Published in final edited form as:

J Inorg Biochem. 2014 May ; 134: 49–56. doi:10.1016/j.jinorgbio.2014.01.021.

Cytotoxicity of Cyclometalated Platinum Complexes Based on Tridentate NCN and CNN-coordinating ligands: Remarkable Coordination Dependence

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Abstract

A series of cyclometalated platinum complexes with diverse coordination patterns and geometries were screened for their anticancer activity. It was discovered that the N Δ C Δ N-coordinated platinum complex based on 1,3-di(pyridyl)benzene displayed much higher cytotoxicity against human lung cancer cells NCI-H522, HCC827, and NCI-H1299, and human prostate cancer cell RV1 than cisplatin. In a sharp contrast, the C Δ N Δ N-coordinated platinum complex based on 6-phenyl-2,2'-bipyridine was ineffective on these cancer cells. This remarkable difference in cytotoxicity displayed by N Δ C Δ N- and C Δ N Δ N-coordinated platinum complexes was related to the trans effect of the carbon donor in the cyclometalated platinum complexes, which played a crucial role in facilitating the dissociation of the chloride ligand to create an active binding site. The DNA binding was studied for the N Δ C Δ N-coordinated platinum complex using electrophoresis and emission titration. The cellular uptake observed by fluorescent microscope showed the complex is largely concentrated in the cytoplasm. The possible pathways for the cell apoptosis was studied by western blot analysis and the activation of PARP via caspase 7 was observed.

Keywords

cytotoxicity; cyclometalated complex; Platinum; anticancer; DNA binding

1. Introduction

Among various treatments to deadly cancer diseases, chemotherapy has played an important role in improving the cancer survival. Chemotherapy is the use of chemical agents to kill the fast-growing cancer cells. Cisplatin, a coordination complex based on platinum, is one of the

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most successful anti-cancer drugs [1-4]. Since its discovery by Rosenberg [5, 6] in 1965 and approval by FDA in 1978, cisplatin has been widely used in treating a variety of cancers including all common cancers: breast, colon/rectum cancer, lung cancer, prostate cancer, and skin cancer. However, there are two major drawbacks with cisplatin: drug-resistance and severe toxicity such as nephrotoxicity and neurotoxicity [7]. These drawbacks have been the impetus for the development of an improved platinum antitumor drug. From thousands of analogous platinum compounds that have been synthesized and evaluated as potential antitumor agents, carboplatin and oxaliplatin emerged as two other drugs that were proved by FDA and have been used clinically for treating cancers.

Aside from the analogues of cisplatin, organometallic compounds that possess a carbon-metal bond offer a potential venue to the discovery of new anticancer drugs [8, 9]. In this context, the antitumor activity of organometallic compounds based on iron [10, 11], titanium [12], cobalt [13], rhodium [14], osmium [15], silver [16], gold [17], iridium [18], rhenium [19], and copper [20] have been extensively investigated. In contrast, organometallic compounds based on platinum have received much less attention [21-24], possibly because the focus has been primarily on the analogues of cisplatin. More recently the platinum complexes based on carbene ligands and cyclometalating C $\wedge$ N $\wedge$ N ligands have been reported to show high antitumor activity and their interaction with DNA was also studied [25]. Cyclometalated compounds are an important class of organometallic compounds with diverse structural variations on the coordination pattern and geometry. Cyclometalated platinum compounds have attracted a great deal of attention in recent years mainly because of their rich photophysical chemistry. Despite this, the cytotoxicity of cyclometalated platinum complexes were initially studied by Che's group [26-28], especially on a series of platinum complexes based on CANAN coordinating ligands. They reported that the platinum complex based on 6-phenyl-2,2'-bipyridine and chloride ligands did not show respectful cytotoxicity against a number of cancer cells; however, when the chloride was replaced with other ligands such as 2,4-diamino-6-(4-pyridyl)-1,3,5-triazine [28] and carbene ligands [25], the cytotoxicity of modified platinum complexes was substantially improved.

Over last several years, our group has developed a number of luminescent platinum complexes based on cyclometalating ligands with diverse coordination patterns and geometries [29-32]. Luminescent platinum complexes have been investigated as imaging and labelling materials in biological applications [33-36]. Such applications require that the materials should exhibit low cytotoxicity. On the other hand, as mentioned above, platinum complexes may have antitumor activity. Therefore, it would be desirable to examine the cytotoxicity of these complexes to determine their suitability for different applications. In this paper, we report a study on the cytotoxicity of series of structurally diverse cyclometalated platinum complexes to reveal its dependence on coordination geometry.

2. Experimental

2.1. Synthesis

All reactions involving moisture- and/or oxygen sensitive organometallic complexes were carried out under argon atmosphere and anhydrous conditions. All anhydrous solvents were purchased from Aldrich Chemical Co. and were used as received. All other reagents were

purchased from chemical companies and were used as received. NMR spectra were measured on a Bruker 400 spectrometer. Spectra were taken in CDCl_3 using tetramethylsilane as standard for ^1H NMR chemical shifts and the solvent peak (CDCl_3 , 77.0 ppm) as standard for ^{13}C NMR chemical shifts. The syntheses of complexes 1 [37], 2-4 [31], 5 [38], 6 [39], 7-8 [30], 9-11 [40], and 12 [29] have been reported previously. Although the complexes 1 and 5 were prepared according to the literature procedures; however, the corresponding cyclometalating ligands 1,3-di(2-pyridyl)benzene (L1) and 6-phenyl-2,2'-bipyridine (L5) was prepared using modified methods involving a Negishi coupling as described below.

Synthesis of 1,3-di(2-pyridyl)benzene (L1) [41]—To a 250 mL, 3-necked, dry, nitrogen flushed flask was charged with n-BuLi (1.6 M in hexanes, 8.5 mL, 13.5 mmol) and the solution was cooled to -78°C . To the flask, a solution of 2-bromopyridine (0.89 mL, 9 mmol) in 10 mL of diethyl ether was added dropwise. After stirring for 30 min, the reaction mixture was warmed to 0°C , and zinc chloride solution (1.0 M in diethyl ether, 13.5 mL, 13.5 mmol) was added dropwise. The mixture was then warmed to room temperature. 1,3-Dibromobenzene (0.354 mL, 3 mmol), $\text{Pd}(\text{PPh}_3)_4$ (350 mg, 0.3 mmol), and 45 mL of THF (tetrahydrofuran) were added and the reaction mixture was refluxed for 24 h. After cooling, the reaction mixture was quenched by an aqueous solution made from EDTA and sodium carbonate and extracted with ethyl acetate (3×75 mL). The combined organic phases were washed with water (100 mL), brine (100 mL), dried over MgSO_4 , filtered, and evaporated. The crude product was purified by column chromatography with hexane and ethyl acetate (2:1) as eluting solvents, colorless oil, 390 mg, yield 88%. ^1H NMR (400 MHz, CDCl_3): δ 8.73 (dm, $J = 4.8$ Hz, 2H), 8.62 (t, $J = 1.8$ Hz, 1H), 8.06 (dd, $J = 7.8$, 1.8 Hz, 2H), 7.85 (dt, $J = 8.0$, 1.0 Hz, 2H), 7.78 (td, $J = 7.4$, 1.8 Hz, 2H), 7.60 (t, $J = 7.7$ Hz, 1 H), 7.28-7.25 (m, 2H). ^{13}C NMR (100 MHz, CDCl_3): δ 120.8 (2C), 122.3 (2C), 125.6, 127.5 (2C), 129.2, 136.8 (2C), 139.9 (2C), 149.7 (2C), 157.2 (2C).

Synthesis of 6-phenyl-2,2'-bipyridine (L5)—This compound was prepared from 2-bromo-6-phenylpyridine and 2-bromopyridine. 2-bromo-6-phenylpyridine was prepared according to the literature method [42]. To a 100 mL, 3-necked, dry, nitrogen-flushed flask was charged with n-BuLi (1.6 M in hexanes, 6.7 mL, 11 mmol) and the solution was cooled to -78°C . To the flask, a solution of 2-bromopyridine (0.89 mL, 9 mmol) in 10 mL of ether was added dropwise. After stirring for 30 min, the reaction mixture was warmed to 0°C and zinc chloride solution (1.0 M in diethyl ether, 9 mL, 9 mmol) was added dropwise. The mixture was then warmed to room temperature. 2-Bromo-6-phenylpyridine (702 mg, 3 mmol), $\text{Pd}(\text{PPh}_3)_4$ (350 mg, 0.3 mmol), and 15 mL of THF were added. The reaction mixture was refluxed for 1 h. After cooling, the reaction mixture was quenched by an aqueous solution made from EDTA and sodium carbonate and extracted with ethyl acetate (3×75 mL). The combined organic phases were washed with water (100 mL), brine (100 mL), dried over NaSO_4 , filtered, and evaporated. The crude product was purified by chromatography on silica gel with a mixture of dichloromethane and ethyl acetate (v/v = 50:1), white color crystals, 397 mg, yield 57%. ^1H NMR (400 MHz, CDCl_3): δ 8.72 (dq, $J = 4.8$ Hz, 0.90 Hz, 1H), 8.67 (dt, $J = 8.0$ Hz, 1.0 Hz, 1H), 8.40 (dd, $J = 7.8$ Hz, 1.0 Hz, 1H), 8.20-8.17 (m, 2 H), 7.92 (t, $J = 7.8$ Hz, 1H), 7.89- 7.85 (m, 1H), 7.80 (dd, $J = 7.8$ Hz, 1.0

Hz, 1H), 7.56-7.51 (m, 2H), 7.49-7.44 (m, 1H), 7.37-7.33 (m, 1H). ^{13}C NMR (100 MHz, CDCl_3): δ 156.4, 156.3, 155.7, 149.0, 139.3, 137.7, 136.8, 130.1, 128.9 (2C), 127.2, 123.7, 121.3 (2C), 120.3, 119.3.

2.2 MTT (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Assay

The cytotoxicity of the platinum complexes was investigated on three lung cancerous cell lines, NCI-H522, NCI-H1299, HCC827, and one prostate cancerous cell line RV1. The cell lines were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 units/mL of penicillin and 100 $\mu\text{g/mL}$ streptomycin at 37 °C under a 5% CO_2 atmosphere. The *in vitro* cytotoxicity of the platinum complexes were investigated by the MTT assay [43]. Cells (10×10^4) were added to each well in a 24 well plate followed by 500 μL of medium. The plates were left in incubator for about 48 h or until 95% confluent. A concentrated stock solution of the platinum complex was prepared in DMSO. For each platinum complex, five different concentrations were prepared from the stock solution by diluting with the cell culture medium prior to the use and the concentration of DMSO is less than 1% in all dilutions. A control was prepared by adding DMSO to the cell culture medium. Each concentration was added to four different wells in the same plate. After incubation of 24 h, the drug solution was aspirated and then 500 μL of MTT solution (1 mg/mL) was added to each well and incubated for 4 h. The MTT media was removed, and 500 μL of DMSO was added to each well to dissolve the formazan crystals at room temperature for 10 min. The optical density of each well was recorded using a Bio-Tek Synergy-HT plate reader at a wavelength of 570 nm. The percentage of cell viability was determined for each concentration by dividing the average absorbance of the concentration with the average absorbance of the control (DMSO). The IC_{50} was determined from the plot of the concentration vs cell viability. The reported IC_{50} (μM) value represents an arithmetic mean of three independent experiments.

2.3 Western Blot

HCC827 and RV1 cells (5×10^5 cells) were seeded in their respective p60 culture plates and incubated until 90% confluent. The cells were treated with platinum complexes at concentration of IC_{25} for 24 h. The dead cells and media were aspirated and washed with ice cold PBS (phosphate buffered saline). A solution of 150 μL of RIPA buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 150 mM NaCl, 10 mM Hepes, pH 7.3, 2 mM EDTA, 10 mM sodium pyrophosphate, 20 mM sodium fluoride) was added to each plate. The cells were scrapped and the lysate was collected. The cell lysate was homogenized by passing through a syringe with a needle for 15 to 20 times on ice and allowed to sit on ice for 15 min, centrifuged at 4 °C and 13,200 RPM for 15 min. The supernatant was harvested. The protein concentration was quantified by BCA (bicinchoninic acid) protein assay (Pierce, Rockford, IL) and adjusted to equal concentration. The samples (20 $\mu\text{g/lane}$) were separated by SDS-PAGE using an 8-16% Tris-Glycine gel (Invitrogen) and followed by transferring proteins onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TBST (Tris buffered saline plus 0.1% Tween 20) for 1 h then incubated with primary antibody at 4 °C overnight. Then the membrane was incubated with a HRP (horseradish peroxidase) conjugated secondary antibody for 1 h at room temperature. The membrane was washed with TBST three times for 10 min. The bands were visualized by

using ECL (enhanced chemiluminescence) detection reagents and exposed on blue autoradiography film (Amersham).

2.4 DNA gel Electrophoresis

pUC DNA (3.1 Kbp) was used for this study. Complex 1 was used for the interaction studies and cisplatin was used as a reference control. A diluted solution of plasmid (500 ng/ μ L) was prepared from stock solution (1900 ng/ μ L). Three different concentrations of complex 1 (30, 75, and 150 μ M) and cisplatin (300, 450, and 600 μ M) were prepared in phosphate buffer (20 mM sodium phosphate, 6 mM NaCl, pH 7.2). In a small MicroAmp reaction tube, 1 μ L of diluted DNA solution, 6 μ L of diluted drug solution, and 12 μ L of phosphate buffer were added. A negative control was used by adding DMSO instead of the complex. All the tubes were incubated at 37 °C for 24 h. After 24 h, the reaction was stopped by adding 3 μ L of 6X DNA loading dye. 1% Agarose gel was prepared in Tris-Acetate-EDTA (TAE. 2.0 M Tris-acetate. 0.05 M EDTA, pH 8.3) buffer by dissolving 1 g of agarose into 100 mL of TAE buffer and then adding 10 μ L of EtBr (ethidium bromide, 10 mg/mL). The samples were loaded into the gel along with 1 kb DNA ladder (New England BioLabs) and then ran at 90 V for 90 min in TAE buffer. The gel was visualized using ChemiDoc™ XRS.

2.5 Emission Titration

The emission titration were performed on a PTI QM-4CW fluorimeter system equipped with a xenon lamp, double monochromator for excitation, and a photon counting R928 PMT detector. Platinum complex 1 (4.6×10^{-5} M), was prepared in a phosphate buffer (20 mM sodium phosphate, 6 mM NaCl, pH 7.2). 2 mL of the solution was taken in a cuvette and the emission spectrum ($\lambda_{\text{ex}} = 375$ nm) was recorded. Calf thymus DNA solution (110 μ L, concentration 1.5×10^{-3} M in base pairs) was prepared from the stock DNA solution. Five μ L of the DNA solution was added to the cuvette and the emission spectrum was recorded after 3 min of incubation time at room temperature. The titration was stopped after addition of 100 μ L DNA. A graph was made by plotting the area of the peak versus the molar ratio of the complex and DNA ([Pt]:[DNA]). The integration of the peak area covers the emissions from 450-740 nm.

2.6 Fluorescence Microscopic Cell Imaging

NCI-H522 cells were chosen for this study. The cells were added into a p60 culture plate containing sterilized cover slips and incubated until 90% confluent. The desired platinum complexes at a concentration of IC_{25} were added to each plate and incubated for 24 h. After 24 h, the cover slips were removed and stained with DAPI (4',6-diamidino-2-phenylindole, nucleus staining agent) and mounted on a microscopic slide using anti-fade medium. The images were captured by using a Zeiss Axio M2 microscope equipped with LED (light-emitting diode) lamps. Phosphorescence was visualized from the platinum complexes by using a LED lamp ($\lambda_{\text{ex}} = 470$ nm) and emission filter ($\lambda_{\text{em}} = 509$ nm). The DAPI was visualized using a different LED lamp ($\lambda_{\text{ex}} = 359$ nm) and emission filter ($\lambda_{\text{em}} = 461$ nm).

3. Results and Discussion

3.1. Cytotoxicity of the platinum complexes

The compounds to be investigated are listed in the Figure 1 and they were prepared according to literature procedures described in the experimental section. Complex 12 is based on a tetradentate ligand with two nitrogen donors and two carbon donors. All other complexes are based on tridentate cycometalating ligand with two nitrogen donors and on carbon donor; however, the coordination geometries of these complexes, including the order of the coordination of carbon and nitrogen donors and the size of the metallacycles, are different. Complexes 1-4 have the same order of NCN coordination, but differ in the size of metallacycles. To facilitate discussion, the coordination of 1 [37] is designated as $N\Delta C\Delta N$ -coordination and that of 2-4 as $N\Delta C^*N$ -coordination [31], where $N\Delta C$ and $C\Delta N$ denote a five-membered chelation to the platinum with the C and N donors while C^*N denotes a six-membered chelation. Likewise, 5 [38] and 6 [39] are $C\Delta N\Delta N$ -coordinated and 7 and 8 are $C\Delta N^*N$ -coordinated [30]. Complexes 9-11 can be designated as $N\Delta N^*C$ -coordinated in a similar way [40]. Complex 12 is $C\Delta N^*N\Delta C$ -coordinated [29].

For cytotoxicity study, the complexes were first dissolved in DMSO to prepare a concentrated stock solution, which was then diluted with the cell culture medium to different concentrations for a dose-dependence study using MTT assay [44]. The half maximum inhibitory concentration (IC_{50}) was determined from the dose-dependence of surviving cells after exposure to the platinum complexes for 24 h. Experiment of each sample was repeated three times. The maximum concentration of the complexes tested was 100 $\mu\text{g/ml}$. An MTT assay test was not performed on the complexes beyond that concentration. The final concentration of DMSO in the sample was $<1\%$. A human lung cancer cell line NCI-H522 was used for the initial screening. Cisplatin was used as the comparison. The IC_{50} values are summarized in Table 1. Among twelve complexes, four showed higher anticancer activity than that of cisplatin. They are complexes 1, 2, 7, and 11. $N\Delta C\Delta N$ -coordinated complex 1 displayed highest potency in killing NCI-H522 lung cancer cells with an IC_{50} value of 21.5 μM , less than half of the IC_{50} value of cisplatin. The $N\Delta C^*N$ -coordinated complex 2 was the second most potent. The other two $N\Delta C^*N$ -coordinated complexes 3 and 4 showed slightly lower cytotoxicity than that of cisplatin. The cytotoxicity of $C\Delta N\Delta N$ -coordinated complex 5 was very low, although $C\Delta N^*N$ -coordinated 7 and $N\Delta N^*C$ -coordinated 11 showed comparable toxicity to that of cisplatin.

The drastic difference in the cytotoxicity of 1 and 5 is noteworthy because the two compounds are constitutional isomers and share essentially the same planar geometry. The other $C\Delta N\Delta N$ -coordinated complex 6 showed much lower cytotoxicity than that of cisplatin. Complexes 8, 10, and 12 also showed much lower cytotoxicity compared with cisplatin.

The complexes that had shown potency to NCI-H522 cells were then tested on cisplatin-resistant RV1 human prostate cancer cells. Complex 5 was also included as a direct comparison to 1. Once again, complex 1 demonstrated superior cytotoxicity with an IC_{50} value of 37.7 μM . Complex 5 contrasted sharply to 1, showing an IC_{50} value greater than $> 236 \mu\text{M}$. $N\Delta C^*N$ -coordinated complex 2 showed somewhat lower cytotoxicity IC_{50} value of 79.8 μM . The IC_{50} value for Complex 7 was 111.9 μM . All other complexes tested were

ineffective. The drastic difference between 1 and 5 prompted us to test them side by side on two other human lung cancer cell lines NCI-H1229 and HCC827. As the results showed (Table 1), 1 showed consistently higher anticancer potency in the two cell lines while 5 remained ineffective. It should be emphasized that cisplatin showed much lower cytotoxicity against NCI-H1299, and was ineffective to HCC827 even at a high concentration of 666 μ M.

Several trends were revealed from the cytotoxicity screening. Firstly, the presence of labile chloride ligand seems to be critical to the antitumor activity, because all three compounds 8, 10, and 12 without a chloride ligand were not effective against NCI-H522 cancer cells. It can be speculated that dissociation of the Pt–Cl bond may be necessary to create an active site for the complex to bind with DNA, a widely accepted biological target for cisplatin and other platinum complexes, and exert its cytotoxicity. Secondly, the $\text{N}\Delta\text{C}\Delta\text{N}$ -coordinated complex 1 is much more potent than $\text{N}\Delta\text{C}^*\text{N}$ -coordinated complexes. Two factors may be considered here, (1) $\text{N}\Delta\text{C}^*\text{N}$ -coordinated complexes 2–4 are less planar than complex 1 and (2) the binding site in the $\text{N}\Delta\text{C}^*\text{N}$ -coordinated complexes is less accessible because of the steric hindrance. After dissociation of the chloride, the cationic platinum in 1 is more widely open as the N–Pt–N angle is 198.9° (161.1°) in 1 [37], while the angle in the $\text{N}\Delta\text{C}^*\text{N}$ -coordinated complex 2 [31] is about 187.1° (173.9°). A planar geometry would facilitate the intercalative interaction with DNA. However, these considerations cannot be used to explain the fact that $\text{C}\Delta\text{N}\Delta\text{N}$ -coordinated complex 5 was less potent than $\text{C}\Delta\text{N}^*\text{N}$ and $\text{N}\Delta\text{N}^*\text{C}$ -coordinated complexes 6, 7, 9, and 11.

Perhaps the drastic difference in cytotoxicity between 1 and 5 is the most striking: 1 was the most potent to the cancer cells tested while 5 was not effective on any of those cancer cells. As mentioned before, geometrically, 1 and 5 have no significant difference because both complexes possess a nearly perfect planar structure, which is conceived to facilitate the interaction with DNA through intercalation. The low cytotoxicity of 5 was also noted by Che's group and modification of 5 by replacing the chloride with other functional ligands was found to substantially improve the cytotoxicity of the modified complexes [28]. Structurally, the only difference in 1 and 5 is the order of the coordination of carbon and nitrogen donors to the platinum, namely $\text{N}\Delta\text{C}\Delta\text{N}$ or $\text{C}\Delta\text{N}\Delta\text{N}$ -coordination. Since the dissociation of the chloride ligand may be critical to the cytotoxicity of the complexes, the bond strength of the chloride may play a role. In $\text{N}\Delta\text{C}\Delta\text{N}$ -coordinated complex 1, strong trans effect of the carbon donor [45] would weaken the Pt–Cl bond and make the dissociation of the chloride more facile. The weaker Pt–Cl bond in $\text{N}\Delta\text{C}\Delta\text{N}$ and $\text{N}\Delta\text{C}^*\text{N}$ -coordinated complexes 1 and 2 than those in $\text{C}\Delta\text{N}\Delta\text{N}$ and $\text{C}\Delta\text{N}^*\text{N}$ or $\text{N}\Delta\text{N}^*\text{C}$ -coordinated complexes 5, 7, and 9 are clearly indicated by their longer bond lengths (Table 2). Indeed, the rate for the displacement of chloride in 1 by thiourea was reported to be 445 times faster than that in 5 [45]. Another difference between $\text{N}\Delta\text{C}\Delta\text{N}$ and $\text{C}\Delta\text{N}\Delta\text{N}$ -coordinated compounds is the electron density distribution on the organic ligands, due to the different order of the connection of the benzene ring and two pyridine rings. Pyridine ring is electron-deficient and can serve as a π electron acceptor, while the benzene ring can serve as a π electron donor. The intercalation into DNA's base pairs is through a π – π stacking interaction, in which the conjugated plane of the intercalator and the plane of the base pair(s) have a face to face interaction. It can be speculated that the electron density distribution should match each other for a favorable

electronic donor-acceptor attraction. It is possible that the electron density distribution in the $N\Delta C\Delta N$ structure is more favorable to the Π – Π stacking with the base pair(s).

3.2. Interaction with DNA

It is generally believed that the primary target of the platinum complexes is DNA. The hydrolysis of platinum complexes yields cationic, highly electrophilic species that can covalently bond to the N7 atom of guanine and adenine bases in DNA. The interactions of the platinum complex 1 with DNA were studied by the gel electrophoresis of plasmid DNA and emission titration with calf thymus DNA (ct-DNA)

Interaction with plasmid DNA—The platinum complexes can interact with supercoiled plasmid DNA and induce a cleavage or nicks in the supercoiled DNA, forming a relaxed DNA. The two forms of DNA could be separated and quantified by using agarose gel electrophoresis. When the electric field is applied to the gel, the supercoiled DNA migrates faster than the nicked/cleaved DNA. Complex 1 was chosen for this study and cisplatin was used as a reference for this experiment. Different concentrations of complex 1 and cisplatin were prepared and incubated with pUC DNA (same concentration of DNA) for 24 h at 37 °C in phosphate buffer. After 24 h, the reaction was stopped and the samples were analyzed by gel electrophoresis. The results indicate that the cyclometalated platinum complex 1 and cisplatin interacted with the DNA at 50 μ M concentration, as seen in Figure 2. However, we did not observe two forms of the plasmid. At a lower concentration, the complex 1 had no drastic effect on the plasmid DNA. But at a concentration of 50 μ M, the intensity of the DNA bands were greatly reduced and smeared, which are different from the bands treated with the drug cisplatin. Higher concentrations of cisplatin were used when compared to the complex 1. The mobility of the cisplatin-treated DNA was increased but no smearing was observed. These results suggest that the mode of interact with DNA might be different for cyclometalated complex 1 and cisplatin. Added features to the $N\Delta C\Delta N$ -coordinated complex such as planar geometry and hydrophobicity may result in other types of additional DNA interaction, especially intercalation. Whether a dual DNA interaction, namely covalent binding and intercalation, exists or one interaction is assisted by the other is unclear.

Emission titration with ct-DNA—Electronic spectroscopy has been utilized as an important tool in analyzing DNA-Pt binding studies. Emission titration studies of the platinum complex 1 with ct-DNA were performed. Briefly, in a cuvette with a fixed concentration of 1 in a phosphate buffer (2 mL), aliquots of known concentration of ct-DNA (5 μ L) were added and the emission spectra were recorded. The emission intensity of the complex alone in the buffer was very low, but increased as the concentration of DNA increased, as shown in Figure 3. The enhancement of emission intensity is the indication of binding of the complex to ct-DNA. The enhancement of the emission intensity can be related to the extent to which the complex entered the hydrophobic environment inside DNA. In addition, the motion of the complex in the binding site is restricted, which can lead to decreased nonradiative vibrational relaxation and increased emission intensity.

In this titration experiment, the first aliquot of the DNA added was twice the concentration of the metal complex ($[Pt]:[DNA] = 1:1.9$) and then gradually increased up to fifteen

equivalents. However, when the titration was started with a very low concentrations of DNA aliquots, ([Pt]:[DNA] = 1:0.08 equivalents), there was an initial decrease in the emission intensity until 0.25 equivalents of DNA was added and then a slow increase in the emission intensity was observed, as shown in Figures 4 by a plot of peak areas versus the concentration ratios. It can be clearly observed that the peak area decreased initially until the molar/ities of platinum complex and DNA was 1:0.25 and then the peak area increased with increase in concentration of DNA. We do not have an explanation to such phenomenon. The emission titration data do not fit the models commonly used to estimate the DNA binding constant of cyclometalated platinum complexes [26, 28]. More investigations are required to gain further information on the DNA binding of compound 1.

3.3. Western blot analysis

Western blot experiments were performed to identify the cell signaling pathways that were activated. In a p60 plate, the desired cells were seeded and cultured until the plate was 90% confluent. A solution of the complex was prepared in growth medium at half the IC₅₀ value of the complex in that particular cell line and added to the plate and incubated for 24 h. A control (cells treated with DMSO) was included in the same experiment. After 24 h, the cells were lysed and the proteins were extracted and stored at -80 °C. All experiments were repeated at least twice in different times. Poly(ADP-ribose) polymerase (PARP), caspase, and p53 proteins were tested. GAPDH was probed as a loading control.

Activation of caspase proteins—Caspases (cysteine-aspartic proteases) are a family of cysteine proteases. These proteins play a prominent role in apoptosis, necrosis, and inflammation [46]. Usually the caspases are in inactive form inside the cell. The activation of caspases requires them to be cleaved into active forms which are then responsible for triggering the desired function. The initiator caspases are activated by different pathways. The activated initiator caspases then activate the effector caspases such as caspase 3, 6, or 7. The activated effector caspases can then activate PARP that triggers the apoptosis.

Activation of caspase 7 was observed in RV1 and HCC827 cell lines when treated with complexes 1, as shown in Figure 5. As a reference, cisplatin is also included in these experiments. A corresponding negative control was included for the platinum complexes and cisplatin. It was very clear that caspase 7 was activated in both RV1 and HCC827 cell lines. In both cell lines, complex 1 was added in two different concentrations to determine the dose-dependence of the activation of caspase 7. There was increased band intensity in lane 3 (complex 1, higher concentration) when compared with lane 2 (complex 1, lower concentration), which indicates a clear dose-dependency in both RV1 and HCC827 cell lines. A strong activation of caspase 7 was also observed in RV1 and HCC827 cells when treated with two different concentrations of *cis*-platin (50 and 100 µg/mL).

Activation of PARP—PARP is a family of proteins involved in a number of cellular processes, mainly DNA repair and apoptosis. PARP is activated during cellular stress and activated PARP can trigger apoptosis. The activation of PARP is triggered by the effector caspases. The activated PARP is responsible for apoptotic cell death.

Activated PARP was observed in the cells when treated with cyclometalated platinum 1, in both RV1 and HCC827 cell lines. Two concentrations of complex 1 (9 and 18 $\mu\text{g/mL}$) were added in RV1 cell line and a dose-dependent increase of PARP activation was observed, as seen in Figure 6 (top). As the concentration of complex 1 increased, the band intensity of the activated (cleaved) PARP (89 KDa) was greatly increased. Cisplatin also activated the PARP, but the concentration of activated PARP did not increase as the concentration of the cisplatin increased (lanes 6 and 7).

The expression of PARP in HCC827 cell line was also tested with complex 1. At two different concentrations (6 and 12 $\mu\text{g/mL}$), the amount of cleaved PARP was significantly low, but was still present when compared with the control as shown in Figure 6 (bottom). A dose-dependent increase was observed with complex 1. Cisplatin also increased the cleaved PARP, when compared with the control.

It can be concluded that PARP is activated in RV1 and HCC827 cell lines when treated with cyclometalated platinum complex 1. In addition, there was a dose-dependent increase in the activation of PARP by complex 1. Since the activation of PARP is the last step in triggering apoptosis, the platinum complex exerted its cytotoxicity to the cells by triggering apoptosis. Cisplatin can also trigger the activation of PARP.

Activation of p53 protein—P53 is a tumor suppressor protein that facilitates DNA repair before DNA replication. It is a transcription factor that is considered as a “guardian of the genome” because it can activate a host of other genes that lead to cell cycle arrest and DNA repair [47]. P53 protein is mutated in more than 50% of all human tumors and therefore plays a central role in chemotherapy-induced apoptosis. Several studies have shown that sensitivity (toxicity) to cisplatin usually correlates with the presence of wild type p53, while the presence of mutant p53 is related to cisplatin resistance. However, a clear relationship between the sensitivity (toxicity) to cisplatin and p53 functioning has not been established yet [48]. RV1 and HCC827 cell lines were used for the western blot experiments as shown in figure 7.

In RV1 cell line, complex 1 was found to activate the phospho-p53 (S15) protein, because the signal of a phosphorylated form of phospho-p53 at S15 was increased. In addition, there was a dose-dependent increase in the activation of phospho-p53 levels when treated with complex 1 (9 and 18 $\mu\text{g/mL}$). Cisplatin in this cell line was found to activate the p53 protein, but as the concentration of cisplatin increased, there was a decrease in activation of p53 although the overall increase in the phospho-p53 level was higher when compared with the cyclometalated platinum complex 1. In HCC827 cell line, there was a strong expression of phospho-p53 (S15) when treated with complex 1. There was a clear dose-dependence observed as shown in Figure 7 (bottom). Cisplatin did not activate the p53 protein in this cell line.

3.4. Fluorescence microscopy study

The cellular uptake of platinum complexes was observed by fluorescent microscope. The fixed cells were observed under the fluorescent microscope and the images of the cells were captured. The NCI-H522 cells treated with complexes 1, 2, and 12 are shown in Figure 8.

The three images in the top row represent the control (incubated with DMSO) in which the phosphorescence is negligible (mostly background), DAPI staining to locate the nucleus, and a merged picture of both the emissions. The phosphorescent emission from the complexes 1, 2, and 12 is very strong, so it can be seen clearly that a majority of the complexes was localized in the cytoplasm.

Complex 12, with a $C\Lambda N^*N\Lambda C$ tetradentate coordination pattern, devoid of a labile Pt–Cl bond, and lack of cytotoxicity, could also enter into the cells and be concentrated in the cytoplasm, suggesting that a Pt–Cl bond is necessary for the complexes to exert the toxicity. On the other hand, complex 12 can be tailored as a molecular tag or an imaging agent. The brighter spots may be attributed to the aggregation of the luminescent complexes.

4. Conclusion

The cytotoxicity of various CNN and NCN-coordinated platinum complexes toward human lung and prostate cancer cells has been studied. The results indicate, in these square planar platinum complexes based on tridentate CNN and NCN ligand, that the dissociation of a labile monodentate ligand such as chloride is critical to exhibiting high cytotoxicity, because the complexes with an acetylide ligand did not show any effectiveness. Most notably, the $N\Lambda C\Lambda N$ -coordinated platinum complex 1 showed the most promising anti-cancer activity against all the cancer cells tested in this study, higher than the isomeric $C\Lambda N\Lambda N$ -coordinated complex 5 was essentially ineffective toward all these cancer cells. This drastic difference was attributed to the different *trans* effect experienced by the labile chloride ligand in complex 1 and 5. The stronger *trans* effect of the carbon donor in 5 makes the chloride ligand more labile so the dissociation could occur readily to generate an active binding site to interacting with DNA. The DNA binding study, cell uptake, and western blot analysis indicated that the platinum complexes can interact with the DNA and trigger the apoptosis by activation of PARP via caspase 7 pathway. Human lung cancer is the second most common cancer with very low 5-year survival rate (16% during 2001–2007). Cisplatin has shown variable cytotoxicity against different types of lung cancer cells and some lung cancers displayed great degree of cisplatin resistance [49,50]. The higher cytotoxicity displayed by the complex 1 against human lung and prostate cancers may open a new avenue to the discovery of new platinum-based drugs that target the cancers showing resistance to traditional cisplatin and its analogues.

Acknowledgments

We thank Zhe Lu and Christi Boykin for their technical assistance. This work was partially supported by National Institute of Health grant ES016888 to Y.-H. Chen.

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Highlights

Higher anticancer activity displayed by the $N\Delta C\Delta N$ -coordinated platinum complex compared to cisplatin

Drastic difference in cytotoxicity displayed by $N\Delta C\Delta N$ - and $C\Delta N\Delta N$ -coordinated platinum complexes

Potentially important role of trans effect in cytotoxicity of $N\Delta C\Delta N$ -coordinated platinum complexes

Activation of PARP via caspase 7 observed in the cell apoptosis.

Figure 1.
Structures of cyclometalated platinum complexes 1-12.

Figure 2.

Gel electrophoresis of plasmid DNA treated with complex 1 and cisplatin.

Figure 3.
Emission titration of the interaction of complex 1 with ct-DNA ([Pt]:[DNA] = 0, 1.9, 3.8, 5.6, 7.5, 8.4, 10.3, 12.2, 15.0 equivalents).

Figure 4.

Titration plot of interaction of complex 1 with calf Aymus DNA (concentration ratio versus area of the peak).

Figure 5.

Activation of caspase 7 in RV1 cell line (top) and HCC827 cell line (bottom) by complex 1 (C represents the negative control).

Figure 6.

Activation of PARP in RV1 cell line (top) and HCC827 cell line (bottom) by complex 1 (C represents the control).

Figure 7.

Phospho-p53(S15) activation in RV1 cell line (top) and HCC827 cell line (bottom) by complex 1 (C represents the control).

Figure 8.

Images of comiplexes in NCI-H522 cells. From top to bottom, control, Complex 1, Complex 2, Complex 12. The platinum complexes were incubated with NCI-H522 cells on a cover slip for 24 h. 24 h, the cover slips were mounted on a slide with anti-fade medium. The nucleus was stained with DAPI.

Table 1

Cytotoxicities of platinum complexes in human carcinoma NCI-H522 (lung cancer), NCI-H1299 (lung cancer), HCC827 (lung cancer), and RV-1 (prostate cancer) cells

Complex	IC ₅₀ (μM), 24 h				Coordination
	NCI-H522	RV1	NCI-H1299	HCC827	
1	21.5 ± 2.1	37.7 ± 3.2	19.4 ± 2.2	22.4 ± 2.0	N ⁺ C ⁻ N
2	35.0 ± 0.4	79.8 ± 1.4			N ⁺ C ⁻ *N
3	59.3 ± 1.3	>184			N ⁺ C ⁻ *N
4	63.4 ± 0.3	>165			N ⁺ C ⁻ *N
5	>236	>236	>236	>236	C ⁻ N ⁺ N
6	85.6 ± 4.3	>204			C ⁻ N ⁺ N
7	45.3 ± 5.7	111.9 ± 2.3			C ⁻ N ⁺ *N
8	>162				C ⁻ N ⁺ *N
9	51.6 ± 5.8	>181			N ⁺ N ⁺ *C
10	>162				N ⁺ N ⁺ *C
11	42.8 ± 2.8	>184			N ⁺ N ⁺ *C
12	>169				C ⁻ N ⁺ *N ⁺ C
cisplatin	49.8 ± 1.6	>333	163.0 ± 4.1	>666	

Table 2

The Pt-Cl bond lengths of NCN and CNN-coordinated complexes determined by X-ray crystallography.

Complex	1	2	5	7	9
Pt-Cl (Å)	2.417 [37]	2.4395 [31]	2.312 [38]	2.3029 [30]	2.3140 [40]