Cell-killing Efficiency and Number of Platinum Atoms Binding to DNA, RNA and Protein Molecules of HeLa Cells Treated with Combinations of *trans*-Diaminedichloroplatinum(II) and Hyperthermia

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The effect of hyperthermia on the cell-killing efficiency of Pt atoms binding to DNA, RNA and protein molecules was examined. HeLa S-3 cells were treated with ^{195m}Pt -radiolabeled *trans*-diaminedichloroplatinum(II) (TDDP) for 60 min at various temperatures, and the relationship between the lethal effect and the number of Pt atoms binding to DNA, RNA and proteins was examined. The mean lethal concentration (D_0) of TDDP for a 60-min treatment at 0, 25, 37, 40, 42 and 44°C was 1714, 1016, 302, 179, 125 and 42.5 μM , respectively. (D_0 is defined as the dose that would give an average of one lethal event per member of the population; for further details, see Fig. 1). By using identically treated cells, the numbers of Pt-atoms combined with DNA, RNA and protein molecules were determined in the subcellular fractions. Thus, the D_0 's given as the drug concentrations were replaced with the number of Pt-atoms combined in each fraction. Then, the cell-killing efficiency of the Pt atom was expressed as the reciprocal of the number of Pt-atoms combined and was calculated for each molecule. The efficiency for DNA was 0.206, 0.273, 0.779, 1.28, 1.77 and 5.14×10^3 nucleotides, respectively, for the conditions described above. It seemed that hyperthermia potentially interacted not only with bifunctional, but also with monofunctional bonds. Thus, it was concluded that TDDP was markedly less cytotoxic than *cis*-diaminedichloroplatinum (CDDP) at 37°C, but was more cytotoxic than CDDP at 44°C.

Key words: *trans*-Diaminedichloroplatinum(II) — ^{195m}Pt -TDDP — HeLa cell — Hyperthermia — Cell-killing efficiency

The cytotoxic effects of many chemotherapeutic agents, such as bleomycin,¹⁾ adriamycin²⁾ and mitomycin C³⁾ have been shown to increase at elevated temperatures. Though the cytotoxicity of *cis*-diaminedichloroplatinum(II) (CDDP) is also strongly potentiated by hyperthermia,⁴⁻⁶⁾ there is little information on the hyperthermic interaction of other Pt-compounds, especially *trans*-diaminedichloroplatinum (TDDP). Several studies have suggested that the molecular lesion responsible for cell killing by CDDP is the interstrand cross-linking of DNA.^{7,8)} However, Pinto and Lippard⁹⁾ provided evidence for the involvement of the intrastrand cross-link on pGpG as an inhibitor of DNA replication.

Two types of Pt-DNA binding have been found, namely, monofunctional and bifunctional. The monofunctional binding is unlikely to be responsible for the cytotoxic effects, since the *trans*-compounds are at least as effective as the *cis*-compounds in forming monofunctional Pt-DNA binding.¹⁰⁾ Recently, using synthesized ^{195m}Pt -radiolabeled CDDP,¹¹⁾ we demonstrated that the supra-additive effect of the combination treatment with hyperthermia could be explained by two mechanisms: increased drug level in DNA (from 37°C to 42°C) and increased cell-killing efficiency of the Pt atoms (>42°C).¹²⁾ Therefore, we investigated whether or not the monofunctional cross-linking of DNA by TDDP is enhanced

by hyperthermia. In addition, the ability of hyperthermia to increase the number of Pt atoms binding to biologically important molecules and the cell-killing efficiency was examined.

MATERIALS AND METHODS

^{195m}Pt -TDDP preparation Platinum-195m was produced by neutron bombardment of 95%-enriched ^{194}Pt (10 mg, purchased from Oak Ridge National Laboratory, Oak Ridge, TN) in the hydraulic conveyor of the KUR (Kyoto University Reactor, Osaka) at a thermal neutron flux of approximately $8.15 \times 10^{13} \text{ n} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$ for 75 h. After a cooling period of about 3 days to eliminate undesired radioactivity, due mainly to ^{191}Pt and ^{197}Pt , ^{195m}Pt -radiolabeled TDDP was synthesized according to the method described elsewhere¹³⁾ (specific activity, $7.4 \times 10^6 \text{ Bq/mg-TDDP}$; chemical and radionuclidic purity, higher than 99%).

Hyperthermia treatment of 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. For hyperthermia, tightly capped plastic flasks

(7 mm in inner diameter; 40 mm in length) with 1 ml of cell suspension containing 1.0×10^7 cells and ^{195}mPt -TDDP (2.5–100 $\mu\text{g/ml}$, viz., 8.4–335 μM) were horizontally submerged in a water bath (Advantec ET-35D) for 60 min at 0, 25, 37, 40, 42 or 44°C. Water temperatures were maintained at $\pm 0.1^\circ\text{C}$ (SD). Thereafter, 10 and 990 μl of the cell suspensions were taken from each flask for the cell survival assay and the measurement of fractional distribution of ^{195}mPt , respectively. For the 0°C treatment, the temperature of the water bath was maintained electrically between 0°C and 1.0°C .

Cell survival assay After appropriate dilution, the cells were subcultured in 6-cm Petri dishes at a density of 1000–50000 cells/plate, depending on the treatment. After 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.

Macromolecule contents and fractional distribution of ^{195}mPt The distribution of ^{195}mPt -radioactivity in the DNA, RNA and protein fractions of treated cells was measured after those fractions were isolated using the method of Schneider¹³⁾ with a minor modification. For the measurement of ^{195}mPt -radioactivity in each fraction, an NaI(Tl)-scintillation counter (Aloka, RLC-551) was employed. On the other hand, DNA, RNA and protein contents of each fraction were determined using the diphenylamine, phloroglucinol and biuret methods, respectively.

Other experimental procedures All other experimental procedures were the same as those described in our previous paper.¹⁴⁾ In brief, we obtained the D_0 values (from the cell-survival assay) as the concentration of TDDP with which the cells were treated, and these values were replaced by the number of Pt-atoms bound to each class of macromolecule (determined by the measurement of the fractional distribution of ^{195}mPt). We divided the number of Pt-atoms by Avogadro's number. Then, the number of Pt-atoms obtained was divided by the number of constituent units of each macromolecule/cell, assuming the molecular weight of the constituent unit to be 350 for DNA and RNA, and 120 for proteins. Finally, we took the reciprocal of the quotient as being the 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 is needed to kill the cell.

RESULTS

Dose-survival curves of HeLa cells Fig. 1 illustrates the dependence of cell survival on the drug concentration

Fig. 1. Dose-survival curves of HeLa S-3 cells treated with ^{195}mPt -TDDP for 60 min at various temperatures. Values given are the means $\pm$ SD of samples from 3 separate experiments. Incubation temperatures: $\bullet$ 0°C , $\circ$ 25°C , $\blacksquare$ 37°C , $\square$ 40°C , $\blacktriangle$ 42°C and $\triangle$ 44°C . When a dose-survival curve is exponential, like that at 44°C , the dose that gives 37% survival is called D_0 (here 42.5 μM).

when the cells were treated with ^{195}mPt -TDDP for 60 min at 0, 25, 37, 40, 42 and 44°C . All the semilogarithmic dose-survival curves are linear. D_0 's determined from the slopes of the curves were 1714, 1016, 302, 179, 125 and 42.5 μM for 0, 25, 37, 40, 42 and 44°C , respectively. The cell-killing effect of TDDP increased with increasing temperature. It should be noted that we found approximately 1.7, 2.4 and 7.2-fold enhancement in cell killing by TDDP at 40, 42 and 44°C compared to the cell killing at 37°C .

^{195}mPt -distribution among various fractions Fig. 2 shows the ^{195}mPt -distribution in the DNA (Fig. 2a), RNA (b) and protein (c) fractions of the cells treated at different temperatures. The binding of Pt atoms to DNA molecules increased with increasing temperature. Namely, for every TDDP dose, about a 1.5-fold increase in binding was observed at 44°C compared with 37°C treatment. It should be emphasized that only a 1.5-fold increase in the

Fig. 2. ^{195m}Pt -distributions in DNA (a), RNA (b) and protein (c) fractions of HeLa S-3 cells treated with TDDP for 60 min at various temperatures. ● 0°C, ○ 25°C, ■ 37°C, □ 40°C, ▲ 42°C and △ 44°C. The dotted line represents the medium level.

binding of the Pt atoms resulted in a 7.2-fold enhancement in the cell killing (D_0). The percentage of Pt atoms (%-Pt) in this case were considerably higher than in the case of CDDP⁶⁾ (especially in the DNA and RNA fractions). From Fig. 2, we can obtain the %-Pt atoms bound to each fraction at the mean lethal concentration. In the case of DNA, they were 0.163, 0.208, 0.245, 0.252, 0.260 and 0.264% at 0, 25, 37, 40, 42 and 44°C, respectively. The differences between 37°C and 44°C are very small in each fraction. For example, it was only 0.019% (0.264–0.245%) in this case, while the corresponding value was 0.037% (0.082–0.045) in the case of CDDP.⁶⁾ Those for RNA were 0.250, 0.283, 0.348, 0.398, 0.406 and 0.471%, while those for proteins were 0.779, 0.900, 0.986, 1.001, 1.204 and 1.402%, respectively. In general, the %-Pt atoms bound to each macromolecule increased with increasing temperature but did not significantly vary with the drug concentration.

Determination of DNA, RNA and protein contents After measurement of the radioactivity, the DNA, RNA and protein contents were determined as described above. The contents of the macromolecules were not decreased by the treatment. The mean values obtained from 30 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. From these values, the numbers of constituent units were calculated to be 3.46×10^{10} , 4.91

$\times 10^{10}$ and 1.23×10^{13} for DNA, RNA and proteins, respectively.

D_0 , %-Pt, number of Pt-atoms bound to the macromolecules and efficiency of cell killing by Pt atoms The number of Pt-atoms binding to DNA, RNA and proteins at the mean lethal concentration under various incubation conditions was calculated using the values taken from Figs. 1 (D_0) and 2 (%-Pt), and 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, the efficiency of cell killing by Pt atoms, were calculated and are also listed in Table I. It was found that the number of Pt-atoms required for an equivalent level of cell killing (D_0) was reduced with increasing temperature. However, the magnitude of the decrease was somewhat smaller than that for the D_0 because the %-Pt atoms bound to those molecules also increased with increasing temperature. The ratio of the maximum to minimum value was about 25 in the case of the number of Pt-atoms (16.76/0.673), while that for D_0 was about 40 (1714/42.5). The corresponding values in the case of CDDP were about 6 (13.98/2.31; the number of Pt-atoms) and 50 (233/4.7; D_0), respectively.⁶⁾ The large difference between TDDP and CDDP is attributable to the fact that the %-Pt atoms bound to DNA increased only 0.101% (0.264–0.163% or about 1.6-fold) from 0°C to 44°C (the corresponding

Table I. D_0 , %-Pt and Number of Pt-atoms Binding to Three Classes of Macromolecules, and the Cell-killing Efficiency of Pt Atoms in HeLa Cells Treated with ^{195m}Pt -TDDP for 60 min

Treatment ($^{\circ}\text{C}$) D_0 (μM) ^{a)}	0	25	37	40	42	44
	1714 $\pm$ 255	1016 $\pm$ 123	302 $\pm$ 53.6	179 $\pm$ 51.2	125 $\pm$ 34.1	42.5 $\pm$ 10.3
DNA						
%-Pt binding ^{a)}	0.163 $\pm$ 0.033	0.208 $\pm$ 0.042	0.245 $\pm$ 0.044	0.252 $\pm$ 0.055	0.260 $\pm$ 0.053	0.264 $\pm$ 0.057
No. of Pt ($\times 10^7$) ^{b)}	16.76	12.68	4.44	2.71	1.95	0.673
Efficiency of Pt ($\times 10^3$ units)	0.206	0.273	0.779	1.28	1.77	5.14
RNA						
%-Pt binding ^{a)}	0.250 $\pm$ 0.025	0.283 $\pm$ 0.027	0.348 $\pm$ 0.055	0.398 $\pm$ 0.061	0.406 $\pm$ 0.087	0.471 $\pm$ 0.090
No. of Pt ($\times 10^7$) ^{b)}	25.71	17.25	6.31	4.28	3.05	1.20
Efficiency of Pt ($\times 10^3$ units)	0.191	0.285	0.778	1.15	1.61	4.09
Protein						
%-Pt binding ^{a)}	0.779 $\pm$ 0.081	0.900 $\pm$ 0.103	0.986 $\pm$ 0.124	1.001 $\pm$ 0.156	1.204 $\pm$ 0.178	1.402 $\pm$ 0.231
No. of Pt ($\times 10^7$) ^{b)}	80.11	54.87	17.88	10.76	9.05	3.58
Efficiency of Pt ($\times 10^4$ units)	1.54	2.24	6.88	11.43	13.59	34.35

a) Mean $\pm$ SE of 3 assays.

b) Number of Pt-atoms/cell at the mean lethal concentration.

value in the case of CDDP was $0.082 - 0.010 = 0.072\%$, namely, 8.2-fold). TDDP seems to penetrate into the cells and bind to biologically important molecules more easily, even at lower temperatures.

DISCUSSION

Many studies have demonstrated the enhancement of cytotoxicity *in vitro* of CDDP at elevated temperatures.^{4,15} However, few studies have demonstrated such enhanced cytotoxicity of other Pt-compounds, especially TDDP. The present study revealed that the slope of the survival curve became steeper with increasing temperature (Fig. 1), that is, the effect of TDDP in combination with hyperthermia on the survival of HeLa S-3 cells was supra-additive. Previously, we determined the efficiency of cell killing by Pt atoms for DNA, RNA and protein molecules from both the D_0 -value and the %-Pt atoms bound to each class of macromolecule according to the "single hit" model.¹⁴ The study indicated that among the three classes, only DNA could be the target molecule for cell killing by CDDP because its molecular weight was high enough to include the calculated efficiency (about 10^5 nucleotides). However, the situation was somewhat different in the case of TDDP. The calculated efficiency for RNA (37 $^{\circ}\text{C}$ treatment) amounted to $4.13 - 25.3 \times 10^2$ nucleotides.¹⁶ This value roughly corresponds to the molecular weight of most RNAs, except t-RNA. Thus, we concluded that although DNA was the most likely target of TDDP, high-molecular-weight RNAs might also be a target from the viewpoint of stoichiometry.

The present experiment demonstrates that the efficiencies (target volumes) for the three kinds of molecules increased with increasing temperature, namely from 0.779×10^3 (37 $^{\circ}\text{C}$) nucleotides to 5.14×10^3 (44 $^{\circ}\text{C}$) nucleotides for DNA, from 0.778×10^3 to 4.09×10^3 nucleotides for RNA and from 6.88×10^4 to 34.35×10^4 amino acids for protein. However, the increase is insufficient to allow the conclusion that the binding of Pt atoms to RNA or protein molecules is the main cause of hyperthermia-TDDP-induced cell death. Namely, one Pt atom per 34.4×10^4 amino acids corresponds to one Pt atom per $3.44 \times 10^2 - 3.44 \times 10^3$ protein molecules, assuming proteins to have a usual molecular weight of the order of $10^4 - 10^5$, corresponding to 100–1000 amino acids. It is very unlikely that the cell would be killed by the inactivation of only one out of $3.44 \times 10^2 - 3.44 \times 10^3$ protein molecules, leaving almost all the proteins intact. This is also the case for RNA, more or less, since its molecular weight is much smaller than that of DNA. The maximum efficiency obtained for RNA in this experiment was 4.09×10^3 nucleotides at 44 $^{\circ}\text{C}$ for a 60-min treatment. Assuming the molecular weight of large RNA to be 10^6 ,¹⁷ the value indicates that 0.70/RNA molecule of Pt-binding is enough to kill the treated cells.

Setting aside the question of the target molecule, the variations in the number of Pt atoms binding to DNA and in the cell-killing efficiency of Pt atoms with temperature, together with those of D_0 and the %-Pt bound to each molecule, are depicted in Fig. 3. From 0 $^{\circ}\text{C}$ to 44 $^{\circ}\text{C}$, the %-Pt atoms bound to DNA increased gradually, while D_0 fell rapidly with a few inflection points. The

Fig. 3. The variations in D_0 , %Pt, number of Pt-atoms binding to DNA, and cell-killing efficiency of Pt atoms with incubation temperature. ● D_0 , ■ %Pt, ○ number of Pt/cell and □ efficiency.

rapid decrease in D_0 corresponded to the rapid decrease in the number of Pt-atoms binding to DNA. This was the reason why the cell-killing efficiency of Pt atoms increased rapidly, especially from 37°C to 42°C, and more rapidly over 42°C. Thus, these findings considerably differ from those in the case of CDDP, for which the supra-additive effect of the combination treatment was explained by two mechanisms: the increased drug level in DNA (from 37°C to 42°C) and the increased cell-killing efficiency of the Pt atoms (>42°C).⁶⁾ The cell-killing efficiency of TDDP increased from 0.206×10^3 (0°C) to 5.14×10^3 nucleotides (44°C) (30-fold) and from $0.779 \times$

10^3 (37°C) to 5.14×10^3 nucleotides (44°C) (6.6-fold), whereas the corresponding values in the case of CDDP were 6.1-fold (15.00/2.47) from 0°C to 44°C and only 1.6-fold (15.00/9.49) from 37°C to 44°C. The cell-killing efficiency of TDDP was lower than that of CDDP at 37°C by a factor of 121.8, but the ratio decreased to 29.2 at 44°C. It seems that hyperthermia potentially interacts not only with bifunctional, but also with mono-functional Pt bonds. Thus, it can be concluded that TDDP was much less toxic than CDDP at 37°C, but showed a greater enhancement of cytotoxicity at 44°C than did CDDP. Similar observations were described for [Pt(Rh-123)₂], platinum-rhodamine 123, by Herman *et al.*¹⁸⁾ They proposed the development of specific "hyperthermia-activated" drugs. In this regard, TDDP is considered to be a potentially useful "hyperthermia-activated" drug. The reasons for this are as follows:

- 1) TDDP has much weaker side effects than CDDP. It may be possible, therefore, to give significantly higher doses to patients.
- 2) The %Pt atoms bound to DNA is considerably higher in the case of TDDP than in CDDP treatment, for instance, 5.4-fold (0.245/0.045) and 3.2-fold (0.264/0.082) higher at 37°C and 44°C, respectively. The use of not CDDP, but TDDP in a recent study¹⁹⁾ to examine the radiotoxic effects of ^{195m}Pt incorporated into cultured mammalian cells (chemo-Auger combination therapy) is therefore reasonable.
- 3) The rate of release of Pt atoms from both the DNA and RNA fractions was considerably higher in the case of TDDP than CDDP.²⁰⁾ Thus, after effects on patients should be less with TDDP. This observation is consistent with that of Plooy *et al.*,²¹⁾ who indicated that TDDP-induced cross-links were repaired faster than CDDP-induced cross-links in CHO cells.

Further *in vitro* and *in vivo* studies to confirm the effectiveness and usefulness of the interaction between TDDP and hyperthermia are needed.

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