Inhibition of Deoxyribonucleic Acid Synthesis *in Vitro* by Anticancer Platinum Pyrimidine Greens against Daudi Cells

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Platinum pyrimidine greens inhibited the deoxyribonucleic acid (DNA) synthesis of tumor cells in the S phase of the cell cycle and exerted antitumor activity. Clear differences were observed in the activity between the samples prepared at 40 °C and at 75 °C. Using ^{3}H-thymidine incorporation assay and cell cycle analysis we confirmed that the former had much stronger and more specific inhibitory activity against DNA synthesis than the latter. Reactivity of the 40 °C sample with deoxyguanosine monophosphate (dGMP) and deoxyadenosine monophosphate (dAMP) was, respectively, two and three times larger than that of the 75 °C sample.

Keywords platinum green; antitumor activity; cell cycle; DNA synthesis inhibition

Previous work from this laboratory demonstrated that platinum pyrimidine greens have strong antitumor activity against mouse leukemia L1210 cells, while Pt–pyrimidine blues do not.[1,2] We therefore established a selective and efficient method for preparing platinum pyrimidine greens by a convenient one-pot reaction.[2,3] We have also found that there is a remarkable difference in the cytotoxic activity of the Pt-greens depending on the temperature used in preparation; the sample synthesized at 40 °C (S40) consistently exhibited greater cytotoxic activity than that at 75°C (S75).[4-6] The structural analysis of both samples showed clear differences in oligomerization and platinum oxidation levels,[1-3]; *i.e.*, (1) both elemental analysis and gel chromatographic behavior of S40 and S75 allowed us to estimate the molecular size of about 7—9 for the former and 13—15 for the latter as the number of Pt in the molecule; (2) the respective oxidation levels of Pt were around 2.33 and 2.45 in S40 and S75. In this report, we examined the effects of Pt-greens on the cell cycle of tumor cells and on deoxyribonucleic acid (DNA) synthesis of the cells in association with anticancer activity.

Materials and Methods

Materials Platinum greens were synthesized according to reported procedures,[3] and the data of elemental analysis[7] were as follows. S40: Calcd for $[Pt_7(C_9H_{11}N_2O_6)_2(NH_3)_{14}(OH)_2(H_2O)_8](SO_4)_6/H_2O$: C, 7.55; H, 2.96; N, 8.81; S, 6.72; Pt, 47.70. Found: C, 7.61; H, 2.84; N, 8.80; S, 6.96; Pt, 47.47%. S75: Calcd for $[Pt_{14}(C_9H_{11}N_2O_6)_{10}(NH_3)_{27}(OH)_2(H_2O)_7](SO_4)_{11}/2H_2O$: C, 15.72; H, 3.09; N, 9.57; S, 5.13; Pt, 39.72. Found: C, 15.91; H, 3.16; N, 9.44; S, 5.09; Pt, 39.43%.

Tumor Cells Daudi (human Burkitt lymphoma) cells were maintained in RPMI-1640 medium (GIBCO), supplemented with 20% fetal calf serum (FCS; IBL, Japan), 2 mM glutamine (Wako Pure Chemicals), 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Wako), penicillin (100 unit/ml; Sigma) and streptomycin (100 μg/ml; Sigma).

^{3}H-Thymidine (^{3}H-TdR) Incorporation Assay ^{3}H-TdR uptake was examined as follows. After the cells were cultured (2×10^5 cells/ml in 96-well plate, Sumitomo) in the presence or absence of Pt-greens for 1 or 2 d, they were treated with 0.5 μCi of ^{3}H-TdR (15 Ci/mmol; New England Nuclear) for 7 h. The cultures were harvested onto glass fiber filter strips, and the incorporated radioactivity was determined by a liquid scintillation counter (Beckmann).

Cell Cycle Analysis[8] Tumor cells (1×10^6) treated with Pt-greens were suspended in the culture medium, and BrdU (5-bromo-2'-deoxyuridine; Sigma) was added to achieve a final concentration of 100 μM. Then the cells were incubated for 30 min in 5% CO_2 at 37 °C. After washing the cells with phosphate buffered saline (PBS) (10 mM) twice, the pellets were resuspended into 100 μl saline, which was followed by the addtion of 2 ml cold 90% ethanol with vigorous mixing, then the cells were fixed at 0 °C for 30 min. Next, 2 ml 4 N HCl was added and the cells were incubated for an additional 30 min at r.t., washed with PBS once, and suspended again in 1 ml 0.1 M $Na_2B_4O_7$. The mixture was incubated at r.t. for 5 min, then 5 ml 0.5% Tween 20 and 20 μl FITC (fluorescein isothiocyanate)-anti-BrdU (Becton Dickinson) were added. After incubating the cells for 30 min at r.t. the pellets were washed with PBS once and treated with 1 ml of propidium iodide (PI, 5 μg/ml, Sigma). Analysis of the cell cycle was then carried out with a cell sorter (EPICS CS).

Results and Discussion

We have shown that the antitumor activity of the Pt-greens differed depending upon the preparation temperature. For example, S40 exerted a higher antitumor activity than S75, and this tendency was similar in all tumor cell lines (Daudi, HeLa, U937, L1210 and S-180) examined.[4] This strongly indicates that there are some differences in biological function between the two samples. Thus, we first examined the effect of Pt-greens on ^{3}H-TdR uptake. As shown in Fig. 1, S40 greatly suppressed the DNA synthesis on day 1, while suppression with S75 was low, especially at low concentrations. On day 2, S40 almost completely inhibited DNA synthesis at all concentrations tested, whereas S75 still allowed the synthesis to a certain extent. Thus, both S40 and S75 exert remarkable inhibition of DNA synthesis, but the activity of the former was clearly stronger than the latter.

To further confirm this suppressive activity, we analyzed

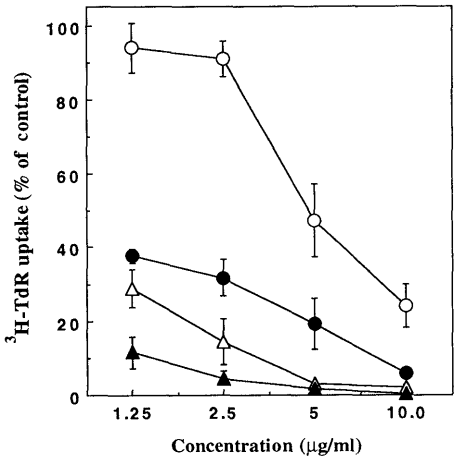

Fig. 1. Effect of Pt-Greens on ^{3}H-TdR Incorporation of Daudi Cells

^{3}H-TdR incorporation of Daudi cells (2×10^5/ml) was examined after the treatment with Pt-greens. Symbols: ○, S75 for 1 d; ●, S40 for 1 d; △, S75 for 2 d; ▲, S40 for 2 d. Bars: S.D.

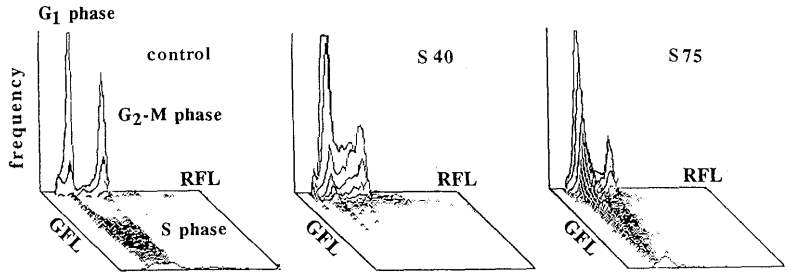

Fig. 2. Effect of Pt-Greens on the Cell Cycle of Dauli Cells

Daudi cells (1×10^5/ml) were treated with Pt-greens (2 μg/ml) for 2 d and the cell cycle was analyzed by a cell sorter. GFL: green fluorescence by FITC. RFL: red fluorescence by PI.

TABLE I. Effect of Pt-Greens on the Cell Cycle of Daudi Cells[a]

Day	Treatment with	% of cells in		
		G1 phase	S phase	G2-M phase
Day 1	Medium	37.5	27.9	34.6
	S75	50.7	32.5	16.8
	S40	60.9	22.0	17.1
Day 2	Medium	30.5	40.7	27.8
	S75	53.8	30.6	15.5
	S40	83.9	6.7	9.4

a) Daudi cells (1×10^5/ml) were treated with Pt-greens (2 μg/ml) for 1 or 2 d and the cell cycle was analyzed by a cell sorter.

the cell cycle of Daudi cells treated with Pt-greens (2 μg/ml). On day 1, the number of the cells in G1 phase had increased following treatment with both S75 and S40, compared to the control, with a greater increase by S40 (Table I). On day 2, the number of S40 treated cells in the G2-M phase had greatly decreased, and those in the S phase were almost non-existent. In contrast, a significant though less remarkable decrease in S phase cells was observed following treatment with S75 (Fig. 2).

These findings corresponded well with the result of ^{3}H-TdR uptake. The results clearly show that both S75 and S40 can block the cell cycle at the S-phase, but the block caused by S40 is the more specific and/or stronger of the two. Lower oligomerization and platinum oxidation levels observed in S40 may be related to this difference in activity, *e.g.* transport through cell membranes.

Cisplatin (CDDP, 2 μg/ml) had almost the same effect on the cell cycle as S40 (data not shown). Jäckel and Köpf-Maier demonstrated that the interaction of CDDP with DNA caused an S or G1/S block,[9] and Pinto and Lippard reported that DNA synthesis was inhibited at nucleotides at which platinum binds to DNA molecules.[10] We therefore speculate that the strong antitumor activity of S40 is probably due to the high inhibitory activity of DNA synthesis, as is true of CDDP.

We then examined the reactivity of Pt-greens towards purine bases, and the reaction was followed by proton nuclear magnetic resonance (^{1}H-NMR) (400 MHz, in D_2O at 5.1 °C, dioxyl sodium sulfosuccinate (DSS) as an internal standard). When deoxyguanosine monophosphate (dGMP) was added to a solution of Pt-green (S75), a downfield shift of the H_8 proton in dGMP instantly resulted; a new peak appeared at 8.63 ppm, and the shift was quite large ($\Delta\delta = 0.47$ ppm). Inherently, the same phenomena were observed with deoxyadenosine monophosphate (dAMP), but the corresponding shifts were even larger, *viz.*, the original proton of H_8 at 8.54 ppm shifted to 9.56 ppm ($\Delta\delta = 1.02$ ppm).

The reactions with CDDP, however, were very slow and gave no new peaks at 5.1 °C, although corresponding peaks with very weak intensities appeared when reacted at 40°C. Outstanding differences in the reactivities were also observed between S40 and S75; the respective ratios of the new peaks after the reaction of the former and the latter with dGMP and dAMP were approximately 2 and 3.

Hemminki and co-workers reported that the H_8 proton of deoxyguanosine (7.99 ppm) shifted downfield (8.45 ppm) upon the binding of CDDP.[11] This is typical of N_7 platination of deoxyguanosine.[12] The change of chemical shift of the H_8 proton of adenosine in ApG from 8.26 ppm to 9.27 ppm, in addtion to the above coresponding shift of guanosine, points to the intramolecular crosslink of CDDP bound to the N_7-atoms of adenine and guanine.[13] Therefore, similar reactions are expected with the Pt-greens at remarkably fast reaction rates.[14] Further detailed studies along this line are in progress, including the isolation and identification of products, and will be reported elsewhere.

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