Cytotoxicity of Asymmetric Platinum Complexes against L-1210 Cells. Effect of Bulky Substituents

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The asymmetric platinum complexes *cis*-Pt(LL')Cl_2 (L = NH_3, L' = CH_3NH_2, $(CH_3)_2NH$, $C_2H_5NH_2$ and $(C_2H_5)_2NH$ and LL' = *N,N*-dimethylethylenediamine), —one of the NH_3 groups of *cis*-Pt$(NH_3)_2Cl_2$ was substituted by alkylamine—, were synthesized and their cytotoxic effects have been measured using L-1210 cells. The IC_{50} values of the asymmetric platinum complexes, —being obtained after 24 h exposure of L-1210 cells to the platinum complexes—, are almost comparable to the corresponding value of *cis*-Pt$(NH_3)_2Cl_2$. In 2 h exposure, however, the IC_{50} values of the platinum complexes were dramatically changed, *i.e.*, a marked difference was observed between those of L' = RNH_2 and L' = R_2NH. On the other hand, the amounts of platinum taken into the L-1210 cells is little affected by the alkylamino substitution. The results suggest that the bifunctional platinum binding to the target molecule may be responsible for the cytotoxicity.

Keywords platinum complex; antitumor activity; cytotoxicity; L1210; cisplatin derivative; structure–activity relationship

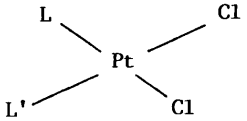
Introduction

It is generally accepted that the bifunctional binding of platinum complexes to deoxyribonucleic acid (DNA) is responsible for various biological activities of antitumor activity, cytotoxicity, mutagenicity and so on.[1] Antitumor active platinum(II) complex is required to have two inert and two labile ligands in its square planar coordination plane, and the two labile ligands must be in *cis*-configuration (general formula *cis*-Pt(amine)$_2Cl_2$).[2] In the present work, we prepared asymmetric platinum complexes (general formula *cis*-Pt(LL')Cl_2; L = NH_3 and L' = alkylamine) which satisfy this prerequisite. Many symmetric platinum complexes (*cis*-Pt(amine)$_2Cl_2$) have been synthesized to data and their biological activities inspected.[2] Little is known, however, about the biological activity of the asymmetric platinum complexes. This study reports the cytotoxic effect of the asymmetric platinum complexes in which one of the two NH_3 groups in *cis*-Pt$(NH_3)_2Cl_2$ was substituted for the alkylamino group (CH_3NH_2, $C_2H_5NH_2$, $(CH_3)_2NH$, and $(C_2H_5)_2NH$).

Experimental

Synthesis of Platinum Complexes The asymmetric platinum complexes *cis*-Pt(NH_3)(L')Cl_2, where L' = alkylamine, were synthesized as described by Rochon and Kong.[3] A slight improvement was found, *i.e.*, on adding ammonium hydroxide to the iododimer $[Pt(L')_2I_2]_2$, a stoichiometric amount was used (instead of the excess advised by the above researchers).

Cell Survival Two hundred thousand L-1210 cells were grown in 2 ml of RPMI 1640 medium, supplemented with 10% fetal calf serum, penicillin and streptomycin, in a 5% CO_2 humidified incubator at 37 °C. After 48 h, the medium was changed and the cells were incubated for another 48 h. After that, the cells were centrifuged at 3000 rpm for 5 min, resuspended in the fresh medium and exposed to the platinum complexes to be tested for 2 or 24 h. The medium was then changed in order to remove the free platinum complexes. Posttreatment incubation was 48 h. The cells were harvested and diluted with Tris–HCl buffer (pH 7.4) containing 0.1% of Trypan blue. Cell numbers were counted in a hemocytometer.



cis-DDP:	L = NH_3,	L' = NH_3
MMA:	L = NH_3,	L' = CH_3NH_2
MEA:	L = NH_3,	L' = $C_2H_5NH_2$
DMA:	L = NH_3,	L' = $(CH_3)_2NH$
DEA:	L = NH_3,	L' = $(C_2H_5)_2NH$
bis-MMA:	L = CH_3NH_2,	L' = CH_3NH_2
bis-MEA:	L = $C_2H_5NH_2$,	L' = $C_2H_5NH_2$
DMEN:	LL' = *N,N*-dimethylethylenediamine	

Fig. 1. Structure of Platinum Compounds and Abbreviations

Inhibition of DNA Synthesis Two hundred thousand L-1210 cells were grown in 2 ml of RPMI 1640 medium as above. After 96 h, the cells were exposed to various concentrations of platinum complex for 2 h, following which the medium was changed in order to remove the free platinum complexes. The cells were labeled by adding 0.1 μCi of [methyl-^{3}H]thymidine and incubated for another 24 h. The cells were harvested in a micro-test tube by centrifugation at 3000 rpm. Harvested cells were solubilized by the addition of 50 μl of 0.5% sodium dodecyl sulfate, followed by sonication with a micro-ultrasonic cell disruptor. Twenty μl of the cell solution was deposited on a filter paper (Whatman 1.9 cm Assay Disc No 3MM) which had previously been acidified with 40 μl of 20% trichloroacetic acid. The paper disc was washed by immersing it in cold 5% trichloroacetic acid for 10 min and subsequently in a cold absolute ethanol for another 10 min. The radioactivity on the paper disc was determined by a liquid scintillation counter.

Determination of Platinum Content in the L-1210 Cells After exposure (2 or 24 h) to 10 μM of platinum complexes, L-1210 cells were collected by centrifugation and washed twice with 0.02 M Tris–HCl saline buffer (pH 7.4). Cells were counted and those collected were solubilized by the addition of 5% sodium dodecyl sulfate, followed by sonication with an ultrasonic cell disruptor. The platinum content of the sample solution thus obtained was determined with a flameless atomic absorption spectrometer under the following conditions (drying 45 s at 100 °C, ashing 60 s at 1300 °C, and atomization 8 s at 2700 °C).

Results and Discussion

Characterization of *cis*-Pt(LL')Cl_2 Complexes The structures of the asymmetric platinum complexes were confirmed on the basis of the data of elemental analyses (data not shown) and infrared (IR) spectra. It was confirmed by IR spectral analysis that Pt(LL')Cl_2 has a *cis*-configuration and not *trans*-configuration. In general, the frequency due to the Pt–N bond would be strongly influenced by the ligand introduced in the position *trans* to the Pt–N bond.[4] The rocking and symmetric deformation vibrations due to the coordinated NH_3 group are also sensitive to such effect.[4] Therefore, we compared the IR spectra of *cis*-Pt(LL')Cl_2 and *cis*-Pt(LL')I_2. When the coordinated halogen ions are substituted by chloride in place of iodide, high frequency shift of 30—40 cm^{-1} was observed for the Pt–N stretching vibration and 40—50 cm^{-1} for the NH_3 rocking vibration. Such a large shift is good evidence showing that the coordinated halogen ions are sitting on the position *trans* to the coordinated NH_3 group, *i.e.*, that Pt(LL')X_2 (X = Cl,

Table I. Infrared Spectral Data of Dichloro- and Diiodo-Platinum Compounds

Compound	ν_{Pt-N}	Δ	ρ (NH_3)	Δ
cis-$Pt(NH_3)_2Cl_2$	517, 508		795	
cis-$Pt(NH_3)_2I_2$	491, 477	36, 31	752	43
trans-$Pt(NH_3)_2Cl_2$	509		819	
trans-$Pt(NH_3)_2I_2$	498	11	806	13
MMA ($L'=CH_3NH_2$)				
cis-$Pt(NH_3)(L')Cl_2$	524, 506		823	
cis-$Pt(NH_3)(L')I_2$	494, 470	30, 36	784	39
DMA ($L'=(CH_3)_2NH$)				
cis-$Pt(NH_3)(L')Cl_2$	531, 520		810	
cis-$Pt(NH_3)(L')I_2$	496, 477	30, 43	762	48
MEA ($L'=C_2H_5NH$)				
cis-$Pt(NH_3)(L')Cl_2$	528, 515		828	
cis-$Pt(NH_3)(L')I_2$	494, 479	34, 36	784	44

Δ = difference in frequency of the dichloro- and the diiodo-platinum complexes.

Table II. IC_{50} Values of Platinum Compounds

Complexes	2 h exposure		24 h exposure
	Cytotoxicity	Inhibition of DNA synthesis	Cytotoxicity
cis-DDP	15	12	12
trans-DDP	250	300	220
MMA	44	62	7
MEA	15	21	13
DMA	160	120	14
DEA	140	260	18
DMEN	170	240	12
bis-MMA	61	—	22
bis-MEA	76	—	37

The IC_{50} values (μmol/l) were determined by counting the number of living cells after 2 or 24 h exposure of L-1210 cells to the platinum complexes, and by drawing a semi-log plot of the cell growth inhibition against the platinum concentration added.

I) has a *cis*-configuration.

The reaction of *cis*-$Pt(NH_3)_2Cl_2$ with dinucleotide GpG is known to result in a single product, *cis*-$Pt(NH_3)_2$(GpG-N7,N7).[5] We examined whether or not the asymmetric platinum complexes react with GpG to yield *cis*-Pt(LL')(GpG-N7,N7). The reaction resulted in two kinds of *cis*-Pt(LL')(GpG-N7,N7) and structures of the two products were determined by nuclear magnetic resonance (NMR) spectroscopy. The presence of two kinds of *cis*-Pt(LL')(GpG-N7,N7) is expected because *cis*-$Pt(LL')Cl_2$ does not possess a C_2 symmetrical element *i.e.*, one with the coordinated NH_3 group *cis* to the 5'-guanine base and the other with the coordinated alkylamino group *cis* to the 5'-guanine base. More detailed structures of *cis*-Pt(LL')(GpG-N7,N7) will be presented elsewhere.

Cytotoxicity of the Platinum Complexes The results of *in vitro* assays are summarized in Table II. The IC_{50} values of these complexes were evaluated after 2 or 24 h exposure of L-1210 cells to the Pt-complexes and the succeeding posttreatment incubation. It was found that the growth of L-1210 cells in culture is significantly inhibited by *cis*-$Pt(LL')Cl_2$ complexes, especially with 24 h exposure. Their IC_{50} values are almost comparable to the IC_{50} value of *cis*-$Pt(NH_3)_2Cl_2$ which is a positive control in our study. In 2 h exposure, only *cis*-$Pt(NH_3)(C_2H_5NH_2)Cl_2$ among *cis*-$Pt(LL')Cl_2$ complexes was comparable to *cis*-$Pt(NH_3)_2Cl_2$. The IC_{50} value of MEA did not show any difference

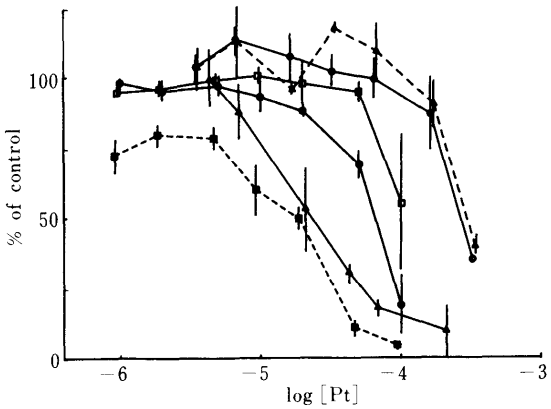


Fig. 2. Inhibition of DNA Synthesis in L-1210 Cells as a Function of Platinum Concentration

—■—, *cis*-DDP; —▲—, MEA; —●—, MMA; —□—, DMA; —○—, DMEN; —△—, DEA.

between 2 h and 24 h exposures. This was also true for *cis*-$Pt(NH_3)_2Cl_2$. The IC_{50} values obtained from 2 h exposure increase in the following order: MEA = *cis*-DDP < MMA < bis-MMA < DMA, DEA, DMEN. That is, platinum complexes in which one of the two non-leaving ligands is substituted by the primary amino ligand show IC_{50} values lower than the corresponding complexes with the secondary and tertially amino ligands. Such tendency seems consistent with the earlier observation of a structure activity relationship, in which the antitumor activity of *cis*-$Pt(amine)_2Cl_2$ decreased along the series $RNH_2 = NH_3 > R_2NH > R_3N$.[6]

In comparing the 2 h exposure with the 24 h exposure, a marked difference is observed in their cytotoxicities. The IC_{50} value of MMA (24 h exposure) was 3 times lower than that obtained from the 2 h exposure. The IC_{50} values of DMA, DEA and DMEN (2 h exposure) increased over 8-fold compared with the values of the 24 h exposure. The cytotoxicity of these platinum complexes tends to increase with the duration of exposure. The IC_{50} value of *cis*-$Pt(NH_3)_2Cl_2$ reported by Macquet and Butour was 2.3 μM after 48 h exposure,[7] while the IC_{50} value of this complex obtained in our study was 5 times greater than that. The IC_{50} values (300 μM) of *trans*-$Pt(NH_3)_2Cl_2$, which was a negative control in our study, was also 5 times greater than that (67 μM) reported by these researchers.[7]

Figure 3 shows the inhibitory effect of *cis*-$Pt(LL')Cl_2$ complexes on the ^{3}H-thymidine incorporation (DNA synthesis), as obtained from a 2 h exposure to the platinum complexes. The curves of the inhibition of DNA synthesis *versus* platinum concentrations correspond roughly to those obtained for the cytotoxicities. The IC_{50} values which correspond to a 50% decrease of DNA synthesis are summarized in Table II.

Pt Uptake into the Cells In order to ascertain the Pt uptake into the cells, L-1210 cells were exposed to 10 μM of the platinum complexes, and the platinum content accumulated inside the cells was determined. As indicated in Table III, the accumulated platinum content inside the cells after 24 h exposure is about 5 fold that after 2 h exposure. For the same duration of exposure, the amount of the Pt-uptake is not significantly affected by the alkylamino substitution. In the case of 2 h exposure, the

Table III. Platinum Content Accumulated inside the L-1210 Cells after 2 or 24 h Exposure to the Platinum Complexes

Complex	2 h exposure	24 h exposure
cis-DDP	0.22±0.04	1.71±0.01
MMA	0.51±0.14	1.85±0.87
MEA	0.29±0.06	2.70±0.01
DMA	0.31±0.01	1.05±0.72
bis-MMA	0.32±0.01	1.01±0.21
bis-MEA	0.19±0.03	1.39±0.06

Numbers indicate the amount of platinum (ng/10^5 cells), as determined by flameless atomic absorption spectrometer.

IC_{50} value of MEA is less than 10 times that of DMA, but there is no difference between the amounts of Pt-uptake of the two platinum compounds. Therefore, it is not likely that there is a relation between the amount of Pt-uptake and cytotoxicity. One should thus consider not only the Pt-uptake but also the binding mode with DNA; for example, monofunctional platinum complex is not effective for the cytotoxicity even though it is taken into the cells.[7]

It has been generally accepted that DNA damage is directly responsible for the cytotoxicity of a platinum drug(s).[1] The mode of action of *cis*-$Pt(NH_3)_2Cl_2$ at the molecular level is conceived to originate from the crosslinking between two bases on DNA, probably adjacent guanine bases on the same DNA strand. Such platinum binding fashion results in a kink of the DNA strands.[8] If such structural change on DNA is related to the cytotoxicity, the cytotoxicity would depend on the amount of the bifunctional adducts on DNA. These bifuctional platinum adducts are formed *via* a monofunctional intermediate. The presence of the alkyl groups on the ligand L′ would then be expected to influence the kinetics of formation of the bifunctional platinum adducts. That is, the bulky group in the L′ is likely to prolong the life-time of the monofunctional intermediate. The alkyl groups in the L′ may also lead to decrease and/or disappearance in hydrogen bonding ability between the coordinated amino group and DNA molecule. Such effects account for the lower cytotoxicity of DMA, DEA and DMEN in the case of 2 h exposure because monofunctional binding of platinum complexes (ex., $[Pt(NH_3)_3Cl]Cl$) to DNA do not inhibit DNA synthesis.[9] Longer incubation times are likely to be required to yield the bifunctional platinum–DNA adducts.

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