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The Number of Platinum Atoms Binding to DNA, RNA and Protein Molecules of HeLa Cells Treated with Cisplatin at Its Mean Lethal Concentration

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HeLa S-3 cells were treated with ^{195m}Pt-radiolabeled *cis*-diamminedichloroplatinum(II) (CDDP) under various conditions, and the relationship between lethal effect and the number of Pt atoms binding to DNA, RNA and proteins was examined. The mean lethal concentrations for the cells treated with CDDP at 37°C for 1, 2 and 3 h were 2.8, 2.0 and 1.1 μg/ml, respectively. By using identically treated cells, the number of Pt atoms combined with DNA, RNA and protein molecules were determined after fractionation of the cells using the method of Schneider. In this way, the D_0 values given as the drug concentration were substituted for the number of Pt atoms combined with each fraction, then the target volumes expressed as the reciprocals of D_0 values were calculated for each fraction. The results provide strong support for the idea that DNA is the primary target for cell killing by CDDP, and the target volumes were 5.17×10^4, 5.71×10^4 and 1.03×10^5 nucleotides for 1, 2 and 3 h treated cells, respectively.

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

Although *cis*-diamminedichloroplatinum(II) (CDDP) is the most effective antitumor drug currently available for many cancers,[1-4] the mechanisms underlying cell killing by CDDP are not yet fully understood. The antitumor activity of CDDP appears to be mediated through modification of DNA bases by forming multiple types of interstrand cross-linking, intrastrand adducts, DNA monoadducts and DNA-protein cross-links.[5-7] For a further understanding of the mechanisms, it is necessary to obtain more quantitative information concerning the number of platinum atoms binding to those biologically important molecules.

To assess the importance of DNA, RNA and proteins as primary targets for CDDP, we synthesized ^{195m}Pt-radiolabeled CDDP to measure the distribution of the drug in biological matrices of the treated cells. The present paper is concerned with determination of the target volume of HeLa S-3 cells for CDDP treatment.

MATERIALS AND METHODS

^{195m}Pt-CDDP ^{195m}Pt was produced by irradiating 95%-enriched ^{194}Pt (10 mg, purchased from Oak Ridge National Laboratory, USA) in the hydraulic conveyer of the KUR at a thermal neutron flux of approximately 8.15×10^{13} n·cm^{-2}·s^{-1} for 75 h. ^{195m}Pt-CDDP was synthesized using the ^{195m}Pt according to the usual method, then was separated and purified by HPLC (column: Shodex 2004). The chemical purity was greater than 99.7%, the radiochemical purity was nearly 100%, and the specific activity was 7.4×10^6 Bq/mg CDDP. For detailed information, see Ref. 8.

HeLa cells HeLa S-3 cells were cultured in monolayer (150 cm^2 flask) in MEM medium 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 in an incubator. Exponentially growing cells were trypsinized, collected and resuspended in MEM medium. One ml of cell suspension containing 1×10^7 cells and ^{195m}Pt-CDDP (0.5–25 μg/ml) was incubated at 37°C for 1, 2 and 3 h. Thereafter, the cell suspension was divided into smaller (10 μl) and larger (990 μl) portions for the cell survival assay and the measurement of fractional distribution of ^{195m}Pt, respectively.

Cell survival After appropriate dilution, the cells were subcultured in 6-cm petri dishes at a density of 1000 cells/plate. After 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 ability was determined. All survival experiments were performed in triplicate for each concentration.

Fractional distribution of ^{195m}Pt The distribution of ^{195m}Pt-radioactivity in DNA, RNA and protein fractions of treated cells was measured by using the fractionation method of Schneider[9] with a minor modification. In brief, the cells were washed with cold fresh medium ("medium") and precipitated with 1 ml of cold 10% trichloroacetic acid (TCA). After two washes and precipitation, the supernatant was collected as the "cold

TCA-soluble fraction." The RNA and DNA fractions were extracted from the precipitated material by alkaline (0.1 *N* NaOH, 80°C) and acid treatments (5% TCA, 90°C), respectively. The residue was dissolved by heating in 0.5 *N* NaOH as a "protein fraction." The radioactivity of ^{195m}Pt in each fraction was measured with an NaI(Tl)-scintillation counter (Aloka, RLC-551). DNA, RNA and protein contents of each fraction were determined by means of the diphenylamine, phloroglucinol and biuret methods, respectively.

RESULTS

Dose-survival curves of HeLa cells Figure 1 shows the dose-survival curves of HeLa cells treated with ^{195m}Pt-CDDP 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 with a small shoulder. From the slopes of the lines, the D_0 values (D_0 is defined as the dose which would given an average of one lethal event per member of the population) for the cells treated under various conditions were 2.8, 2.0 and 1.1 μg/ml at 37°C for 1, 2 and 3 h incubation respectively, and 66 μg/ml for 0°C with a 3 h incubation. Roughly speaking, D_0 is inversely proportional to the incubation time.

^{195m}Pt-distribution among various fractions Figure 2 shows the fractional distribution of ^{195m}Pt among the various fractions of HeLa cells treated with different concentrations of ^{195m}Pt-CDDP for 1 h at 37°C. An almost linear relationship can be seen between the incorporation into all the fractions and the concentration of the drug. The amount of ^{195m}Pt incorporated into the DNA fraction was always somewhat lower than that into the RNA fraction. The incorporation of ^{195m}Pt into the cold-TCA soluble and protein fractions was about 10-fold higher than that into the DNA and RNA fractions. The figure demonstrates that the incorporation into the DNA and RNA fractions was of the order of 0.1%, and that into the cold-TCA soluble and protein fractions was about 1% of the total radioactivity. Figure 3-a shows the ^{195m}Pt-distribution in the DNA fractions from differently treated cells. The incorporation increases with increasing incubation time, although it is not exactly proportional. This phenomenon is an important characteristic found only in the DNA fraction (see other fractions in Fig. 3-b (RNA) and -c (protein)), and in CDDP treatment (other data not shown). Also, the incorporation into the RNA fraction at 0°C is much higher than that in the other two fractions (Fig. 3-b).

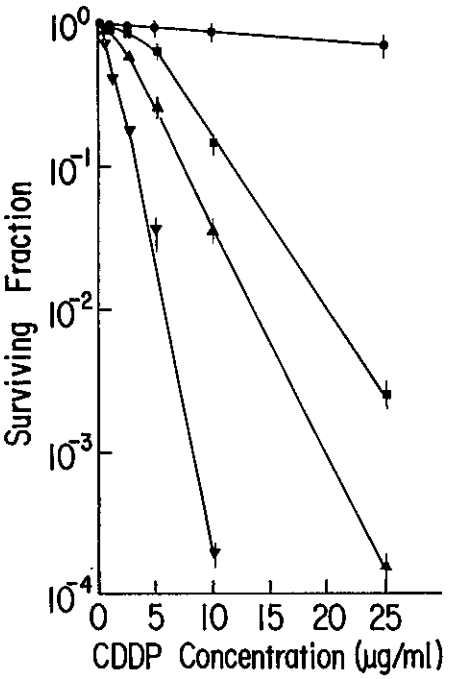

Fig. 1. Dose-survival curves of HeLa S-3 cells treated with ^{195m}Pt-CDDP under various incubation conditions. Values given are mean±SD of samples from 3 separate experiments. Incubation conditions: ● 0°C-3 h, ■ 37°C-1 h, ▲ 37°C-2 h and ▼ 37°C-3 h.

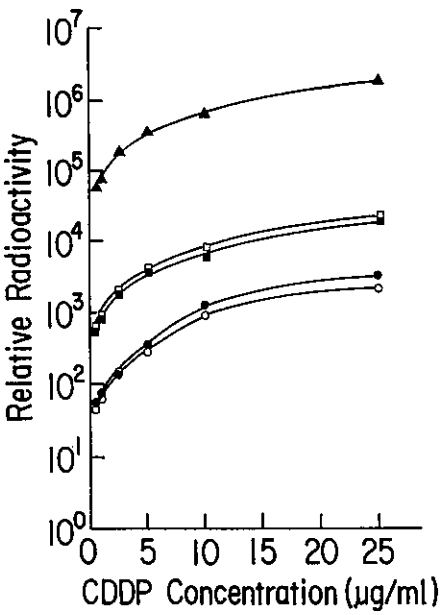

Fig. 2. ^{195m}Pt distribution among various fractions of HeLa S-3 cells treated with CDDP at 37°C for 1 h. ▲ medium, □ cold TCA soluble, ■ protein, ● RNA and ○ DNA.

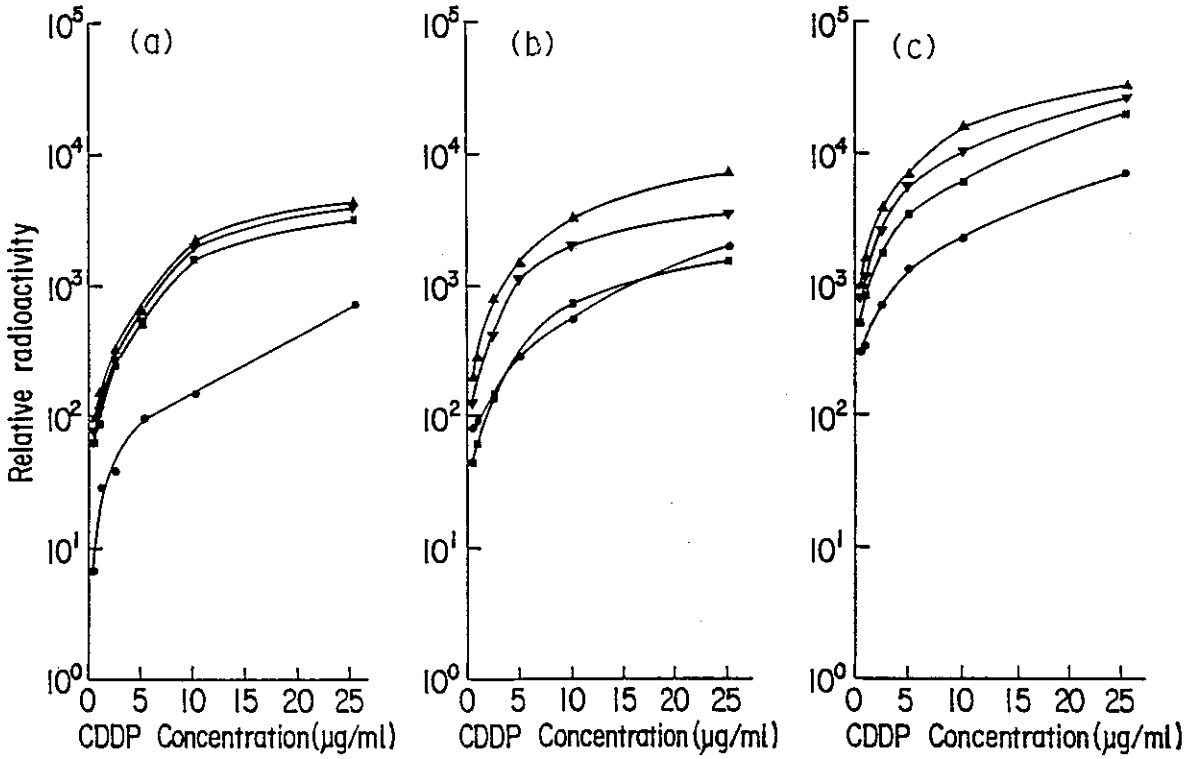

Fig. 3. ^{195m}Pt-distribution in DNA (a), RNA (b) and protein (c) fractions of HeLa S-3 cells treated with CDDP under the different incubation conditions. ● 0°C-3 h, ■ 37°C-1 h, ▼ 37°C-2 h and ▲ 37°C-3 h.

Determination of DNA, RNA and protein contents After the radioactivity measurement, the DNA, RNA and protein contents of cells were determined as described above. There was no tendency for the contents of the macromolecules to decrease with the treatments so far examined. The mean values obtained from the 24 samples each of DNA, RNA and protein fractions were $1.90\pm0.04\times10^{-11}$, $2.72\pm0.06\times10^{-11}$ and $2.47\pm0.08\times10^{-9}$ g/cell, respectively.

DISCUSSION

The D_0 value we obtained for HeLa cells treated with ^{195m}Pt-CDDP (from 2.8 to 1.1 μg/ml) at different incubation times is in close agreement with those reported elsewhere.[10-12] The D_0 has a tendency to decrease with increasingly severe conditions, such as longer incubation times. The fact that all the dose-survival curves decayed exponentially provided the basis upon which to analyze the target volume for CDDP binding to biologically important macromolecules of treated cells. This was done according to the "single hit" model[13] as in the case of radiation-induced cell mortality.

According to the model, the surviving fraction N/N_0 (here, N is defined as the number of cells remaining after the treatment, and N_0, that originally present) after dose D will be given by the equation

$$N/N_0=e^{-D/D0} \text{ or } e^{-\alpha D}$$
$$\ln N/N_0=-D/D_0 \text{ or } -\alpha D \text{ (where } \alpha=1/D_0).$$

In this work, we can obtain the D_0 values as the concentration of CDDP with which the cells were treated, and they can be easily replaced by the numbers of Pt atoms binding to cellular macromolecules by reading the radioactivity at various D_0 concentrations. Actually, the number of Pt atoms binding to the macromolecules was obtained as the product of D_0 (concentration in mole) and the % binding of Pt to the macromolecules. In the case of DNA, the latter values were estimated to be 0.12, 0.15 and 0.18% of total radioactivity (see Fig. 2 and Fig. 3-a). Table I shows the procedures to deduce the target volumes (α) using, as an example, the DNA fractions in various treatments. From the DNA content per cell, which we obtained separately, the reciprocals of the D_0 values, namely the inactivation constants α (here we regard it as target volume), can be calculated for each

Table I. D_0, Number of Pt Atoms Binding to DNA and Target Volume for HeLa S-3 Cells Treated with CDDP under Different Conditions

Treatment	0°C-3 h	37°C-1 h	37°C-2 h	37°C-3 h
D_0 (μg/ml)	66	2.8	2.0	1.1
(μM) (A)	221	9.3	6.7	3.2
% Pt binding to DNA (B)	0.03	0.12	0.15	0.18
No. of Pt atoms binding/cell (C) ($\times 10^5$)	39.8	6.69	6.06	3.45
Target volume (D) ($\times 10^4$ nucleotides)	0.87	5.17	5.71	10.3

(A)$\times$(B)$\times$Avogadro No.$=$(C), and the target volume (D) can be obtained by dividing the number of constituent units (in the case of DNA, 1.9×10^{-11} g/cell, corresponding to 3.46×10^{10} nucleotides/cell) by (C).

Table II. Calculated Target Volumes of Each Macromolecule of HeLa Cells for CDDP Treatments (No. of Units)

Treatment	0°C-3 h	37°C-1 h	37°C-2 h	37°C-3 h
DNA	8.5×10^3	6.7×10^4	8.7×10^4	1.3×10^5
RNA	3.4×10^3	9.9×10^4	6.4×10^4	7.0×10^4
Protein	2.1×10^5	3.0×10^6	2.6×10^6	3.7×10^6

treatment, and expressed as the number of nucleotides. The target volumes calculated for the three kinds of macromolecules under the supposition that they are the targets for CDDP-induced cell killing are listed in Table II. In the calculation, we assumed the molecular weight of the constituent units to be 350 for DNA and RNA, and 120 for proteins. From the table, it can be seen that the target volume of proteins is 3.0×10^6 amino acids for 37°C-1 h treatment. Proteins usually have molecular weights of the order of 10^4–10^5, corresponding to 100–1000 amino acids. This means that about 1 out of 3×10^4–3×10^5 protein molecules combines 1 Pt atom at the mean lethal concentration. It seems unreasonable to assume that the cell would be killed by the inactivation of only one out of 3×10^3–3×10^4 protein molecules, when almost all the proteins still remain intact. This is also the case for RNA, since the target volume is 9.9×10^4 nucleotides, if RNA is supposed to be the target for cell death due to CDDP. The value is too large to allow one hit on all RNA molecules in the cells at the mean lethal concentration (it is reasonable to assume that most RNA molecules consist of 10^2–10^4 nucleotides, and hence the ratio, number of constituent units/target volume will be between 1/1000 and 1/10). Thus it is apparent that RNA and proteins are unlikely to be the target of CDDP to cause cell death. On the other hand, 5.17×10^4 nucleotides, the target volume for DNA, correspond to more than 9 Pt atoms binding per one molecules at the mean lethal concentration if the molecular weight of DNA is assumed to be 3×10^8, and this would be consistent with the hypothesis that DNA is the target for cell death.

Thus the present results provide strong support for the suggestion that DNA is the target molecule for cell killing by CDDP.[14-16] A question arises as to why the target volume changes according to the treatment, namely the longer the incubation time, the larger the target volume. This is an important problem to be solved in the near future.

A few papers have appeared on the direct assay of Pt binding to DNA molecules in CDDP-treated mammalian cells. Pascoe and Roberts[17] first demonstrated that the number of Pt atoms bound to DNA when the surviving fraction of HeLa cells was reduced to 37%, was 1 per 1.3×10^5 nucleotides. The target volume that we determined seems somewhat smaller than that they proposed (under the same incubation conditions, 2 h at 37°C, our value is 8.7×10^4 nucleotides). Unfortunately, as no detailed information about the drug and cell concentrations used for the measurement is given in their report, we can not, for example, compare the target volumes and the detection limits between the two results. Another study has been reported on V79 cells by Roberts (one of the previous authors) and Friedlos.[18] In both studies, Pt was measured by atomic absorption. They reported that the measurement of DNA-binding Pt atoms needed 2×10^8 V79 cells which were treated with 10 μM CDDP at 37°C for over 2 h, and that the detection limit was around 10 ng. It should be emphasized that our measurements were performed using 1×10^7 cells treated with less than 1 μM CDDP at 37°C for 1 h. Another attempt directly to assay Pt-content in animal tissues was made using X-ray fluorescence spectrometry by Stewart *et al.*[19] They required a CDDP concentration of 7 μg/ml for sensitivity testing *in vitro*. Our detection limit was theoretically 1 ng, if the radioactivity that is approximately equal to the background activity is set at the lowest. However, in fact, we could obtain a reliable Pt value of less than 0.2 ng by prolonging the measurement time. Recently, though it would not be a direct Pt assay, a greatly improved method using a monoclonal antibody was described by Tilby *et al.*[20] They detected 3 nmol Pt/g-DNA (i.e., 1 Pt adduct/10^6 bases). Direct comparison of the sensitivity is somewhat difficult, but it can be supposed that the detection limit of the two assays is of the same order. Thus it can be stated that the use of radioactive Pt is the most sensitive direct assay of Pt, except for the immunoassay method.

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