Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Medicinal Chemistry 40 (2005) 772–781
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
www.elsevier.com/locate/ejmech

Synthesis, characterization, activities, cell uptake and DNA binding of trinuclear complex: [{*trans*-PtCl(NH_3)}$_2$$\mu$-{*trans*-Pt($NH_3$)(2-hydroxypyridine)-($H_2N(CH_2)_6NH_2)_2$]$Cl_4$

Howard Cheng [a], Fazlul Huq [a,*], Philip Beale [b], Keith Fisher [c]

[a] *School of Biomedical Sciences, Cumberland Campus, C42, The University of Sydney, East Street, PO Box 170, Lidcombe, NSW 1825, Australia*
[b] *RPAH, Missenden Road, Camperdown, NSW, Australia*
[c] *School of Chemistry, The University of Sydney, Sydney, NSW 2006, Australia*

Received 7 December 2004; received in revised form 28 February 2005; accepted 7 March 2005

Available online 20 April 2005

Abstract

The trinuclear complex: [{*trans*-PtCl(NH_3)}$_2$$\mu$-{*trans*-Pt($NH_3$)(2-hydroxypyridine)-($H_2N(CH_2)_6NH_2)_2$]$Cl_4$ (code named CH9) has been synthesized and characterized. The activity of the compound against human ovarian cancer cell lines: A2780, A2780^{cisR} and A2780^{ZD0473R}, cell up take, level of binding with DNA and nature of its interaction with pBR322 plasmid DNA have been determined. Although the compound is found to be less active (about a half time as active as cisplatin) against the parent ovary cell line A2780, it is found to be more active than cisplatin against resistant cell lines: A2780^{cisR} (3.6 times more) and A2780^{ZD0473R} (3.4 times more). The higher activity of CH9 against the resistant cell lines suggests that the compound has been able to overcome multiple mechanisms of resistance operating in A2780^{cisR} and A2780^{ZD0473R} cell lines. Like other multicentered complexes, the compound is believed to form a range of interstrand GG adducts with duplex DNA that induces permanent global changes in the DNA conformation. This binding is different from that of cisplatin and ZD0473 that form mainly intrastrand adducts, inducing a local kink in a DNA strand. Increasing prevention of BamH1 digestion of form I and form II pBR322 plasmid DNA with the increase in concentration of CH9 provides support to the idea that global changes in DNA conformation are induced as a result of its interaction with the compound.

Keywords: Platinum; Anticancer activity; Cell culture; Cell uptake; DNA binding; Gel electrophoresis

1. Introduction

Although cisplatin is a widely used anticancer drug [1,2], it has a limited spectrum of activity due to the development of drug resistance [3,4] and a number of side effects including neurotoxicity, nephrotoxicity, ototoxicity, myelosuppression, nausea and vomiting [5,6]. The success of cisplatin and its drawback have stimulated the search for newer and better tumor active platinum compounds with improved pharmacological properties [7]. Although thousands of platinum compounds have been synthesized in the past 30 years, only over 28 have entered clinical trials [8]. Currently attention is given to rule breaker platinum compounds primarily with the aim of widening the spectrum of activity [9,10]. One such class of compounds are the polynuclear platinum complexes [11] that contain two or more platinum units linked together by diaminoalkane chains [12]. A notable example is BBR3464 that has been found to circumvent the inherent or acquired cisplatin-resistance in vitro and in vivo in a panel of human adult tumor models [13]. It consists of three *trans*-platinum units joined together by two 1,6-diaminohexane chains. Only the two terminal platinum units in

Abbreviations: AAS, atomic absorption spectrophotometry; CH9, [*trans*-PtCl(NH_3)}$_2$$\mu$-{*trans*-Pt($NH_3$)(2-hydroxypyridine)-($H_2N(CH_2)_6NH_2)_2$]$Cl_4$ Salmon sperm DNA; Cisplatin, *cis*-dichlorodiammineplatinum(II); DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; EDTA, ethylene diamine tetraacetic acid; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; 1 × TAE buffer, 0.05 M Tris base + 0.05 M glacial acetic acid + 1 mM EDTA, pH 8.0; PBS, phosphate-buffered saline.

* Corresponding author. Tel.: +61 2 9351 9522; fax: +61 2 9351 9520.
E-mail address: f.huq@fhs.usyd.edu.au (F. Huq).

0223-5234/$ - see front matter © 2005 Elsevier SAS. All rights reserved.
doi:10.1016/j.ejmech.2005.03.009

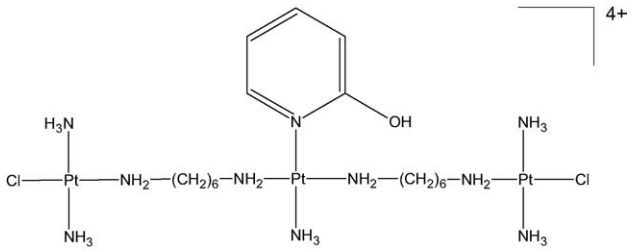


Fig. 1. Structure of CH9: [{*trans*-PtCl(NH_3)}$_2\mu$-{*trans*-Pt(NH_3)(2-hydroxypyridine)-($H_2N(CH_2)_6NH_2)_2$]Cl_4. Only the tetrapositive ion is shown, the valencing anions (4 Cl^-) are not included.

BBR3464 undergo covalent binding with DNA whereas the central platinum unit undergoes only non-covalent interactions such as electrostatic interaction and hydrogen bonding [14–16]. Based on the idea that the replacement of the central platinum unit with other suitable metal units may not significantly alter the covalent interactions of the terminal platinum units (although it could have a subtle effect on the non-covalent interactions), Daghriri et al. [17] synthesized a number of Pt–Pd–Pt trinuclear complexes all of which were found to display significant activity against human ovarian cancer cell lines (one being about twenty times as active as cisplatin against A2780^{cisR} cell line). The compounds are believed to overcome multiple mechanisms of resistance operating in A2780^{cisR} and A2780^{ZD0473R} cell lines. Since the central metal unit in trinuclear complexes takes part only in non-covalent interactions, Huq et al. hypothesized that the presence of one or more planaramine ligands bonded to the central metal ion would introduce additional types of non-covalent interactions such as stacking interaction with nucleobases in DNA that may influence the level and spectrum of activity of the compounds. Thus, they prepared the compound: [{*trans*-PtCl(NH_3)}$_2\mu$-{*trans*-Pd(NH_3)(2-hydroxypyridine)-($H_2N(CH_2)_6NH_2)_2$]Cl_4 (code named CH25) which was found to exhibit significant anticancer activity against ovarian cancer cell lines: A2780, A2780^{cisR} and A2780^{ZD0473R}—about 45 times as active as cisplatin against A2780 cell line, about 76 times as active as cisplatin against A2780^{cisR} cell line and about seven times as active as cisplatin against A2780^{ZD0473R} cell line [18]. In this paper, we report on the synthesis, characterization, activity, cell uptake, and level and nature of binding with DNA of [{*trans*-PtCl(NH_3)}$_2\mu$-{*trans*-Pt(NH_3)(2-hydroxypyridine)-($H_2N(CH_2)_6NH_2)_2$]Cl_4 denoted as CH9 (Fig. 1).

2. Materials and methods

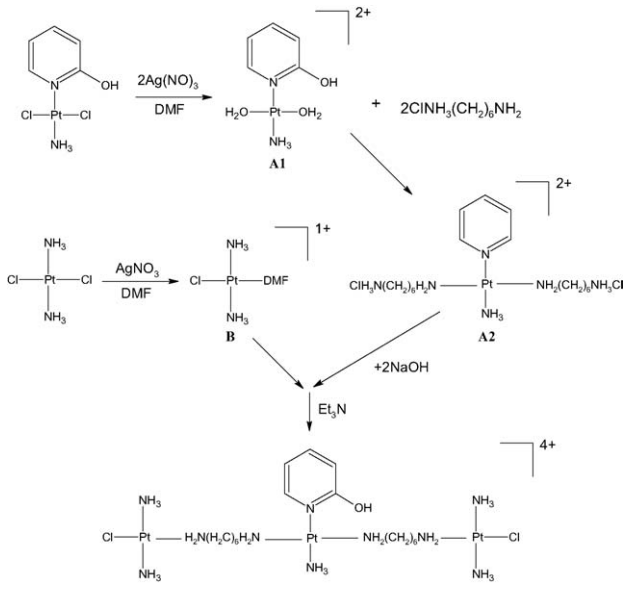
2.1. Materials

Potassium tetrachloroplatinate $K_2[PtCl_4]$, *N*,*N*-dimethylformamide (DMF) [C_3H_7NO], 2-hydroxypyridine and 1,6-diaminohexane were obtained from Sigma Chemical Company, St. Louise, USA; acetone [$(CH_3)_2CO$] and silver nitrate ($AgNO_3$) were obtained from Ajax Chemicals, Auburn, NSW, Australia; methanol [CH_3OH], ethanol [C_2H_5OH], dichloromethane [CH_2Cl_2] were obtained from Merck Pty. Limited, Kilsyth, Vic., Australia. pBR322 plasmid DNA was purchased from ICN Biomedicals, Ohio, USA. Foetal calf serum, 5 × RPMI 1640, 200 mM L-glutamine and 5.6% sodium bicarbonate were obtained from Trace Biosciences Pty Ltd., Australia. Other reagents were obtained from Sigma-Aldrich Pty Ltd., NSW, Australia. Commercially available JETQUICK Blood DNA Spin Kit/50 used to isolate high molecular weight DNA from cell pellet was obtained from Astral Scientific, Australia.

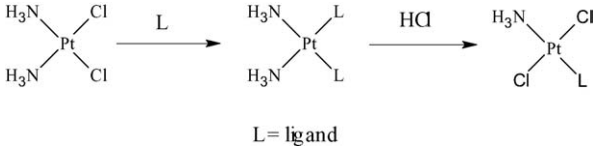
2.2. Synthesis

CH9 was prepared using step-up method of synthesis starting with the compound *trans*-(2-hydroxypyridine)(ammine)dichloroplatinum(II) corresponding to the central platinum unit, as shown in Scheme 1.

The first step in the synthesis of CH9 was the preparation of *trans*-(2-hydroxypyridine)(ammine)dichloroplatinum(II) to serve as the starting material for CH9. The compound was prepared according to previously reported procedure [19] described below. The method used is based on the difference in *trans* effect of halide and amine ligands in platinum(II) complexes, allowing selective substitution and hence control of the stereochemistry [20] (Scheme 2).



Scheme 1. Steps in the synthesis of CH9.



Scheme 2. Synthesis of *trans*-platinum complexes-L stands for a ligand.

2.2.1. Preparation of trans-(2-hydroxypyridine)(ammine)dichloroplatinum(II)

One mmol of potassium tetrachloropalladate(II) was dissolved in 5 ml of DMF. The solution was stirred and heated at 60 °C until it became clear. One mmol of 2-hydroxypyridine was added to the solution. The mixture was stirred for 6 h at 60 °C after which the solution became dark green in color. Then 0.14 ml of concentrated ammonia solution was added slowly to the solution with stirring. The mixture was stirred for further 17 h at room temperature. The precipitate of *trans*-(2-hydroxypyridine)(ammine)dichloropalladium(II) was collected by filtration at the pump, washed with mQ water and ice cold ethanol and air-dried.

2.2.2. Preparation of CH9

It may be noted that all the steps in the preparation of CH9 (given in Scheme 1) were carried out in the dark [21,22]. The method is described as follows.

One millimol of *trans*-(2-hydroxypyridine)(ammine)-dichloroplatinum(II) was dissolved in 5 ml of DMF. Silver nitrate (2 mmol; 0.34 g) was added to the mixture with stirring. Stirring was continued overnight at room temperature. The mixture was centrifuged at 4800 rpm for 15 min to collect the light yellow supernatant containing *trans*-(2-hydroxypyridine)(ammine)bis(aquo)platinum(II)$^{2+}$ that would serve as the starting material for the synthesis of CH9. This solution was marked as **A1.** Two millimols (0.232 g) of 1,6-diaminohexane was dissolved in 2 ml of mQ to which was added 0.2 ml 10 M hydrochloric acid to change one of the two amino groups into hydrochloride form. This was added to solution **A1** prepared earlier. The mixture was stirred at room temperature overnight to produce solution marked as **A2**.

2.2.3. Preparation of terminal units

Two millimols (0.6 g) of transplatin was dissolved in 5 ml of DMF to produce a yellow solution. Two millimols (0.34 g) of sliver nitrate was added to the solution with stirring. The mixture was stirred overnight at room temperature. It was then centrifuged at 4800 rpm for 15 min to collect the supernatant (marked as **B**) which was kept at –16 °C for use in a later step. The low temperature prevented any hydrolysis [23]. Two milliliters of 1 M NaOH were added to solution **A2** with stirring to unblock the two terminal amino groups. Stirring was continued for 30 min. Solution **B** was added to solution **A2** with stirring at room temperature. Stirring was continued until precipitate appeared. Equal volume of dichloromethane as that of the solution was added to reduce the solubility of CH9. The precipitate of CH9 was collected by filtration at the pump, washed with mQ water and ice cold ethanol.

2.3. Methods

2.3.1. Characterization

C, H, N and Cl were determined out using the facility at the Australian National University. Platinum was determined by graphite furnace atomic absorption spectroscopy (AAS) using the Varian Spectraa-20 Atomic Absorption Spectrophotometer. Infrared spectra were collected using a Bruker IFS66 spectrometer equipped with a Spectra-Tech Diffuse Reflectance Accessory (DRA), an air-cooled DTGS detector, a KBr beamsplitter with a spectral range of 4000–650 cm^{-1}. The instrument was run under a vacuum during spectral acquisition. Spectra were recorded at a resolution of 4 cm^{-1}, with the co-addition of 128 scans and a Blackman–Harris 3-Term apodization function was applied. Prior to analysis the samples were mixed, and lightly ground, with finely ground spectroscopic grade KBr. The spectra were then manipulated using the Kubelka–Munk mathematical function in the OPUS™ software to convert the spectra from reflectance into absorbance. Raman spectra were collected using a Bruker RFS100 Raman spectrometer equipped with an air-cooled Nd:YAG laser emitting at a wavelength of 1064 nm, and a liquid nitrogen cooled germanium detector with an extended spectral band range of 3500–50 cm^{-1}. One hundred eighty degree sampling geometry was employed. Spectra were recorded at a resolution of 4 cm^{-1}, with the co-addition of 100 scans at a laser power of 0.065 mW. A Blackman–Harris 4-Term apodization function was applied and the spectra were not corrected for instrument response. To obtain mass spectra, solution of CH9, made in 10% DMF and 90% methanol, were sprayed into a Finnigan LCQ ion trap mass spectrometer. It was not possible to obtain ^{1}H NMR spectrum of CH9 because of its poor solubility in dimethyl sulfoxide-d_6 (DMSO-d_6) and deuterated water.

2.3.2. Molar conductivity

The molar conductivity value for CH9 was determined using PW9506 digital conductivity meter available at the School of Chemistry, University of Sydney. The compound was dissolved in 1:5 mixture of DMSO and water. The measurements were done at concentrations: 0.5, 0.25, 0.1 and 0.01 mM. The molar conductivity (Λ_m) was calculated as $\Lambda_m = k/c$ where k is the specific conductivity and c is the concentration [24]. The molar conductivity values were plotted against concentration and the curve was extrapolated to zero concentration to give the limiting value.

2.3.3. Interaction with pBR322 plasmid DNA

Interaction of CH9 and cisplatin with pBR322 plasmid DNA was studied by agarose gel electrophoresis. The method used was a modification of that described by Stellawagen [25]. Solutions of pBR322 plasmid DNA (at concentration 0.5 μg ml^{-1}) were incubated with increasing concentrations of compounds ranging from 1.25 to 15 μM in a shaking water bath at 37 °C for 4 h 16 μl aliquots of drug–DNA mixtures containing 0.6 μg of DNA was loaded onto the 1% gel and electrophoresis was carried under TAE buffer for 2 h at 5 V cm^{-1}. At the end of electrophoresis, the gel was stained in the same buffer containing ethidium bromide (0.5 mg ml^{-1}). The gel was visualized under UV light using the Bio-Rad Trans illuminator IEC 1010. The illuminated gel was photo-

graphed with a Polaroid camera (a red filter and Polaroid type of film was used).

2.3.4. BamH1 digestion

BamH1 is known to recognize the sequence G/GATCC and hydrolyze the phosphodiester bond between adjacent guanine sites [26]. pBR322 contains a single restriction site for BamH1 [27] which converts pBR322 plasmid DNA from supercoiled form I and singly-nicked circular form II to linear form III. In this experiment, a same set of drug–DNA mixtures as that described previously, was first incubated for 4 h in a shaking water bath at 37 °C and then subjected to BamH1 (10 units μl^{-1}) digestion. To each 20 μl of incubated drug–DNA mixtures were added 3 μl of 10 × digestion buffer SB followed by the addition of 0.2 μl BamH1 (2 units). The mixtures were left in a shaking water bath at 37 °C for 1 h at the end of which the reaction was terminated by rapid cooling. The gel was subsequently stained with ethidium bromide, visualized by UV light then a photograph of the gel was taken as described previously.

2.3.5. Cytotoxicity assays

The human ovarian cancer cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ were grown in 25 cm^2 tissue culture flasks in an incubator at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air. The cells were maintained in logarithmic growth phase in complete medium consisting of RPMI 1640, 10% heat-inactivated fetal calf serum, 20 mM hepes, 0.112% bicarbonate, and 2 mM glutamine without antibiotics [28]. Cytotoxicity was determined using MTT growth inhibition assay [29]. Between 8000 and 12,000 cells, depending on the growth characteristics of the cell line, were seeded into the wells of the flat-bottomed 96-well culture plate in 10% FCS/RPMI 1640 culture medium. The plate was then incubated for 24 h at 37 °C in a humidified atmosphere to allow them to attach. CH9 and cisplatin were first dissolved in a minimum amount of DMSO and DMF, respectively, then diluted to the required concentrations by adding mQ water and finally filtered to sterilize. A serial fivefold dilutions of the drugs (ranging from 0.004 to 16 μM in the case of CH9 and 0.05–10 μM in the case of cisplatin) in 10% FCS/RPMI 1640 medium were prepared and added to equal volumes of cell culture in quadruplicate wells, then left to incubate under normal growth conditions for 72 h. The inhibition of the cell growth was determined using the MTT reduction assay [29]. Four hours after the addition of MTT solution (50 μl per well of 1 mg ml^{-1} MTT solution), the cells were dissolved in 150 ml of DMSO and read with a plate reader (Bio-Rad Model 3550 Microplate Reader). The IC_{50} values were obtained from the results of quadruplicate determinations of at least three independent experiments.

2.3.6. Drug uptake and binding with DNA

The method used for cell subculture was a modification of that described by Freshney [28] and the method used for cell treatment as applied to the determination drug uptake and DNA was a modification of that described by Di Blasi et al. [6]. The platinum complexes (at 50 μM final concentration) were added to culture plates containing exponentially growing A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ cells in 10 ml 10% FCS/RPMI 1640 culture medium (cell density = 1×10^6 cells ml^{-1}). The cells containing the drugs were incubated for 2, 4 and 24 h at the end of which cell monolayers were trypsinized and cell suspensions (10 ml) was transferred to centrifuge tube and spun at 3500 rpm for 2 min at 4 °C. The experiment was carried out for both CH9 and cisplatin. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and the pellets were stored at –20 °C until assayed. At least three independent experiments were performed.

2.3.7. Drug accumulation in cells

Following drug incubation the cell pellets were suspended in 0.5 ml 1% triton-X, held on ice then sonicated. Total intracellular platinum contents were determined by graphite furnace AAS using a variant of standard addition technique [30].

2.3.8. Drug–DNA binding

Following drug incubation high molecular weight DNA was isolated from cell pellet using JETQUICK Blood DNA Spin Kit/50 according to the modified protocol of Bowtell [31]. The cell pellets were resuspended in PBS to a final volume of 200 μl and mixed with 10 μl of RNase A, incubated for 4 min at 37 °C. Twenty-five microliters of proteinase K and 200 μl buffer K1 (containing guanidine hydrochloride and a detergent) were then added to the mixture followed by incubation for 10 min at 70 °C. Two hundred microliters of absolute ethanol was added and mixed thoroughly to prevent any precipitation of nucleic acids due to high local alcohol concentrations. The samples were centrifuged for 1 min at 10,600 rpm through the silica membrane using JETQUICK micro-spin column. The columns containing the samples were then washed with 500 μl buffer KX (containing high-salt buffer to remove residual contaminations) and centrifuged for 1 min at 10,600 rpm, again washed with 500 μl buffer K2 (containing low-salt buffer to change the high-salt conditions on the silica membrane to low-salt) and centrifuged for 1 min at 10,600 rpm. To further clear the silica membrane from residual liquid, the sample columns were centrifuged again for 2 min at full speed (13,000 rpm). The column receivers were changed and the purified DNA in the column was eluted from the membrane with 200 μl of 10 mM Tris–HCl buffer (pH 8.5). Platinum contents were determined by graphite furnace AAS. A_{260}/A_{280} ratios were found to be between 1.75 and 1.8 for all samples indicating high purity of the DNA [32].

3. Results and discussion

3.1. Characterization

CH9: Formula: $C_{17}H_{52}N_{10}Cl_6OPt_3$.
Molar mass: 1210.80 g mol^{-1}.

Table 1
Molar conductivity values (in Ω^{-1} cm^2 mol^{-1}) of CH9

Concentration	CH9
0.5	11.54
0.25	12.34
0.1	17.31
0.01	35.7
0	78

Elemental composition

Calculated: C: 17.3, H: 4.3, Cl: 11.4, N: 12.4, Pt: 46.9.

Observed: C: 17.8 ± 0.4, H: 4.5 ± 0.4, Cl: 11.2 ± 0.4, N: 12.6 ± 0.4, Pt: 46.8 ± 0.4.

It can be seen that the observed values for the elements C, H, N, Cl, Pt agree with the calculated values within the limits of the measurement.

3.2. Molar conductivity

Table 1 gives the molar conductivity values (in Ω^{-1} cm^2 mol^{-1}) of CH9 at different concentrations of the compound. From the plot of molar conductivity against concentration (plot not given), it was found that the limiting value of the molar conductivity of CH9 at zero concentration was 78 Ω^{-1} cm^2 mol^{-1}. The result suggests that in the 1:5 mixture of DMSO and water, the compound remains largely undissociated. However, this may not be true in biological system where the degree of dissociation may be greater because of higher water content. Even then, it is very likely that a very significant fraction of the 'molecules' remains as undissociated ionic aggregate so that they can cross the cell membrane by passive diffusion.

3.3. IR, Raman and mass spectral analyses

The bands observed in IR, Raman and mass spectra of CH9 are given in Table 2. The interpretation of the bands has been based on published spectra [33–38]. It can be seen that the prominent peaks in the IR and Raman spectra of the compound correspond to the suggested structure. For example, the frequency at 406 cm^{-1} is due to Pd–N(NH_3) stretching vibration, that at 212 cm^{-1} is due to (Pt–N(2-hydroxypyridine) stretching vibration and that at 524 cm^{-1} is due to Pt–Cl stretching vibration.

3.3.1. IR

Although the IR spectrum alone of a complex molecule like CH9 cannot provide conclusive evidence for its structure, it can be seen that the peaks listed in Table 2 and the description given are in agreement with the suggested structure. In particular, it indicates the presence of groups such as CH, NH and 'aromatic ring', and Pt–N bonds.

The band at 2858 cm^{-1} is due to aliphatic C–H stretching vibration. The band at 1203 cm^{-1} is due to ring C–O stretching vibration. The band at 994 cm^{-1} is believed to be due to C=C and C=N in-plane bending associated with the pyridine ring. The band at 825 cm^{-1} is believed to be due to C–N stretching vibration in –CH_2–NH_2 chain. The band at 776 cm^{-1} is due to C–H bending vibration associated with *o*-disubstituted ring. The band at 418 cm^{-1} is associated with the vibration of the pyridine ring. The band at 406 cm^{-1} is associated with the Pt–N(NH_3) stretching vibration.

3.3.2. Raman

The band at 2866 cm^{-1} is due NH_3 stretching vibration whereas that at 2850 cm^{-1} is due to C–H stretching vibration of an A–CH_2–NH_2–A chain. The band at 2449 cm^{-1} is believed to be due to stretching vibration of A–NH_2–A chain. The band at 1441 cm^{-1} is believed to be due to C–H bending vibration in *o*-disubstituted ring. The band at 1288 cm^{-1} is believed to be due to N–H bending vibration. The band at 1039 cm^{-1} is due to CH_2 wagging. The band at 843 cm^{-1} is believed to be due to Ph–OH bending vibration. The band at 524 cm^{-1} is due to Pt–N(NH_3) stretching vibration. The band at 212 cm^{-1} is believed to be due to Pt–N(2-hydroxypyridine) ring stretching vibration.

3.3.3. Mass

The mass spectrum of CH9 appears to be highly complex. This is to be expected from the nature of the compound. It is an ionic compound composed of a long polyvalent cation and four valencing anions. A part of the polyvalent cation is flexible and can be broken easily whereas another part is rigid. Thus it is not surprising the mass spectrum of the CH9 does not have the molecular peak. The peaks observed described below can be seen to provide support for the suggested structure of the compound.

Table 2
Prominent bands found in the IR, Raman, mass and ^{1}H NMR spectra of CH9

IR (cm^{-1})	2858 (alkanes R–$(CH_2)_4$–R, s*), 1203 (Ph–OH, s), 994 (C=C and C=N in-plane, pyridine ring, w-m), 825 (A–CH_2–NH_2–A, s), 776 (C–H in *o*-disubstituted ring, s), 418 (pyridine ring, s-m), 406 (Pt–N, s)
Raman (cm^{-1})	2866 (NH_3^+, m), 2850 (A–CH_2–NH_2–A, s-m), 2449 (A–NH_2–A, m), 1441 (C–H in *o*-disubstituted ring, m), 1288 (NH, w), 1039 (CH, s), 843 (Ph–OH, s), 524 (Pt–N, s), 212 (Pt–N(2-hydroxypyridine), w)
Mass (*m*/*z*)#	*m*/*z*: M = 1208; (M–5Cl = 1031) (26); (M – 6Cl – NH_3 + 3H= 983) (18); $Cl(NH_3)_2$Pt -μ-{$NH_2(CH_2)_6NH_2$}Pt(2-hydroxypyridine)(NH_3)-μ $NH_2(CH_2)_5CH_3$ = 788 (19); $Cl(NH_3)_2$ Pt-μ-{$NH_2(CH_2)_6NH_2$}Pt(2-hydroxypyridine)(NH_3)-μ-$NH_2(CH_2)_3CH$ = 744 (23); {$(NH_3)_2$Pt -μ-{$NH_2(CH_2)_6NH_2$}Pt(2-hydroxypyridine)(NH_3)-μ-$NH_2(CH_2)_3CH_3$ + 2H} = 727 (21), $(NH_3)_2$Pt-μ-{$NH_2(CH_2)_6NH_2$}Pt(2-hydroxypyridine)(NH_3)(NH_2) = 668 (27), $(CH_2)_5(NH_2)$Pt(2-hydroxypyridine)(NH_3)–$NH_2(CH_2)_5$ = 479 (32), Pt(2-hydroxypyridine)(NH_3){$NH_2(CH_2)_6NH_2$} = 423 (20.5); $Cl(NH_3)_2$Pt{$NH_2(CH_2)_6NH_2$} = 381 (19.5), $Cl(NH_3)_2$Pt–$NH_2(CH_2)_2CH_3$ = 323 (11); $Cl(NH_3)_2$Pt{$NH_2CH_2CH_3$} = 309 (100), Pt(NH_3)(2-hydroxypyridine) = 307 (65)

* The letters 's', 'm', 'w' and 'o' stand for 'strong', 'medium', 'weak' and 'ortho', respectively. The letter 'A' stands for 'anything'. # The number in parentheses after *m*/*z* value indicates the percent intensity.

The peak observed in the mass spectrum of CH9 with $m/z = 1031$ may be due to (M – 5Cl) and that with $m/z = 983$ may be due to (M – 6Cl – NH_3 + 3H). The peak with $m/z = 788$ may be due to $Cl(NH_3)_2Pt$-μ-$\{NH_2(CH_2)_6NH_2\}Pt$(2-hydroxypyridine)(NH_3)-μ-$NH_2(CH_2)_5CH_3$ that may be produced in situ from the fragmentation of CH9. The peak observed in the mass spectrum of CH9 with $m/z = 744$ may be due to $Cl(NH_3)_2Pt$-μ-$\{NH_2(CH_2)_6NH_2\}Pt$(2-hydroxypyridine)(NH_3)-μ-$NH_2(CH_2)_3CH$, produced in situ from the fragmentation of CH9. The peak with $m/z = 668$ may be due to $(NH_3)_2Pt$-μ-$\{NH_2(CH_2)_6NH_2\}Pt(NH_3)$(2-hydroxypyridine)$(NH_2)$ produced from the fragmentation of CH9. The peak observed in the mass spectrum of CH9 with $m/z = 479$ may be due to $\{CH_2)_5NH_2\}Pt$(2-hydroxypyridine)$(NH_3)\{NH_2(CH_2)_5\}$ and that with $m/z = 423$ may be due to Pt(2-hydroxypyridine)$(NH_3)\{NH_2(CH_2)_6NH_2\}$ The peak observed in the mass spectrum of CH9 with $m/z = 381$ may be due to $Cl(NH_3)_2Pt\{NH_2(CH_2)_6NH_2\}$ and that $m/z = 323$ may be due to $Cl(NH_3)_2Pt\{NH_2(CH_2)_2CH_3\}$. The peak with $m/z = 309$ may be due to $Cl(NH_3)_2Pt\{NH_2CH_2CH_3\}$ and that with $m/z = 307$ may be due to $Pt(NH_3)$(2-hydroxypyridine).

3.4. Gel electrophoresis

Interaction between pBR322 plasmid DNA and compounds (CH9 and cisplatin).

Fig. 2 gives the electrophoretograms applying to the interaction of pBR322 plasmid DNA with increasing concentrations of CH9 and cisplatin, for 4 h at 37 °C—(a) and (b) apply to CH9 without and with BamH1 digestion, respectively, (c) and (d) apply to cisplatin without and with BamH1 digestion, respectively. In (a) the concentration range is 0–20 μM, in (b), (c) and (d) it is 0–10 μM.

As pBR322 plasmid DNA which was initially found to be mixture of super coiled form I (band at the front) and singly-nicked form II, was allowed to interact with CH9 (Fig. 2 (a)), the mobility of form I plasmid DNA band decreased whereas that of the form II band remained essentially unchanged so that the two bands came closer together. At higher concentrations of CH9, the intensity of both form I and form II bands decreased to produce an elongated smudged band at 10 μM and no band was observed above the concentration. It may be noted that the analogous Pt–Pd–Pt complex code named CH25 was found to be more damaging to DNA and also more active against the ovary cancer cell lines [18]. When pBR322 plasmid was allowed to interact with increasing concentrations of cisplatin (Fig. 2 (c)), the mobility of both form I and form II bands increased essentially at the same rates so that bands remained parallel at all concentrations of the compound. There was a slight decrease in the intensity of the form I band with the increase in concentration of cisplatin.

The change in mobility of plasmid DNA bands as a result of interaction with CH9 is believed to be due interstrand ladder-like binding of the trinuclear cation primarily to the GG sites of DNA [39]. The high flexibility of the CH9 means that a range of interstrand adducts of varying lengths dictated by the sequence of nucleobases could be formed [40], caus-

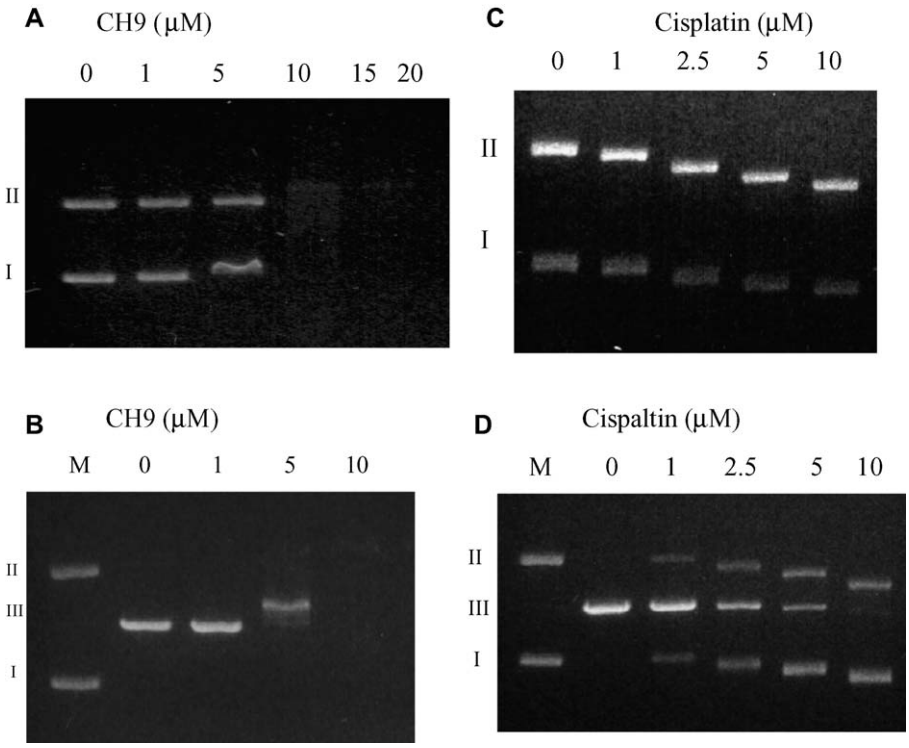


Fig. 2. (a) Interaction between pBR322 plasmid DNA and increasing concentrations of CH9, (b) interaction between pBR322 plasmid DNA and increasing concentrations of CH9 followed by BamH1 digestion, (c) interaction between pBR322 plasma DNA and increasing concentrations of cisplatin, (d) interaction between pBR322 plasmid DNA and increasing concentrations of cisplatin followed by BamH1 digestion.

ing a permanent B to Z transformation of DNA conformation [7]. Since the length of CH9 is long enough to cover a distance of over eight nucleotides, depending on the sequence of nucleobases in DNA, the compound (like BBR3464) can potentially form a number of GG interstrand adducts, made possible by the flexibility of the molecule. CH9 can also interhelical GG adducts with DNA especially at higher concentrations of the compounds.

In contrast, the change in mobility of plasmid DNA bands as a result of interaction with cisplatin is believed to be due to the formation of mainly monofunctional Pt(G) and intrastrand bifunctional Pt(GG) adducts, the latter causing a local distortion of DNA. The decrease in intensity at higher concentrations of compounds indicates the occurrence of DNA damage caused by the binding of the compounds. When CH9 binds covalently with DNA, the planaramine ligand (2-hydroxypyridine) bonded to the central metal ion (Pt^{2+}) can undergo stacking interaction with nucleobases in DNA. It is believed that such stacking interaction may be playing a significant role in causing DNA damage.

3.4.1. BamH1 digestion

As stated earlier, Fig. 2 (b), (d) give the electrophoretograms applying to the BamH1 digested incubated mixtures of pBR322 plasmid DNA and varying concentrations of CH9 and cisplatin ranging from 0 μM to 10 μM. Lane 1 applies to untreated and undigested pBR322 plasmid DNA, lane 2 applies to untreated but BamH1 digested pBR322 plasmid DNA, lanes 3–6 apply to pBR322 plasmid DNA interacted with increasing concentrations of compounds (3: 1.0 μM, 4: 5 μM, 5: 10 μM for CH9 μM as against 3: 1.0 μM, 4: 2.5 μM, 5: 5 μM, 6: 10 μM for cisplatin).

When untreated pBR322 plasmid DNA was digested with BamH1, only one band corresponding to form III band was observed (lane 2). In the untreated and undigested pBR322 plasmid DNA (lane 1), two bands corresponding to form I and form II were observed (form I band has the highest velocity, form II has the lowest velocity and form III band has the intermediate velocity).

In the case of BamH1 digested incubated mixtures of pBR322 plasmid DNA and CH9 (Fig. 2 (b)), only form III band was observed at concentration of CH9 = 1.0 μM and form II band and a small amount of form III band were observed at 5 μM and no band was observed at 10 μM. In the case of cisplatin (Fig. 2 (d)), three bands corresponding to forms I, II, III were observed for concentrations of cisplatin ranging from 1.0 μM to 5 μM, two bands corresponding to forms I and II were observed for concentration of cisplatin = 10 μM. Table 2 summarizes the above results. The results show that whereas for the untreated pBR322 plasmid DNA BamH1 digestion at the specific GG site is not prevented thus producing only form III DNA, in presence of increasing concentrations of cisplatin, there is a corresponding increase in prevention of BamH1 digestion at the specific GG site but not as much in the case of CH9. Like CH25, CH9 is expected to form a range of interstrand GG adducts dictated by the sequence of nucleobases in the DNA whereas cisplatin is expected to form mainly intrastrand bifunctional GG adducts with DNA that cause a local pronounced bending of the DNA strand. On the other hand, formation of long-range GG adducts is likely to cause a global change in the conformation of DNA (B–Z transformation) in which the conformations of the intervening not directly involved in the cross-link are also altered [40,41]. The reason why CH9 is found to be less able to prevent BamH1 digestion as compared to cisplatin, is because of the two opposing roles played by the compound. On the one hand, binding of the compound causes conformational change in DNA and on the other it causes DNA damage (which discussed earlier could be due to stacking interaction). Both CH9 and CH25 are believed to cause DNA damage—CH25 more than CH9. As to why then CH9 is less able to prevent BamH1 digestion than CH25, it is suggested that CH9 may be binding less than CH25 with DNA Table 3.

Table 3
Bands observed in the incubated mixtures of pBR322 plasmid DNA and varying concentrations of CH9 and cisplatin followed by BamH1 digestion

Drug	0	1.0	5.0	5 (10)	10
CH9	III	III	II, III	No band#	Not applicable
Cisplatin	III	I, II, III	I, II, III	I, II, III	I, II

#: Total DNA damage.

3.5. Anticancer activity

Figs. 3 and 4 give percentage cell survival versus concentrations curves as applied to the cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ when the cells were treated with increasing concentrations of CH9 and cisplatin. Table 4 gives the IC_{50} values and resistance factor of CH9 and cisplatin for the cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$. It can be seen from the cell survival curves and IC_{50} values that CH9 is less active than cisplatin against the parent cell line: A2780 but more active than cisplatin against the resistant cell lines: $A2780^{cisR}$ and $A2780^{ZD0473R}$ (about 0.5 time in A2780, 3.6 times in $A2780^{cisR}$ and 3.4 times in $A2780^{ZD0473R}$). The results suggest that compared to cisplatin CH9 has been better able to overcome mechanisms of resistance operating in

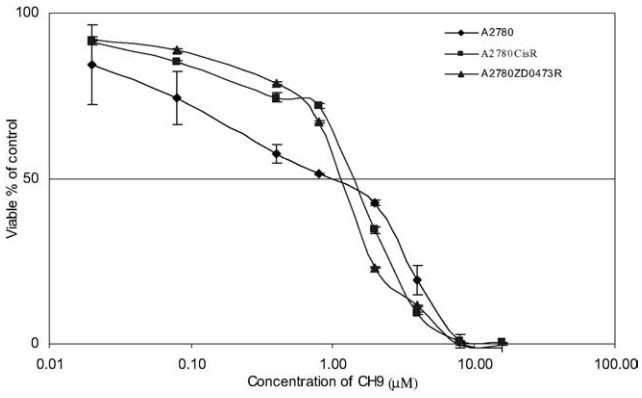


Fig. 3. Survival curve for the cell lines, A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ when the cells were treated with increasing concentrations of CH9.

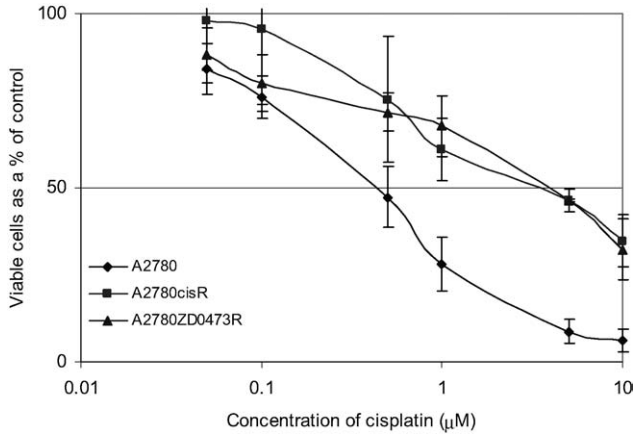


Fig. 4. Survival curve for the cell lines, A2780, A2780^{cisR} and A2780^{ZD0473R} when the cells were treated with increasing concentrations of cisplatin.

the cell lines: A2780^{cisR} and A2780^{ZD0473R} but not in A2780. The higher activity of CH9 as compared to cisplatin in the resistant cell lines, is believed to be difference in the nature of interaction with DNA. As stated earlier, whereas cisplatin forms mainly monofunctional Pt(G) and intrastrand bifunctional Pt(GG) adducts, CH9 would form a range of interstrand GG adducts dictated by the sequence of nucleobases. The differences in nature of binding mean that the two compounds would produce different conformational changes in DNA.

3.5.1. Cell uptake and binding with DNA

3.5.1.1. Cell uptake. In this study, the cellular accumulation of platinum has been used as a measure of the cell uptake of compounds. Fig. 5 gives the uptakes in 2, 4 and 24 h of CH9 and cisplatin in the human ovarian cell lines: A2780 and A2780^{cisR} and A2780^{ZD0473R}.

It is found that the cellular uptake of CH9 is much greater than that of cisplatin in all the three cell lines: A2780 and A2780^{cisR} and A2780^{ZD0473R}. As stated earlier, whereas CH9 is found to be more active than cisplatin against the cell lines: A2780^{cisR} and A2780^{ZD0473R}, it is less active than cisplatin against the cell line: A2780. The results illustrate that cell uptake per se may not reflect the antitumor activity of the compounds as it is known that platinum compounds may be deactivated by a number of means inside the cell (e.g. due to binding with sulfur containing ligands such as glutathione present in the cell) [37] before they have a chance to bind with DNA. Thus it is more appropriate to consider the level and nature of binding with DNA.

As stated before, whereas cisplatin is expected to form mainly intrastrand GG, AG and GNG adducts, CH9 is expected to form a plethora of long-range interstrand GG adducts. Whereas intrastrand 1,2-bifunctional binding of cisplatin would cause mainly local bending of DNA, the long-range interstrand adducts formed by CH9 would cause long-range distortion of DNA in which the conformations of the intervening bases not directly involved in the cross-link are also altered [40]. DNA binding of multinuclear platinum complexes is characterized by flexible, non-directional DNA adducts and a greater percentage of interstrand to intrastrand adducts and the ability to induce conformational changes to both A- and Z-type DNA [40,41].

3.5.1.2. Platinum binding with DNA. Fig. 6 gives the levels of platinum binding (in nanomoles per milligram of DNA) in 2, 4 and 24 h in A2780 and A2780^{cisR} and A2780^{ZD0473R} cells as applied to CH9 and cisplatin.

It is found the level of DNA binding for CH9 is greater than that for cisplatin as applied to all the three cell lines: A2780 and A2780^{cisR} and A2780^{ZD0473R}. The higher activity

Table 4

	A2780 IC_{50} (μM)	A2780^{cisR} IC_{50} (μM)	IC_{50} A2780^{cisR} IC_{50}A2780 (RF)	A2780^{ZD0473R} IC_{50} (μM)	IC_{50}A2780^{ZD0473R} IC_{50}A2780 (RF)
Cisplatin	0.46 ± 0.10	4.96 ± 0.63	10.78	4.97 ± 0.94	10.80
CH9	0.93 ± 0.04	1.37 ± 0.05	1.47	1.45 ± 0.46	1.56

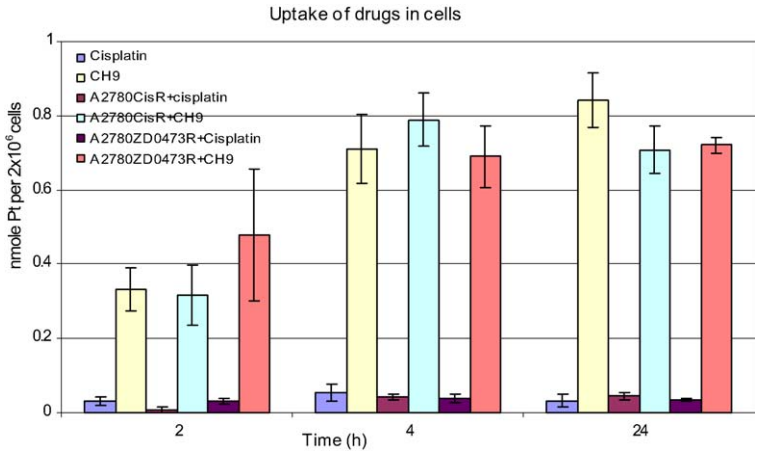


Fig. 5. Pt accumulation in A2780, A2780^{cisR} and A2780^{ZD0473R} cells as applied to cisplatin, transplatin and CH9 in 2, 4 and 24 h.

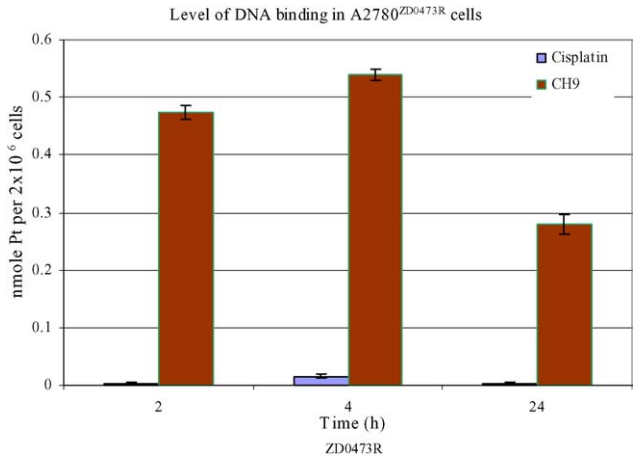


Fig. 6. Levels of Pt-DNA binding in $A2780^{ZD0473R}$.

of CH9 than cisplatin against the cell lines: $A2780^{cisR}$ and $A2780^{ZD0473R}$ is in line with its greater level of binding with DNA. The lower activity of CH9 but higher level of DNA binding as applied to the cell line: A2780 is difficult to explain except to note that the nature of binding of the two compounds (CH9 and cisplatin) with DNA is different (as discussed earlier). Perhaps, the results point to the complex nature of drug resistance.

The bending induced in DNA by binding of cisplatin is recognized by high-mobility group (HMG) domain proteins, which is believed to be the pathway for processing and differential repair of cellular cisplatin-DNA adducts [42]. In contrast, when long-range interstrand adducts are formed, DNA is not sufficiently bent to be recognized by HMG1 proteins [43]. Instead, the long-range cross links are very effective in inducing B–A or B–Z transformations. Cellular alkaline elution studies have shown that interstrand cross links formed by BBR3464 persist over time, suggesting a lack of DNA repair [44,45]. It has been suggested that the induction of Z-DNA within the cell would have serious consequences with regard to transcription and DNA replication. As noted earlier, formation of 1,4-GG interstrand adduct by BBR3464 with the self complementary 5′-d(ATG*TACAT)$_2$-3′ octamer the *syn* conformation is induced in the adenine moieties not just within the strand bounded by the two platinum binding sites but also those at the end of the strand [40]. It is found that Watson–Crick pairing is essentially maintained and that the central linker is situated in the minor groove of the DNA. The authors pointed out that the cooperative nature of the B to Z transformation lends itself easily to the delocalization of the lesions beyond the binding site. It was suggested that the factors that might contribute to the delocalization would include the linking of the two separated platinating sites and the presence of charge and electrostatic interactions introduced after incorporation of BBR3464 into the oligonucleotide. The contacts between the lipophilic backbone of BBR3464 and DNA may be especially effective in displacing water from within DNA and thus facilitating conformational transitions. It has been suggested [46] that in canonical poly(dGdC). poly(dGdC), polynuclear platinum complexes can form a plethora of intra- and interstrand cross links.

When fully stretched, the length of CH9 but with the chlorides replaced by nitrogens would be 2.68 nm. When fully stretched the CH9 would form G(1) to G(9) interstrand adduct. It should however be noted that, because of the flexibility of the diamine linkers, a range of interstrand adducts of varying lengths can be formed by CH9.

The high charge on the multicentered cation produced from CH9 would facilitate rapid binding with DNA. For BBR3464, $t_{1/2}$ was reported to be about 40 min, significantly faster than that of the neutral cisplatin. It was suggested that the rapid binding of BBR3464 could affect sequence specificity—the high charge could lead to initial electrostatic interactions very different from those in small molecules such as cisplatin and the alkylating agents, leading to enhanced sequence specificity. It is generally accepted that *cis*-$[Pt(NH_3)_2(Cl)(H_2O]^+$, formed by hydrolysis of one Pt–Cl, pre-associates with DNA [47,48] before binding to specific nucleobases in DNA. Wheatle et al. point out that since pre-association is stabilized largely by electrostatic forces, the pre-association of cationic multicentered platinum complexes with DNA would be even stronger and therefore more important. It has been suggested that the pre-association of multinuclear platinum complexes with polyanionic DNA will significantly affect the rate and site of platination because an increased local concentration will increase the probability of a covalent interaction at these sites. Also, pre-association may induce a local conformational change in the DNA that may influence binding at a specific site. The above idea may be applicable to the interaction of CH9 with DNA.

The high activity of CH9 in $A2780^{CisR}$ and $A2780^{ZD0473R}$ cells as compared to that for cisplatin also indicates that the compound has been able to overcome multiple mechanisms of cisplatin-resistance in $A2780^{CisR}$ cells and ZD0473 resistance operating in the $A2780^{ZD0473R}$ cells.

4. Conclusion

A tumor active multicentered metal complex code named CH9 has been synthesized and studied. The anticancer activity of the compound against ovarian cancer cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$ has been determined. The cell uptake and the level of binding with DNA have also been determined. The nature of interaction with pBR322 plasmid DNA has also been determined. The gel electrophoresis results show that CH9 has been able to cause conformational changes in DNA and DNA damage at higher concentrations. This is believed to be due to covalent interstrand binding of the compound with DNA through the terminal metal centers. Like other multicentered compounds [10], because of the flexible nature, it is believed that the compound would form a range of interstrand GG adducts dictated by the sequence of DNA bases. Non-covalent interactions are believed to play a role in DNA damage. For example, the planaramine ligand may be involved in stacking interaction with DNA bases. The compound displays significant anticancer activity against ovarian

cancer cell lines—much greater than cisplatin in A2780^{cisR} and A2780^{ZD0473R} cells. The higher activity of CH9 suggests that the compound has been able to overcome resistance in A2780^{cisR} and A2780^{ZD0473R} cell lines. CH9 is believed to form long-range interstrand adducts with DNA, causing long-range deformation of the DNA.

Acknowledgements

Howard Cheng is grateful to the University of Sydney for the award of International Merit Scholarship.

References

[1] P. Pil, S.J. Lippard, Cisplatin and related drug, in: J.R. Bertini (Ed.), Encyclopaedia of Cancer, Academic Press, San Diego, 1997, pp. 392–410.
[2] U. Bierbach, Y. Qu, T.W. Hambley, J. Peroutka, H.L. Nguyen, M. Doedee, N. Farrell, Inorg. Chem. 38 (1999) 3535–3542.
[3] R.W. Hay, S. Miller, Polyhedron 17 (1998) 2337–2343.
[4] P. Perego, C. Caserini, L. Gatti, N. Carenini, S. Romanelli, R. Supino, D. Colangelo, L. Vino, R. Leone, S. Spinelli, G. Pezzoni, C. Monzotti, N. Farrell, F. Zunino, Mol. Pharm. 55 (1999) 528–534.
[5] P.J. Loehrer, L.H. Einhorn, Ann. Intern. Med. 100 (1984) 704–713.
[6] P. Di Blasi, A. Bernareggi, G. Beggiolin, L. Piazzoni, E. Menta, M.I.L. Formento, Cytotoxicity, Anticancer Res. 18 (1998) 3113–3117.
[7] M.A. Fuertes, J. Castilla, P.A. Nguewa, C. Alonso, J.M. Perez, Novel concepts in the development of platinum antitumour drugs: an update, Med. Chem. Rev.—online 1 (2004) 187–198.
[8] R.B. Weiss, M.C. Cristian, Drugs 46 (1993) 360.
[9] M.J. Bloemink, J. Reedijk J, in: A. Sigel, H. Sigel (Eds.), Metal Ions Biological Systems, vol. 32, Marcel Dekker, New York, 1996, pp. 641–685 (Chapter 19).
[10] M. Adams, R.P. A'Hern, A.H. Calvert, J. Carmichael, P.I. Clark, R.E. Coleman, H.M. Earl, C.J. Gallagher, M.E. Gore, J.D. Graham, P.G. Harper, G.C. Jayson, S.B. Kaye, J.A. Ledermann, R.J. Osborne, T.J. Perren, C.J. Poole, J.A. Radford, G.J. Rustin, M.L. Slevin, J.F. Smyth, H. Thomas, P.M. Wilkinson, Br. J. Cancer 78 (1998) 1404–1406.
[11] P.R. Perez, P.J. O'Dwyer, L.M. Handel, R.F. Ozols, T.C. Hamilton, Int. J. Cancer 48 (1991) 265–269.
[12] S.W. Johnson, K.V. Ferry, T.C. Hamilton, Drug Resistance Update 1 (1998) 243–254.
[13] M.S. Ali, K.H. Whitmire, T. Toyomasu, Z.H. Siddik, A.R. Khokhar, J. Inorg. Biochem. 77 (1999) 231–238.
[14] N. Farrell, Y. Qu, M.P. Hacker, J. Med. Chem. 33 (1990) 2179–2184.
[15] L.R. Kelland, B.A. Murrer, G. Abel, C.M. Giandomenico, P. Mistry, K.R. Harrap, Cancer Res. 52 (1992) 822–828.
[16] P. Perego, L. Gatti, C. Caserini, R. Supino, D. Colangelo, R. Leone, et al., J. Inorg. Biochem. 77 (1999) 59–64.
[17] H. Daghriri, F. Huq, P. Beale, J. Inorg. Biochem. 98 (2004) 1722–1733.
[18] H. Cheng, F. Huq, P. Beale, K. Fisher, Synthesis, characterisation, activities, cell uptake and DNA binding of a trinuclear complex: [{*trans*-PtCl(NH_3)}$_2$μ-{*trans*-Pd(NH_3)(2-hydroxypyridine)-($H_2N(CH_2)_6NH_2)_2$]Cl_4, J. Inorg. Biochem. (2005) (under review).
[19] F. Huq, H. Daghriri, J.Q. Yu, P. Beale, K. Fisher, Studies on the synthesis and characterization of four *trans*-planaramineplatinum(II) complexes of the form *trans*-PtL(NH_3)Cl_2 where L = 2-hydroxypyridine, 3-hydroxypyridine, imidazole, and imidazo(1,2-α)pyridine, Eur. J. Med. Chem. 39 (8) (2004) 691–697.
[20] G.B. Kauffman, D.O. Cowan, *cis*- and *trans*-dichlorodiammine-platinum (II), Inorg. Synth. 7 (1963) 239–245.
[21] H. Rauter, R. Didomenico, et al., Inorg. Chem. 36 (18) (1997) 3919–3927.
[22] G. Zhao, H. Lin, et al., Anticancer Drug Des. 13 (7) (1998) 769–777.
[23] M. Meroueh, J. Kjellstrom, et al., Inorg. Chim. Acta 297 (1–2) (2000) 145–155.
[24] P.W. Atkins, Physical Chemistry, Oxford University Press, Oxford, Melbourne, Tokyo, 1998.
[25] N. Stellawagen, Nucleic Acid Electrophoresis, Springer, Berlin, New York, 1998 1–53.
[26] G. Collela, M. Nennati, R. Leone, D. Colangelo, C. Manzotti, M.G. Daidone, et al., Br. J. Cancer 84 (2001) 1387–1390.
[27] M.S. Davies, D.S. Thomas, A. Hegmans, S.J. Berners-Price, N. Farrell, Inorg. Chem. 41 (2002) 1101–1109.
[28] I.R. Freshney, Culture of Animal Cells: A Manual of Basic Technique, forth ed, Wily-Liss, New York, 2000.
[29] T. Mosmann, J. Immun. Methods 65 (1983) 55–63.
[30] N. Farrell, L.R. Kelland, Cancer Res. 52 (1992) 5065–5072.
[31] D.D. Bowtell, Anal. Biochem. 162 (2) (1987) 463–465.
[32] J. Heptinstall, R. Rapley, in: R. Rapley (Ed.), The Nucleic Acid Protocols Handbook, Humana Press, Totowa, NJ, 2002, pp. 57–60.
[33] R.M. Silverstein, G.C. Bassler, T.C. Morrill, Spectrometric Identification of Organic Compounds, fifth ed, Wiley & Sons, 1991.
[34] N. Farrell, Y. Qu, L. Feg, B. Van Houten, Biochemistry 29 (1990) 9522–9531.
[35] K. Nakamoto, Infrared and Raman Spectra of Inorganic and Coordination Compounds Part B: Applications in Coordination, Organometallic and Bioinorganic Chemistry, Wiley, New York, 1997.
[36] S.J. Berners-Price, P.J. Sadler Coord, Chem. Rev. 151 (1996) 1–40.
[37] H.E. Gottlieb, V. Kotlyar, A. Nudelman, J. Org. Chem. 62 (21) (1997) 7512–7515.
[38] R.S. Macomber, A Complete Introduction to Modern NMR Spectroscopy, Wiley, New York, 1998.
[39] Y. Qu, N.J. Scarsdale, M.C. Tran, N. Farrell, J. Biol. Inorg. Chem. 8 (2003) 19–28.
[40] N. Farrell, S. Spinell, in: N. Farrell (Ed.), Uses of Inorganic Chemistry in Medicine, Royal Society of Chemistry, Cambridge, 1999, pp. 124–134.
[41] F. Huq, H. Daghriri, J.Q. Yu, P. Beale, M. Zhang, Eur. J. Med. Chem. 39 (2004) 947–958.
[42] E. Jamieson, S.J. Lippard, Chem. Rev. 99 (1999) 2467–2498.
[43] J. Zehunlova, J. Kasparkova, J. Biol. Chem. 276 (25) (2001) 22191–22199.
[44] R.J. Roberts, G.A. Wilson, F.E. Young, Nature 265 (1977) 82–84.
[45] J.D. Roberts, J. Peroutka, G. Beggiolon, C. Manzotti, L. Piazzoni, N. Farrell, J. Inorg. Biochem. 77 (1999) 47–50.
[46] T.D. McGregor, Z. Balcarova, J. Inorg. Biochem. 77 (1–2) (1999) 43–46.
[47] N.J. Wheate, J.G. Collin, Coord. Chem. Rev. 241 (1–2) (2003) 133–145.
[48] Y. Wang, N. Farrell, N.J.D. Burgess, J. Am. Chem. Soc. 123 (23) (2001) 5576–5577.