pt atom in killing the cells (target volume), 254-S was only 1.3 times less toxic than CDDP and 1.2, 4.5, and 30.5 times more toxic than DWA2114R, carboplatin, and TDDP. It should be noted that, in terms of D_0, the cell killing effect of 254-S was less than that of CDDP by a factor of 5.1, while it was higher than those of DWA2114R, carboplatin, and TDDP by factors of 1.3, 11.8, and 3.5, respectively. The discrepancies between the target volumes and D_0 values can be attributed unequivocally to the difference in the %-Pt atoms combined. For example, with CDDP, 0.12% of Pt atoms were bound to DNA at 37°C on 1-h treatment, while in the present case, the corresponding value was only 0.031%. Namely, the target volume directly represents the cell-killing efficiency of DNA-binding Pt atoms, while the D_0 indicates the concentration of the Pt-compound in the medium required to kill the cells. It should also be noted that the relative efficiency of 254-S compared with that of CDDP varied depending on the treatment.

Fig. 3 shows the variations in the D_0, in the percentage and number of Pt atoms binding to the DNA molecule, and in the target volume under different incubation conditions for 254-S treatment (from Table I), and the corresponding values for CDDP,[9] DWA2114R,[11] carboplatin,[12] and TDDP.[10] The relative efficiency of 254-S compared with CDDP increased from 0.77 (3.96/5.17) to 0.87 (4.97/5.71) for 2-h incubation and to 1.14 (11.77/10.3) for 3-h incubation. It was also observed that the efficiency of 254-S was higher than that of CDDP at 0°C for 3-h treatment. It seems that prolonged incubation with 254-S increased the %-Pt atoms, and hence resulted in enhanced cell killing efficiency. This was proved by the finding that the %-Pt atoms increased from 0.031 at 37°C for 1 h to 0.062 at 37°C for 3 h (that is, 2 times) with 254-S treatment, while the corresponding increase was only 1.5 times with CDDP treatment. The cell killing efficiency of 254-S was considerably higher than that of the other Pt-compounds examined. For example, at 37°C with 1 h treatment, the values were 1.2, 4.4, and 30.2 times those for DWA2114R, carboplatin, and TDDP, while the values at 37°C with 3-h treatment were 2.9, 3.0, and 18.8 times those of DWA2114R, carboplatin, and TDDP, respectively. At 0°C for 3-h treatment, the efficiency of 254-S was lower than that of DWA2114R (0.53 times). The present results show the cell-killing efficiency of 254-S to be comparable to that of CDDP in cultured HeLa cells under all tested conditions. *In vivo* confirmation of these *in vitro* results is required.

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