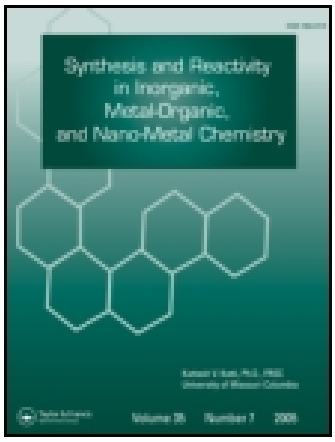

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Synthesis, Cytotoxicity, and DNA-Binding Levels of Ammine/Cyclohexylamine Platinum(II) Complexes with Dicarboxylates

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Synthesis, Cytotoxicity, and DNA-Binding Levels of Ammine/Cyclohexylamine Platinum(II) Complexes with Dicarboxylates

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Four new ammine/cyclohexylamine platinum(II) complexes with dicarboxylates (a–d) have been synthesized and characterized by elemental analysis, conductivity, IR, UV, and ^{1}HNMR spectra techniques. The cytotoxicity of the complexes was tested by MTT assay. The cell cycle analysis and the levels of total platinum bound to DNA were measured by flow cytometry and ICP-MS. The results show that the cytotoxicity of complexes (a–d) against EJ, HCT-8, BGC-823, HL-60, and MCF-7 cell lines decreases in the sequence: b > a > c > d. Complexes (a–d) have better cytotoxicity against EJ and HL-60 and complex (b) demonstrates cytotoxicity superior to that of the clinically established cisplatin. The complexes (a–d) induced a concentration-dependent accumulation of HL-60 cells in the G_2/M phase of the cell cycle as cisplatin. The levels of total platinum bound to DNA in HL-60 and EJ cells decrease in the sequence: b > cisplatin > a > c > d under the same experimental conditions.

Keywords ammine/cyclohexylamine platinum(II) complexes, synthesis, cytotoxicity, cell cycle, DNA binding

INTRODUCTION

By now, cisplatin is one of the most active chemotherapeutic agents available for the treatment of a variety of malignancies, especially testicular and ovarian. Despite its success, cisplatin has several disadvantages that include severe toxicity, relatively narrow range of tumors and resistance to cisplatin. These drawbacks have been the impetus for the development of an improved Pt antitumor drug, thousands of Pt compounds have been synthesized and evaluated as potential antitumor agents. Among the 33 platinum agents that have entered clinical trials after the onset of clinical studies with cisplatin in the early 1970s, only one (carboplatin) has received worldwide approval so far. Four drugs (oxaliplatin, nedaplatin, lobaplatin and SKI2053R) have gained regionally limited approval and another eight continue to be evaluated in clinical studies. Two of these (JM-216, ZD0473) have recently entered phase III studies. Therefore, research work is still worthwhile.[1–5]

The structure-activity relationships summarized by Cleare and Hoeschele dominated Pt drug design for over 20 years and remained valid until relatively recently. This is reflected in the fact that all Pt compounds that have entered clinical trials so far adhere to this set of guidelines. However, it has become quite evident that mere analogues of cisplatin or carboplatin will probably not offer any substantial clinical advantages over the existing drugs. A number of researchers have taken a completely different approach to Pt drug design and have prepared compounds that violate the structure-activity relationships but show antitumor activities. Therefore, the search continues for an improved Pt antitumor agent, motivated by the desire to design a less toxic, orally active compound that is non-cross-resistant with cisplatin and carboplatin.[2,4,6,7]

The mixed ammine/amine platinum complexes with chloro have been reported and demonstrated better activity against cisplatin-resistant cells in vitro and more less toxicity than the parent cisplatin.[8,9] For example, JM-216 has recently entered phase III studies. The possible advantage of platinum anticancer drugs with decreased reactivity of leaving group is an established approach which commenced with the clinical success of carboplatin. It is reported that the decreased reactivity of carboplatin reduce the nephrotoxic and neurotoxic side effects of cisplatin. Moreover, decrease in reactivity of leaving groups may also lead to reduced detoxification reactions by intracellular thiols. This may increase the efficacy of the drug and help to circumvent resistance mechanism such as overexpression of glutathione. So, carboxylate platinum complexes seem to be quite promising than the corresponding chloro analogs. Thus far, many mixed ammine/amine platinum complexes with chloro have been reported. But few mixed ammine/amine platinum complexes with carboxylates were reported.[8–11] Previously

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we reported the synthesis, characterization and antitumor activity of platinum(II) complexes of mixed ammine/methylamine with bidentate carboxylates.[12] In the present work, the synthesis, cytotoxicity and DNA-binding levels of new mixed ammine/cyclohexylamine platinum(II) complexes with dicarboxylates are reported and discussed.

EXPERIMENTAL

Reagents

All reagents and solvents were analytical reagent grade. MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), TrisHCl (tris-(hydroxymethyl)-aminomethane, hydrochloride), PI (propidium iodide), RNase and Genomic DNA extraction kit were purchased from Sigma (USA). RPMI 1640 culture medium and FBS (fetal bovine serum) was from Gibco. Cisplatin was purchased from Qi Lu Pharmaceutical Factory in China.

Instrumentation and Measurement

Elemental analyses were determined on a EA-1110 elemental analyzer. The thermal analysis was determined using RI-GAKU 8150 meter (Ar, $10^{\circ}C \cdot min^{-1}$, Al_2O_3). Molar conductances at room temperature were measured in 10^{-3} M aqueous solutions using a DSS-11A type conductivity meter. The IR spectra were recorded in the 400–4000 cm^{-1} range using KBr pellets and a Perkin-Elmer Model-683 spectrophotometer. The electronic spectra in H_2O were measured on an UV-3400 Toshniwal spectrophotometer. The ^{1}HNMR spectra in D_2O were recorded on a Brucker AV 400 NMR spectrometer. Cell cycle analysis was performed on a BECScan Flow Cytometer. The levels of total platinum bound to DNA were measured by PE Elan-5000 ICP-MS (Inductively Coupled Plasma Mass Spectrometry).

Preparation of Complexes

Precursor complexes cis-[Pt(〈〉–NH_2)$_2$$I_2$] **(i)**, [Pt(〈〉–$NH_2$)$I_2$]$_2$ **(ii)** and cis-[Pt(〈〉–NH_2)(NH_3)I_2] **(iii)** were synthesized according to the literatures.[13,14] [Pt(NH_3)(〈〉–NH_2)$(OOC)_2$] **(a)**: cis-[Pt(〈〉–NH_2)(NH_3)I_2] (0.57 g, 1 mmol) was mixed with $AgNO_3$ (0.331 g, 1.95 mmol) in 15 mL of water. The mixture was allowed to stir overnight in the dark. The AgI precipitate was removed by filtration and 1–2 drops of NaCl was added to the filtrate. If a precipitate (AgCl) appeared immediately, it was filtered out after 10 min and again 1 drop of NaCl was added to the filtrate. The procedure was repeated until there was no immediate precipitate after adding one drop of NaCl solution. When all the silver ions have been removed, a slight excess of the sodium salt of the oxalic acid was added to the filtrate. After 6 hr, the mixture was evaporated to dryness and washed a few times with a minimum quantity of very cold water. The final product [Pt(NH_3)(〈〉–NH_2)$(OOC)_2$] was dried over P_2O_5 under vacuum. Yield: 65%.

The synthetic procedure for [Pt(NH_3)(〈〉–NH_2)(OOC-CH_2-COO)] **(b)**, [Pt(NH_3)(〈〉–NH_2)(OOC-CH_2-CH_2-COO)] **(c)** and [Pt(NH_3)(〈〉–NH_2)(OOC-CH=CH-COO)] **(d)** are similar. The synthetic routines of the mixed ammine/cyclohexylamine platinum(II) complexes with dicarboxylates are given here (Figure 1).

Cytotoxicity Analysis

The complexes were dissolved in phosphate-buffered saline (PBS) and diluted to the required concentration with culture medium when used. The cytotoxicity was evaluated by MTT assay.[15] Briefly, cells were plated in 96-well microassay culture plates (10^4 cells per well) and grown overnight at 37°C in a 5% CO_2 incubator. Compounds were then added to the wells to achieve final concentrations ranging from 10^{-7} to 10^{-4} M. Control wells were prepared by addition of culture medium. Wells containing culture medium without cells were used as blanks. The plates were incubated at 37°C in a 5% CO_2 incubator for 48 hr. Upon completion of the incubation, stock MTT dye solution (20 μL, 5 mg/mL) was added to each well. After 4 hr incubation, 2-propanol (100 μL) was added to solubilize the MTT formazan. The optical density of each well was then measured on a microplate spectrophotometer at a wavelength of 570 nm. The IC_{50} value was determined from plots of % viability against dose of compound added. Five different human carcinomas were the subjects in this study: EJ (bladder carcinoma), HCT-8 (colon carcinoma), BGC-823 (gastric carcinoma), HL-60 (immature granulocyte leukemia) and MCF-7 (galactophore carcinoma).

Flow Cytometry Analysis

The cell cycle analysis was performed as described by Ferlini et al.[16] HL-60 cells were treated with platinum complexes for the indicated times and harvested by centrifugation at 1200 rpm/min for 5 min at room temperature. Cell pellets were rinsed with PBS, suspended in a 1:1 (v/v) solution of PBS and 0.2 M Na_2HPO_4 − 0.1 M citric acid (pH = 7.5), and fixed with cold ethanol at 4°C for 1 hr. Fixed cells were washed with PBS and resuspended in a staining solution containing PI (10 μg/mL) and DNase-free RNase (100 μg/mL). The cell suspensions were incubated at 37°C for 1 hr in the

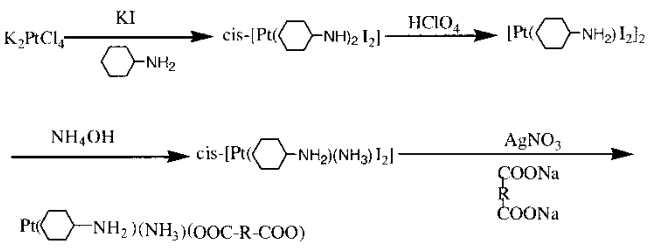

FIG. 1. Synthetic routines of the mixed ammine/cyclohexylamine platinum(II) complexes with dicarboxylates.

TABLE 1
Physical properties of the complexes

Complex	Found (calcd.) (%) C	N	H	Pt	Ω
(**i**)	22.20 (22.27)	4.30 (4.33)	4.01 (4.05)	—	—
(**ii**)	13.09 (13.15)	2.50 (2.56)	2.34 (2.39)	—	—
(**iii**)	12.70 (12.75)	4.80 (4.96)	2.70 (2.85)	—	—
a	24.12 (24.06)	7.23 (7.02)	3.98 (4.04)	48.96 (48.86)	5.36
b	26.31 (26.15)	6.87 (6.78)	4.29 (4.39)	47.32 (47.20)	5.68
c	28.32 (28.10)	6.58 (6.56)	4.87 (4.72)	45.87 (45.65)	5.79
d	28.36 (28.23)	6.69 (6.59)	4.32 (4.27)	45.86 (45.87)	6.00

dark and analyzed on a flow cytometry. Data were collected by ModFit LT 2.0 for power software.

DNA Binding

The levels of total platinum bound to DNA in HL-60 and EJ cells were performed as described by Mellish et al.[17] Briefly, approximately 5×10^7 HL-60 or EJ cells were seeded in tissue-culture flasks, then the complexes were added in a concentration gradient, each concentration in triplicate, and the final concentrations were maintained at 10, 25, 50 and 100 μM, respectively. They were incubated at 37°C in 5% CO_2 for 4 hr. Cells were then harvested, and DNA was extracted according to DNA extraction kit procedure. The DNA was dissolved in 300 μL water. The purity and concentration of DNA was measured by UV spectroscopy. An aliquot of the remaining sample was sonicated and subjected to platinum analysis by ICP-MS.

Statistical Analysis

Data were collected from at least three separate experiments. The results are expressed as means ± sd. The statistical differences were analyzed using SPSS′ *t*-test. p values less than 0.05 were considered to indicated statistical differences.

RESULTS AND DISCUSSION

Physical Properties of the Complexes

The physical properties of the complexes are presented in Table 1. There is good agreement between the calculated and the found values. Low molar conductances for the complexes correspond to non-electrolytes.[18]

IR Spectra

The IR spectra of the complexes (**a**–**d**) are similar, the main bands with tentative assignments are listed in Table 2. The bands υ_{NH} and δ_{NH} in the precursor complexes (**i**–**iii**) and new complexes (**a**–**d**) shift to lower frequencies than those of free ammine and cyclohexylamine. New band appears at 444–483 cm^{-1} and is assigned to Pt-N stretching. Thus it indicates that they are coordinated with platinum through nitrogen atoms. The carboxylate group of the complexes (**a**–**d**) shows two bands, an intense antisymmetric carboxylate stretching $\upsilon_{(as,\,coo^-)}$ and a symmetric stretching $\upsilon_{(s,coo^-)}$, at about 1650 and 1400 cm^{-1}, respectively. The values of $\Delta\upsilon_{(coo^-)}(\upsilon_{(as,\,coo^-)}$-$\upsilon_{(s,\,coo^-)})$ of the complexes (**a**–**d**) are in the range 248–294 cm^{-1}, which is greater than $\Delta\upsilon_{(coo^-)}$ of the corresponding sodium dicarboxylates, so we may suggest that the carboxylate group is monodentate coordinated through oxygen atoms.[19] This is further confirmed by the appearance of the peaks of $\upsilon_{Pt\text{-}O}$.

Electronic Spectra

No absorption peaks appear for the malonic acid and succinic acid, one peak appears at 212.0 ($n \rightarrow \pi^*$) and 212.0 nm ($\pi \rightarrow \pi^*$) in the spectra of the oxalic acid and maleic acid respectively. After formation of the complexes, blue shifts by ca. 18.0 and 7.0 nm in the spectra of the complex **(a)** and **(d)** respectively, one new peak appears at

TABLE 2
IR data (cm^{-1}) of the complexes

Complex	υ_{NH}	δ_{NH}	$\upsilon_{(as,\,coo^-)}$	$\upsilon_{(s,\,coo^-)}$	$\Delta\upsilon_{(coo^-)}$	$\upsilon_{Pt\text{-}O}$	$\upsilon_{Pt\text{-}N}$
(**i**)	3195, 3109	1566					444
(**ii**)	3236, 3200	1570					470
(**iii**)	3270, 3200	1510					480
a	3260, 3165	1550	1679	1385	294	586	470
b	3258, 3156	1549	1669	1401	268	569	465
c	3209, 3125	1535	1647	1399	248	578	470
d	3268, 3158	1523	1656	1405	251	574	483

TABLE 3
^{1}HNMR spectra of the ligands and complexes

Compound	Chemical shift (δ, ppm)
Cyclohexylamine	2.52 (br, 1H, CH (methine)), 0.9–2.0 (m, 10H, CH(alkyl))
Oxalic acid	—
Malonic acid	3.26 (s, 2H, -CH_2-)
Succinic acid	2.67 (s, 4H, -CH_2-CH_2-)
Maleic acid	6.28 (s, 2H, -CH=CH-)
a	2.91 (br, 1H, CH (methine)), 1.0–2.2 (m, 10H, CH(alkyl))
b	2.92 (br, 1H, CH (methine)), 1.1–2.4 (m, 10H, CH(alkyl)), 3.38 (s, 2H, -CH_2-)
c	2.88 (br, 1H, CH (methine)), 1.0–2.4 (m, 10H, CH(alkyl)), 2.82 (s, 4H, -CH_2-CH_2-)
d	2.85 (br, 1H, CH (methine)), 1.0–2.3 (m, 10H, CH(alkyl)), 6.35 (s, 2H, -CH=CH-)

206.0 nm in the spectra of the complex **(b)**, no absorption peak appears for the complex **(c)**.

^{1}HNMR

As listed in Table 3, after formation of the complexes, the protons of the complexes shift to low field compared with those of free ligands. This is also further confirmed that the dicarboxylate and cyclohexylamine are coordinated with platinum through oxygen and nitrogen atoms.

Thermal Stability

Thermal analytical data was listed in Table 4. The thermal behavior of the complexes (**a**–**d**) is similar. They have a fixed melting point, there is an endothermic peak at 320–342°C coinciding with the melting point. There is a big endothermic peak (405–800°C), corresponding to 50.25–55.06% weight loss.

Based on the preceding studies, we propose a tentative coordination structure for the complexes (Figure 2).

Cytotoxicity Effect

As listed in Figure 3, the cytotoxicity against EJ, HCT-8, BGC-823, HL-60, and MCF-7 cell lines decreases in the sequence: **b** > **a** > **c** > **d**. Complexes (**a**–**d**) have better cytotoxicity against EJ and HL-60 and complex **(b)** demonstrates cytotoxicity superior to that of the clinically established cisplatin.

The mode of action of platinum anticancer drugs is still not completely understood but it is thought to depend on hydrolysis reactions where the leaving group is replaced by a water molecule adding a positive charge on the molecule. The hydrolysis product is believed to be the active species reacting mainly with glutathione in the cytoplasm and the DNA in the nucleus, thus inhibiting replication, transcription

TABLE 4
Thermal analytical data of the complexes

Complex	m.p. (°C)	Dec. temp. (°C) T_1	Dec. temp. (°C) T_2	Total wt. loss (%)	Residue
a	334	410	800	50.25	Pt
b	342	405	786	51.36	Pt
c	328	421	765	53.47	Pt
d	320	419	776	55.06	Pt

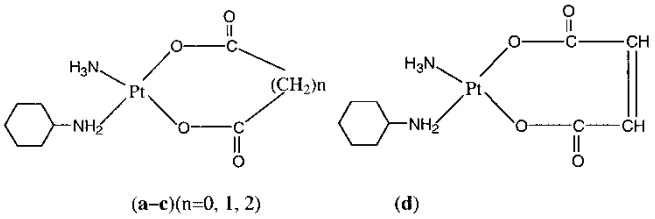

FIG. 2. Possible structure of the complexes (**a**–**d**).

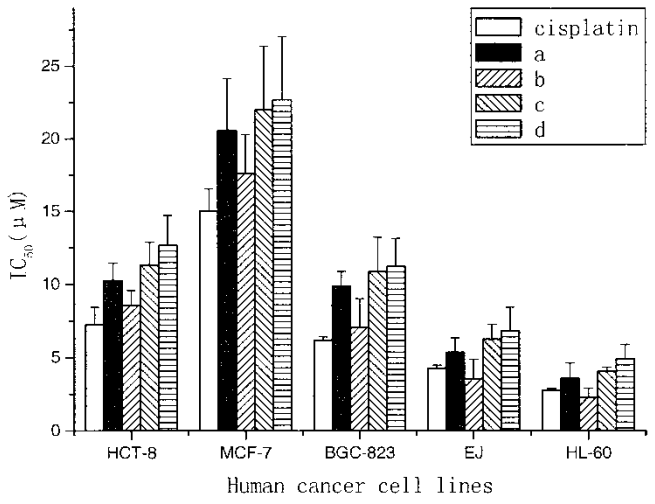

FIG. 3. Cytotoxicty of complexes (**a**–**d**) against various human carcinomas.

and other nuclear functions and arresting cancer cell proliferation and tumor growth. So the reactivity of leaving groups is an important factor to affect anticancer activity.[5] In the present work, for ammine/cyclohexylamine platinum(II) complexes with dicarboxylates, dicarboxylates coordinating with platinum through oxygen atoms form different chelate cycle, cycle size affects the reactivity of leaving groups, and further affect their cytotoxicity.

Cell Cycle Analysis

The effect of the complexes on cell cycle was given in Table 5. The complexes (**a**–**d**) induced a concentration-dependent accumulation of HL-60 cells in the G_2/M phase of the cell cycle as cisplatin.

DNA Binding

As listed in Figures 4 and 5, the levels of total platinum bound to DNA in HL-60 and EJ cells decrease in the sequence: **b** > cisplatin **> a** > **c** > **d** under the same experimental conditions. It is generally been accepted that platinum-based drugs exert their cytotoxic effects through the formation of platinum-DNA adducts. This occurs predominantly at the N7 position of guanosine. The vast majority of cisplatin-DNA adducts are of the intrastrand type between adjacent guanines.[20] In general, the degree of cytotoxicity of cisplatin correlates with the amount of DNA-platination.[21] However, a study in testicular cancer germ cell lines found no association between cisplatin DNA-platination and drug sensitivity and a similar observation was made in a breast cancer cell line.[22,23] With respect to a correlation between the cytotoxicity of oxaliplatin and DNA-platination, the literature is limited. No correlation could be found between DNA-platination and cytotoxicity. Mellish et al.[17] reported that no significant correlation was found between total DNA platination levels and cytotoxicity of the seven platinum-based drugs in SKOV-3 or in CH1 cell lines. Roberts et al.[24] reported that significant correlation was found between total DNA platination levels and cytotoxicity of polynuclear platinum complex in L1210/0 cell line. In our work, we found that there was also significant correlation between total DNA platination levels and cytotoxicity of four ammine/cyclohexylamine platinum complexes with dicarboxylates in HL-60 and EJ cell lines. The total DNA platination levels contains some kinds of Pt-DNA adducts formed by platinum complex such as 1,2-intrastrand adducts between adjacent guanines or adjacent adenine. This suggests that it is probably the level of specific DNA adducts that is important in determining the cytotoxicity of platinum-based drugs.[17] In addition,

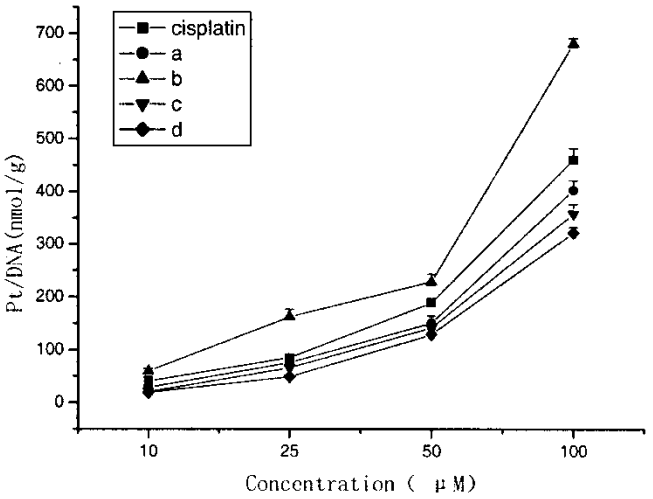

FIG. 4. Levels of total platinum bound to DNA in HL-60 cell after 4-hr exposure to platinum complexes.

TABLE 5
Effect of the platinum complexes on cell cycle in HL-60 cells

Complex	Concentration (μM)	Cell cycle (%) ($\bar{x}_{\pm s}$)		
		G_1	S	G_2/M
Control	0.0	31.34 ± 1.06	60.85 ± 1.41	7.80 ± 1.18
Cisplatin	1.5	25.36 ± 1.25**	50.36 ± 1.35**	24.28 ± 2.35**
	3.0	18.98 ± 1.02**	45.68 ± 2.87**	35.34 ± 2.87**
a	1.5	26.38 ± 1.06*	51.35 ± 4.06*	22.27 ± 2.35*
	3.0	22.65 ± 1.65*	48.36 ± 5.02*	28.99 ± 2.65**
b	1.5	22.78 ± 2.03*	47.65 ± 4.65*	29.57 ± 2.06*
	3.0	14.65 ± 1.23*	43.25 ± 3.98*	42.10 ± 3.75**
c	1.5	27.56 ± 2.13**	53.46 ± 4.79**	18.98 ± 0.35**
	3.0	24.58 ± 1.65**	49.68 ± 4.75**	25.74 ± 2.03**
d	1.5	29.68 ± 2.35**	56.98 ± 5.98**	13.34 ± 1.32**
	3.0	26.78 ± 1.25**	52.36 ± 2.65**	20.86 ± 2.98**

*$P < 0.05$, **$P < 0.01$ compared with the control group.

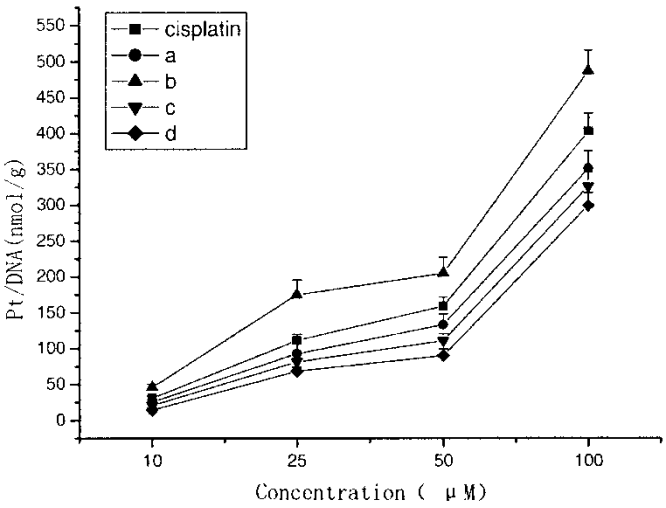

FIG. 5. Levels of total platinum bound to DNA in EJ cell after 4-hr exposure to platinum complexes.

platinum-based drugs might have other important targets apart from nuclear DNA. It has been demonstrated that cisplatin and oxaliplatin can induce apoptosis independent of the cell nucleus. It is interesting to note that mitochondrial DNA has been shown to have a 2 to 50 times greater propensity to be platinated than nuclear DNA. Cisplatin reacts with phospholipids, inhibits amino acid transport, protein synthesis, ATPases, uncouples oxidative phosphorylation, causes calcium efflux from the mitochondria and selectively alters the intracellular concentrations of calcium and potassium.[25–27] Although the importance of these other targets in relation to cytotoxicity is completely unknown, these other targets may have important effect on cytotoxicity. It remains to be further studied.

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