Synthesis, Activity and Binding with DNA of [{trans-$PtCl(NH_3)_2$}$_2$μ-{trans-$Pd(4\text{-hydroxypyridine})_2(H_2N(CH_2)_6NH_2)_2]Cl_4$ (TH8)

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Abstract: This paper describes the synthesis, characterization, cytotoxicity of a new trinuclear Pt-Pd-Pt complex code named TH8 containing two 4-hydroxypyridine ligands bound to the central metal ion. In addition to its activity against human ovarian cancer cell lines: A2780, A2780^{cisR} and A2780^{ZD0473R}, cell uptake, level of DNA-binding and nature of interaction of the compound with pBR322 plasmid DNA have also been determined. TH8 is found to be less active than cisplatin against the parent cell line A2780 but is more active against the cisplatin-resistant cell line A2780^{cisR}. Whereas the resistance factors for cisplatin as applied to the cell lines A2780 and A2780^{cisR}, and A2780 and A2780^{ZD0473R} are 12.9 and 3.0 respectively, the corresponding values for TH8 are 1.4 and 2.1. The results suggest that TH8 has been better able to overcome the resistance operating in A2780^{cisR} cell line. Whereas cisplatin binds with DNA forming mainly intrastrand GG adduct that causes local bending of a DNA strand, TH8 is expected to bind with DNA forming mainly interstrand GG adducts that would cause more of a global change in DNA conformation.

Key Words: Cisplatin, BBR3464, 4-hydroxypyridine, Ovarian cancer, MTT.

1. INTRODUCTION

Currently much attention is given to rule-breaker platinum compounds with the aim of widening the spectrum of activity and reducing the side effects associated with platinum-based chemotherapy [1, 2]. One such compound code named BBR3464 has shown much higher activity than cisplatin in a large number of tumour models but in clinical trials it has shown dose-limiting toxicity [3, 4]. Recently we reported on the synthesis, activity and binding with DNA of a novel trinuclear platinum complex code named TH1 that contains two 3-hydroxypyridine ligands bound to the central platinum [5]. The compound is found to be significantly more active than cisplatin against a number of human ovarian cancer cell lines. Earlier, we also reported on the synthesis and activity of a number of Pt-Pd-Pt and Pt-Pt-Pt complexes [6-9]. Here we report on the synthesis, activity and nature of binding with DNA of trinuclear Pt-Pd-Pt complex code named TH8 in which the central metal ion is bound to two 4-hydroxypyridine ligands (Fig. **1**). Although palladium is much more reactive than platinum, it is believed that the caged-in effect or steric hindrance provided by the planaramine ligand and diaminohexane chains would reduce greatly the availability of central Pd^{2+} to solvent molecules and other ligands.

2. CHEMISTRY

Materials

Potassium tetrachloroplatinate $K_2[PtCl_4]$, potassium tetrachloropalladate $K_2[PdCl_4]$, *N,N'*-dimethylformamide [DMF] [$(CH_3)_2NCHO$], 4-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 x 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.

Synthesis

*TH7 [*trans-*$PdCl_2$(4-hydroxypyridine)$_2$]*

TH7 denoting *trans*-$PdCl_2(4\text{-hydroxypyridine})_2$, required for the synthesis of TH8 was prepared according to previously published method [10].

Yellow solid; Anal. Calcd. for $C_{10}H_{10}N_2Cl_2O_2Pd$ (367.5): C, 32.8; H, 2.7; N, 7.6; Cl, 19.3; Pd, 29.2. Found: C, 32.8 ± 0.4; H, 2.9±0.4; N, 7.6 ± 0.4; Cl, 19.3 ± 0.4; Pd, 28.1 ± 1.2.

TH8

TH8 was synthesized starting with the central unit following a procedure similar to that used for the synthesis of TH1 [5]. Briefly the method was as follows. To transplatin (0.3 g, 1 mmol) dissolved in DMF (20 mL) was added silver nitrate (0.169 g, 0.99 mmol). The mixture was stirred at room temperature for 24 h in the dark followed centrifugation at 4800 rpm for 30 min to remove AgCl. The super-

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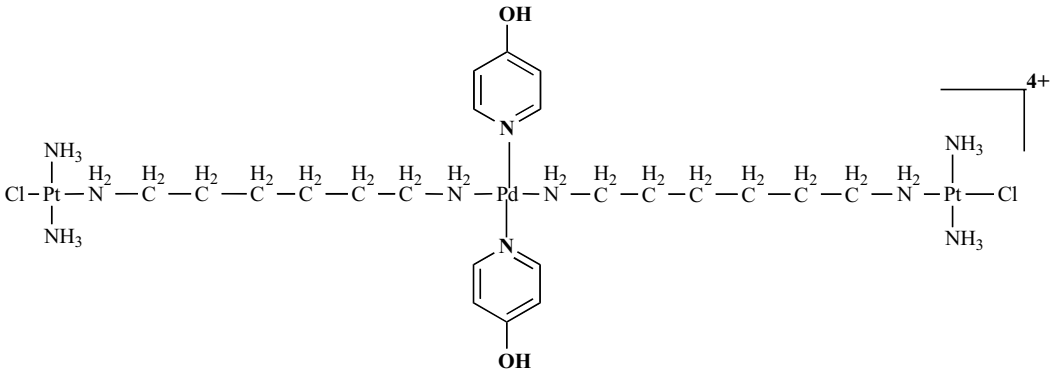

Fig. (1). Representation of TH8 showing linking of atoms.

natant containing *trans*-$[Pt(NH_3)_2(DMF)Cl]^+$ was collected and kept at -16ºC. A suspension of TH7 (0.18 g, 0.5 mmol) in DMF (6 ml) was gently heated with stirring to 39ºC to which was added dropwise a freshly prepared solution of 1,6-diaminohexane (0.116 g, 1 mmol) in mQ water (6 ml) followed by the addition of 1 M HCl (1 ml). The mixture was stirred for 20 min at 39ºC, then at room temperature for further 2 h and 40 min. The temperature of the mixture was raised again to 39ºC followed by the addition of 1 M NaOH (0.4 ml) during which the mixture was constantly stirred. Stirring was continued for further 24 min at 36ºC. The transplatin supernatant (0.5 mmol) was then added with stirring to the light yellow solution at 39ºC followed by stirring for a further 27 min at room temp. The temperature of the mixture was again raised to 39ºC followed by adding of 1 M NaOH (300 μl). The mixture was stirred for a further 5 min at 39ºC followed by the addition with stirring of another portion of transplatin supernatant (0.5 mmol) prepared earlier. Stirring was continued for a further 5 min at 39°C and then for 1 h at room temperature. The light yellow precipitate produced was collected by filtration at the pump, washed first with ice-cold water, then with methanol and ethanol and air-dried. Repeated dissolution in DMF followed by precipitation by adding dichloromethane was carried out to improve the purity of the product. The final weight of the product was 0.085 g giving a yield of 70.8 %. The identity of the compound was confirmed by microanalyses and spectral studies. Steps in synthesis are shown in Fig. (**2**).

Characterization

C, H, N and Cl were determined out using the facility at the Australian National University. Pt and Pd were determined by graphite furnace atomic absorption spectroscopy (AAS) using the Varian Spectraa-20 Atomic Absorption Spectrophotometer. Yellow solid; Anal. Calcd. for $C_{22}H_{54}C_{l6}N_{10}O_2PdPt_2$ (1199.5): C, 22.0; H, 4.5; N, 11.7; Cl, 17.7; Pt, 32.5; Pd, 8.9. Found: C, 21.6 ± 0.4; H, 4.0 ± 0.4; N, 11.3 ± 0.4; Cl, 16.8 ± 0.4; Pt, 30.9 ± 1.2; Pd, 9.2 ± 1.2. Percentage yield 80 %. The achieved purity of TH8 is estimated to be about 94% even after repeated purification by dissolution and precipitation. This was found to be the case with other trinuclear complexes [4, 5, 7-9]. One likely reason for the low purity is the formation of some dinuclear complexes along with trinuclear complex. Another reason for the low purity could be due to co-precipitation of other molecules such as DMF or dichloromethane that was used to reduce solubility.

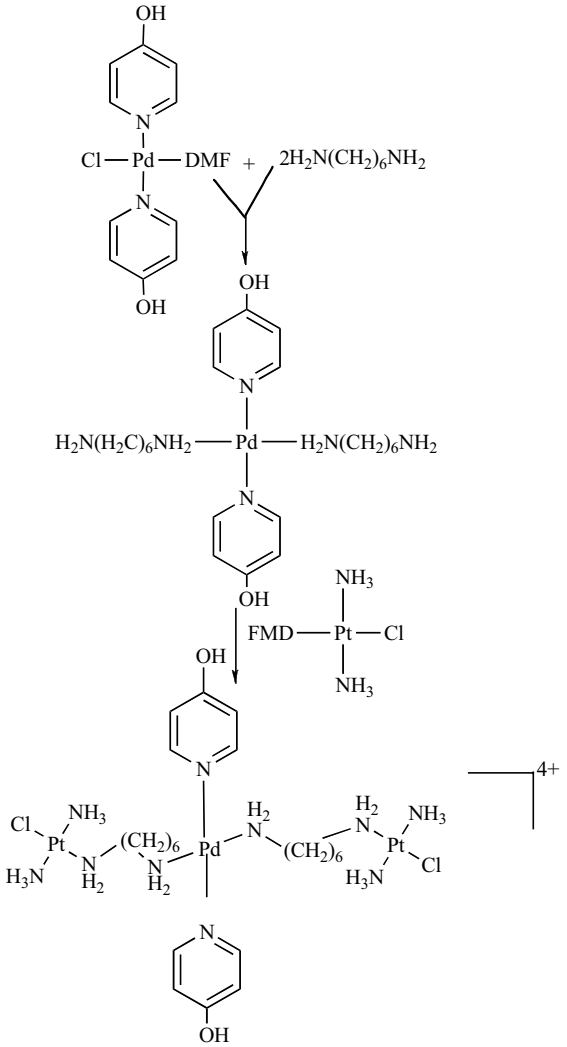

Fig. (2). Steps in the synthesis of TH8.

Spectral Analyses

The infrared spectrum of TH8 was collected using a Bruker IFS66 spectrometer equipped with a Spectra-Tech

Diffuse Reflectance Accessory (DRA), an air-cooled DTGS detector, a KBr beamsplitter. Spectrum was recorded at a resolution of 4 cm^{-1}, with the co-addition of 128 scans and a Blackman-Harris 3-Term apodisation function was applied. To obtain ^1H NMR spectrum of TH8, the compound was dissolved in deuterated DMSO then prepared in a 5 mm high precision Wilmad NMR tube. A DPX400 spectrometer was used with frequency of 400.2 MHz. Spectrum was recorded at 300 K (± 1 K) and referenced to internal solvent residues. To obtain mass spectrum, solution of TH8, made in 10% DMF and 90% methanol, was sprayed into a Finnigan LCQ ion trap mass spectrometer.

Molar Conductivity

The molar conductivity for TH8 was determined using a PW9506 digital conductivity meter. The compound was first dissolved in 1:1 mixture of DMF and water to obtain a 1 mM solution, which was then progressively diluted with mQ water to obtain solutions at concentrations ranging from 0.5 mM to 0.0625 mM. Molar conductivity values were then plotted against concentration to obtain the limiting value i.e. the value at zero concentration or infinite dilution [11]. The limiting molar conductivity of TH8 at zero concentration was 270 ohm^{-1} cm^2 mol^{-1} a value much lower than that expected for 1:4 electrolyte consisting one tetrapositive cation and four uninegative anions, indicating that in the solution in mixture of DMF and water MH8 is far from being dissociated fully into ions.

Cytotoxic Assay

Cytotoxicity of available TH8 (94% pure) against human ovarian cancer cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ along with that for cisplatin (that served as the reference) was determined using MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) reduction assay [12, 13]. The method used for cell subculture was a modification of that described by Freshney [12] and the method used for cell treatment as applied to the determination of drug uptake and DNA was a modification of that described by Di Blasi *et al*. [14]. Between 8000 to 12000 cells (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), 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 incubated for 24 h at 37°C in a humidified atmosphere to allow cells to attach. TH8 and cisplatin were first dissolved in a minimum amount of DMF and then diluted to the required concentrations with mQ water and finally filtered to sterilize. A serial five fold dilutions of the drugs (ranging from 0.004 μM to 40 μM) in 10% FCS/RPMI 1640 medium were added to equal volumes of the cell culture in quadruplicate wells that were then left to incubate under normal growth conditions for 72 h. The inhibition of the cell growth was determined using the MTT reduction assay. Four hours after the addition of the MTT solution (50 μl per well of 1 mg ml^{-1} MTT solution), the yellow formazan crystals produced from the reduction of MTT were dissolved in 150 μl of DMSO and read with a Bio-Rad Model 3550 Microplate Reader. The IC_{50} values were obtained from the results of quadruplicate determinations of at least three independent experiments.

Drug Uptake and Binding with DNA

TH8 and cisplatin (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 x 10^6 cells ml^{-1}). The cells containing the drugs were incubated for 2, 4, 24 and 72 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 TH8 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.

Drug Accumulation in Cells

Following drug incubation the cell pellets were suspended in 0.5 ml of 1% triton-X, held on ice then sonicated. Total intracellular platinum contents were determined by graphite furnace AAS.

Drug-DNA Binding

Following drug incubation high molecular weight DNA from a cell pellet was isolated using JETQUICK Blood DNA Spin Kit/50 according to the modified protocol of Bowtell [15]. 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. Proteinase K (25 μl) and buffer K1 (200 μl) (containing guanidine hydrochloride and a detergent) were added to the mixture followed by incubation for 10 min at 70°C. Absolute ethanol (200 μl) 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 [16].

Interaction with pBR322 Plasmid DNA

The interaction of TH8 and cisplatin with pBR322 plasmid DNA was studied by agarose gel electrophoresis based on a method described by Stellwagen [17]. In the study, the amount of DNA was kept constant while the concentrations of compounds were varied. Exactly, 1.5 μl of supplied pBR322 plasmid DNA in solution was added to varied amounts of solutions of the compounds at different concentrations ranging from: 0.25 μM to 4.5 μM. The total volume

was made up to 20 μl by adding mQ water. The DNA blank was prepared by adding 18.5 μl mQ water to 1.5 μl of pBR322 plasmid DNA. The samples including the DNA blank were incubated for 4 h on a shaking water bath at 37 ºC in the dark. At the end of incubation, the reaction was quenched by rapid cooling to 0 ºC for 20 min. The samples were thawed then mixed with 4 μl of marker dye (0.25% bromophenol blue and 40% of sucrose). 17 μl of each sample was loaded onto 1% agarose gel made in TAE buffer that contained ethidium bromide (1 mg/ml). The gel was stained in the same buffer [18]. Electrophoresis was carried out also in TAE buffer containing ethidium bromide at 80 V for 3 h at room temperature. The bands of the plasmid DNA were viewed under short wave UV light using the BIO-RAD Trans illuminator IEC1010 and photographed with a Polaroid camera (orange filter) using Polaroid black-and-white print film, type 667.

BamH1 Digestion

BamH1 is known to recognize the sequence G/GATCC and hydrolyse the phosphodiester bond between adjacent guanine sites [19]. pBR322 contains a single restriction site for BamH1 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^{o}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 10x 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^{o}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.

HPLC

High pressure liquid chromatography (HPLC) combined with UV-visible spectrophotometry and graphite furnace spectroscopy was used to investigate the binding between TH8 and guanine. Equal volumes of 1 mM solution of TH8 and 2 mM solution of guanine were mixed together and incubated at 37^{o}C in a shaking water bath for 2 h. To dissolve guanine in mQ water, pH was increased to about 10 by adding a tiny drop of 0.1 M NaOH. After incubation, 5-20 μl of each of the mixtures and appropriate components was injected separately into a Waters HPLC system, consisting of a Waters 600 controller, a Waters 600 pump, a Waters 746 data module, a Waters Dual λ absorbance detector and Waters Nova-Pak C18 column consisting of Waters RCM 8 x 10 Module and Resolve^{TM} Cartridge set at a pressure of 17 Mpa. The wavelength was set at 260 nm. The mobile phase consisted of 5 % methanol and 95% of ammonium acetate (0.1 M at pH 5.5) with a flow rate of 1 ml/min. The retention times of the peaks applying to the mixtures and the components were recorded and the peak fractions were collected and analyzed for platinum and guanine contents to obtain binding ratio between platinum and guanine.

3. RESULTS AND DISCUSSION

IR, Mass and 1H NMR Spectral Results

Since suitable crystal of MH8 was not available, IR, ^{1}H NMR and mass spectral results combined with microanalyses results were used to characterize the identity of TH8.

The mass and ^{1}H NMR spectra of TH8 are given in Figs. (**3** and **4**) respectively.

The major peaks observed in the IR, mass and ^{1}H NMR spectra are listed in Table **1**. The letters ‘s’, ‘m’, ‘w’, ‘br’ and ‘d’ applying to bands in IR spectrum denote strong, me-

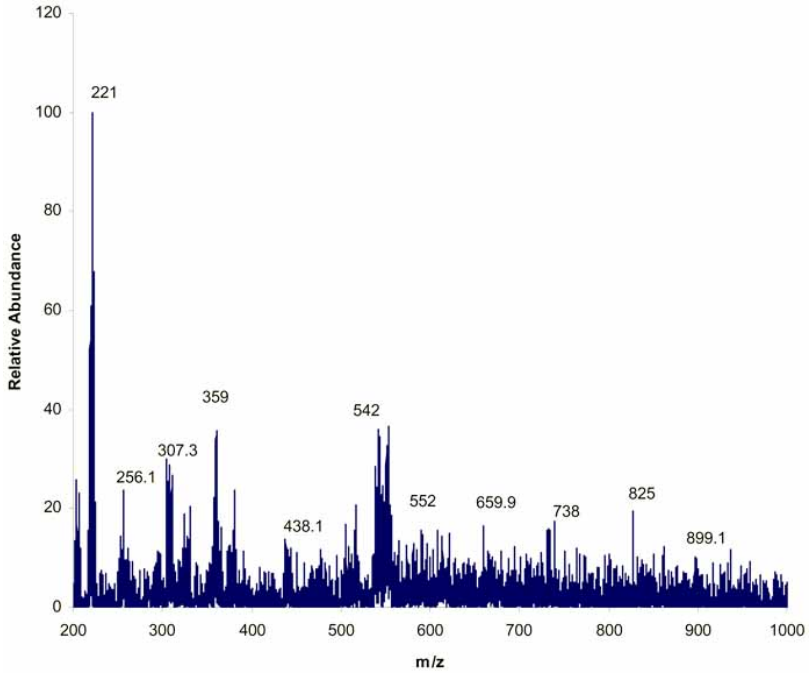

Fig. (3). Mass spectrum of TH8.

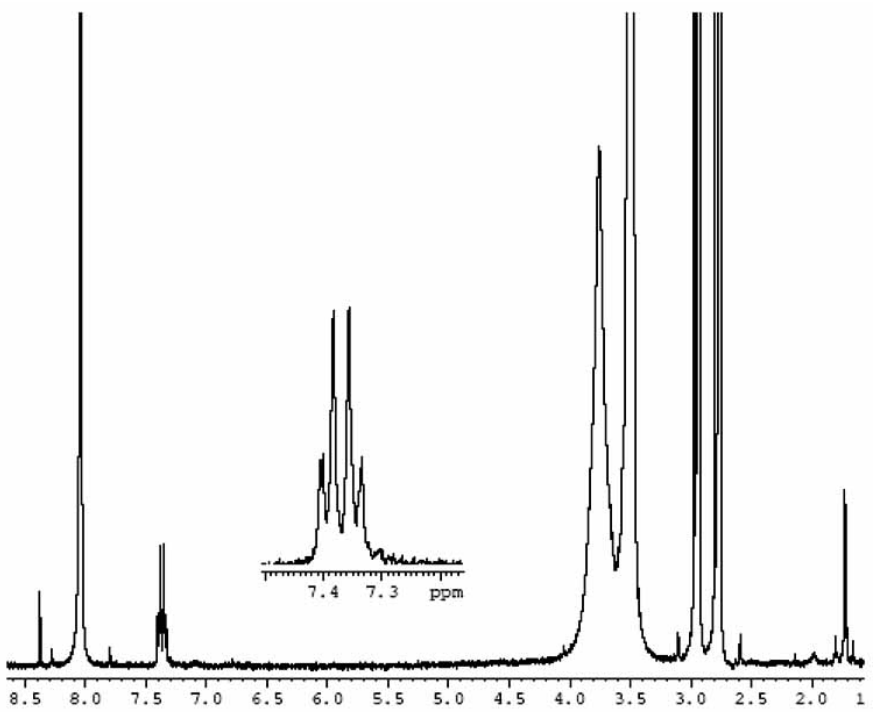

Fig. (4). ^{1}H NMR spectrum of TH8.

dium, weak, broad and doublet respectively. As applied to the peaks in mass spectrum, the number in parentheses after each m/z value indicates the relative intensity. As applied to resonances in ^{1}H NMR spectrum, the letters 's', 'd', 't' and 'q' stand for 'singlet', 'doublet', 'triplet' and 'quartet' respectively.

IR

The bands at 3726, 3625 and 3559 cm^{-1} are believed to be due to O–H stretching vibrations whereas that at 3301 cm^{-1} is believed to be due to N–H stretching vibration. The band at 2348 cm^{-1} is due to N–H and C–H stretching vibrations. The band at 1660 cm^{-1} is due to ring stretching vibration, that at 1284 cm^{-1} is due to N–H and C–H bending vibrations. Finally the bands at 350 and 324 cm^{-1} are due to Pt–Cl stretching vibrations whereas that at 302 cm^{-1} is due to Pd–Cl stretching vibration.

Mass

No peak was observed corresponding to M indicating that the molecule broke down in solution or in the spectrometer. The peak at m/z = 825 corresponds to the fragment produced from the loss of two 4-hydroxy pyridine units from TH8, that at m/z = 738 corresponds to the fragment produced from the loss of one 4-hydroxypyridine ligand and one chloro ligand from TH8, that at m/z = 552 corresponds to [M – Pt(4-hydroxy pyridine)$_2$($NH_2(CH_2)_6NH_2$),] that at m/z = 542 corresponds to [M – Pd(4-hydroxypyridine)$_2$ $(NH_3)_2(NH_2(CH_2)_6NH_2)_2$] and that at m/z = 359 corresponds to [Pt_2(4-hydroxy pyridine)$(NH_3)_2Cl_2$].

^{1}H NMR

The resonance at δ=8.3 ppm (singlet) is believed to be due to OH, that at δ=8.0 ppm is due to C_3H and C_5H. ortho and that at δ=7.4 ppm is also believed due to C_2H and

Table 1. Prominent Peaks or Resonances Found in IR, Mass and ^{1}H NMR Spectra of TH8

	Observed Peaks or Resonances
IR (cm^{-1})	3726 (s, O–H stretching vibration); 3625 (w, O–H stretching vibration); 3559 (m, O–H stretching vibration); 3301 (w, N–H stretching vibration); 2348 (b, C–H stretching vibration); 1660 (n, ring stretching vibration); 1284 (b, OH and CH bending vibration); 350 (w, Pt–Cl); 324 (m, Pt–Cl); 302 (s, Pd–Cl)
ESI-MS (DMF)(m/z: M = 1199.46	EIS-MS (DMF) (m/z: M = 1199.46); [M – (4-hydroxy pyridine)$_2$] = 825, [M – Pt(4-hydroxypyridine)Cl] = 738; [M – Pt (4-hydroxy pyridine)$_2$($NH_2(CH_2)_6NH_2$)] = 552; [M – Pd (4-hydroxypyridine)$_2$$(NH_3)_2(NH_2(CH_2)_6NH_2)_2$] = 542, [M – $PtPd(NH_3)_4(NH_2(CH_2)_6NH_2)_2$(4-hydroxypyridine)] = 359
^{1}H NMR	^{1}H NMR DMF δ ppm: 8.0 (due to C_3H and C_5H); 7.4 (d, due to meta C_1H and C_6H); 3.8 (s, due to NH_2); 3.6 (s, due to NH_2); 2.9 (t, due to CH_2); 2.8 (q, due to CH_2); 1.7 (s, due to CH_2)

Table 2. IC_{50} Values and Resistance Factors (RF) for TH8 and Cisplatin as Applied to the Human Ovary Cancer Cell Lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ (The Results are Averages of Those Obtained from 4 Identical Wells with 6000 to 9000 Cells Per Well)

IC_{50} (uM) ± SD and Resistant Factors					
Compound	**A2780**	**$A2780^{cisR}$**	**RF**	**$A2780^{ZD0473R}$**	**RF**
TH8	4.5 ± 0.4	6.2 ± 0.2	1.39	9.1 ± 1.1	2.05
Cisplatin	1.0 ± 0.4	12.9 ± 0.4	12.8	3.1 ± 1.2	3.0

C_6H.The resonances at δ=3.8 ppm (singlet) and 3.6 ppm (singlet) are believed to be due to NH_2. The resonances at δ=2.9, 2.8 and 1.3 ppm are believed to be due to CH_2 protons of 1,6-diaminohexane.

Finally, it should be noted that although none of the spectral results (IR, mass or ^1H NMR) on its own could completely characterize the compound, together the results provided a greater support for the suggested structure. If suitable crystals were available, the structure could have been confirmed by single crystal x-ray diffractometry. However, no suitable crystals of any of TH8 could have been grown.

Cytotoxicity

Table **2** lists the IC_{50} values and resistance factors (RF) of TH8 and cisplatin as applied to the human ovary cancer cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$. The IC_{50} value stands for drug concentration required for 50% cell kill whereas resistance factor indicates the ratio of the IC_{50} value applying to the resistant cell line to that applying to the parent cell line.

TH8 is found to be less active than cisplatin against the ovarian cancer cell lines: A2780 and $A2780^{ZD0473R}$ but more so against the cell line $A2780^{cisR}$, indicating that the compound has been better able to overcome mechanisms of resistance operating in the $A2780^{cisR}$ cell line. Three main mechanisms of resistance as applied to platinum drugs are (1) reduced cell uptake, (2) increased deactivation within the cell and (3) increased DNA repair. TH8 being a trinuclear complex in which the two terminal platinum centres only bind covalently with DNA forming a range of interstrand Pt(GG)Pt, may cause a global change in DNA conformation from B- to Z- and A-forms. In contrast, cisplatin is known to form mainly intrastrand Pt(GG) adduct that causes a local bending of a DNA strand. It should be noted that as some decomposition of MH8 is expected, binding of the decomposition products with DNA is also likely and if so the consequences of such binding with DNA cannot be ruled out.

Cell Uptake

The cellular accumulation of platinum and palladium were used as measures of cell uptake of TH8 and that of platinum for cisplatin. It may be noted that for the trinuclear complex TH8, both platinum and palladium levels should provide equivalent measures of the drug uptake provided the metal centres remain bound together. Table **3** gives the total intracellular platinum and palladium levels (expressed as nanomol Pt or Pd per 2 $x10^6$ cells) found in the cell lines:

Table 3. Pd and Pt Accumulation in A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ Cells in 2, 4, 24 and 72 h as Applied to TH8 and Cisplatin. – The Symbol Within Parentheses in Column 1 Indicates the Metal to which it Applies

Time (h)	**Compound**	**A2780**	**$A2780^{cisR}$**	**$A2780^{ZD0473R}$**
		nmol Pd or Pt per $2x10^6$ Cells	**nmol Pd or Pt per $2x10^6$ Cells**	**nmol Pd or Pt per $2x10^6$ Cells**
2	TH8 (Pd)	3.13 ± 0.53	0.02 ± 0.00	0.90 ± 0.01
	TH8 (Pt)	0.55 ± 0.13	0.06 ± 0.07	0.49 ± 0.09
	Cisplatin (Pt)	0.21 ± 0.26	0.09 ± 0.12	0.24 ± 0.26
4	TH8 (Pd)	1.43 ± 0.06	0.16 ± 0.02	2.63 ± 0.89
	TH8 (Pt)	0.44 ± 0.03	0.61 ± 0.18	1.84 ± 1.43
	Cisplatin (Pt)	0.26 ± 0.26	0.03 ± 0.02	0.20 ± 0.09
24	TH8 (Pd)	5.15 ± 6.00	0.64 ± 0.21	1.32 ± 0.07
	TH8 (Pt)	2.45 ± 1.32	0.30 ± 0.05	0.24 ± 0.23
	Cisplatin (Pt)	0.50 ± 0.34	0.21 ± 0.19	1.30 ± 0.26
72	TH8 (Pd)	0.70 ± 0.16	0.15 ± 0.02	0.11 ± 0.08
	TH8 (Pt)	0.21 ± 0.02	0.06 ± 0.06	0.10 ± 0.01
	Cisplatin (Pt)	0.36 ± 0.07	0.29 ± 0.03	0.11 ± 0.08

A2780, A2780^{cisR} and A2780^{ZD0473R} after exposure to 50 μM concentrations of TH8 and cisplatin for 2, 4, 24 and 72 h.

It can be seen that that the uptake of TH8 in terms of both Pt and Pd is highest at 24 h in all the three ovarian cancer cell lines A2780 and A2780^{cisR} and at 72 h there is a sharp drop in uptake. However, large errors observed for a number of measurements make the results less reliable. Not withstanding uncertainty associated with large error, Pt uptake from cisplatin is found to be lower than that from TH8 at 2, 4 and 24 h in the cell lines A2780 and A2780^{cisR} whereas in the cell line A2780^{ZD0473R} it is higher for cisplatin than for TH8. The decrease in uptake at 72 h as compared to that at 24 h suggests that increased efflux of the drug may be a mechanism of resistance operating in all the three ovarian cancer cell lines. When cell uptakes of Pt and Pd from TH8 are compared it is found that the Pt:Pd ratios are generally different from the expected value of 2:1; in fact at 72 h Pd uptakes are greater than Pt values in A2780 and A2780^{cisR} cell lines and similar in A2780^{ZD0473R} cell line. Noting that the trinuclear compound TH8 has one Pd and two Pt centres, the higher or comparable uptake of Pd over Pt clearly suggests that the compound must have undergone significant dissociation within and/or before entry into the cell. However, uncertainty remains as there are large errors associated with the measurements.

When the activity of TH8 in human ovarian cancer cell lines A2780, A2780^{cisR} and A2780^{ZD0473R} is compared with that of analogous Pt-Pd-Pt or Pt-Pt-Pt complexes such as CH25 (in which the central metal ion is bound to one 2-hydroxypyridine ligand and one ammonia ligand) [8], DH6Cl (in which the central metal ion is bound to two ammonia ligands) [7], and TH1 (in which the central metal ion is bound to two 3-hydroxypyridine ligands) [5], TH8 is found to possess much lower activity than CH25, DH6Cl or TH1. The results suggest that in terms of cytotoxicity, 4-hydroxypyridine is a deactivating ligand whereas 2-hydroxypyridine, 3-hydroxypyridine and ammonia may be activating, thus illustrating structure-activity relationship in cytotoxicity.

DNA Binding

Table **4** gives the levels of platinum and palladium DNA binding expressed as nanomol of Pt and Pd per milligram of DNA in A2780, A2780^{cisR} and A2780^{ZD0473R} cells in 2, 4, 24 and 72 h. for TH8 and cisplatin. For TH8, the highest level of Pt-DNA binding is observed at 4 h and lowest at 72 h for all the three cell lines. The decrease in the level of Pt-DNA binding with the increase in time (especially during the period 24 to 72 h) observed for TH8 as applied to all the three ovarian cancer cell lines: A2780, A2780^{cisR} and A2780^{ZD0473R} can be seen to support the idea that increased DNA repair is likely to be a mechanism of resistance operating in all the three cell lines. It may be noted that for the trinuclear compound BBR3464 increased DNA repair was also found to be a dominant mechanism of resistance operating in A2780/BBR3464 resistant cell line (Paola, Laura *et al.* 2003). As applied to cisplatin-resistant cell line A2780^{cisR}, level of Pt-DNA binding was found to decrease progressively during the period 2 to 72 h indicating that increased DNA repair may be dominant mechanism of cisplatin resistance operating in the cell line.

Table 4. Levels of Pd and Pt Binding with DNA in 2, 4, 24 and 72 h in A2780, A2780^{cisR} and A2780^{ZD0473R} Cells as Applied to TH8 and Cisplatin. – The Symbol within Parentheses in Column 1 Indicates the Metal to which it Applies

Time (h)	Compound	A2780	A2780^{cisR}	A2780^{ZD0473R}
		nmol Pd or Pt per 2x10^6 cells	nmol Pd or Pt per 2x10^6 cells	nmol Pd or Pt per 2x10^6 cells
2	TH8 (Pd)	2.6 ± 0.2	5.5 ± 0.8	28.0 ± 1.9
	TH8 (Pt)	11.0 ± 0.4	17.2 ± 1.2	1.0 ± 0.1
	Cisplatin (Pt)	13.8 ± 4.3	62.5 ± 6.4	0.5 ± 0.0
4	TH8 (Pd)	118.6 ± 2.1	109.0 ± 3.7	49.1 ± 1.7
	TH8 (Pt)	28.2 ± 12.5	25.3 ± 3.2	2.1 ± 0.1
	Cisplatin (Pt)	3.2 ± 0.2	15.7 ± 2.9	1.3 ± 0.4
24	TH8 (Pd)	30.2 ± 2.5	46.7 ± 11.5	6.3 ± 0.0
	TH8 (Pt)	26.6 ± 2.2	4.1 ± 1.0	1.4 ± 0.3
	Cisplatin (Pt)	25.4 ± 3.4	1.3 ± 0.5	1.7 ± 0.1
72	TH8 (Pd)	16.7 ± 0.7	28.4 ± 0.9	10.4 ± 0.6
	TH8 (Pt)	2.8 ± 0.1	0.9 ± 0.0	0.6 ± 0.1
	Cisplatin (Pt)	2.8 ± 0.0	1.0 ± 0.0	1.4 ± 0.0

It was suggested earlier that trinuclear cation in TH8 must have undergone significant dissociation.

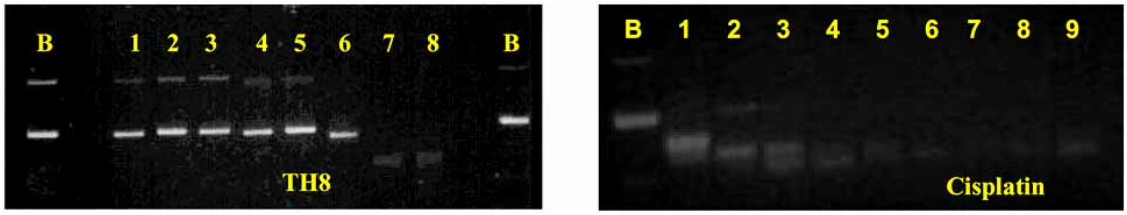

Fig. (5). Electrophoretograms applying to interaction of pBR322 plasmid DNA with increasing concentrations of TH8 and cisplatin. Lane B in both sides in the electrophoretograms applies to untreated pBR322 plasmid DNA to serve as a control, lanes 1 to 9 apply to plasmid DNA interacted with increasing concentrations of TH8 and cisplatin. For TH8: lane 1: 6.25 μM, lane 2: 12.5 μM, lane 3: 15 μM, lane 4: 20 μM, lane 5: 25 μM, lane 6: 35 μM, lane 7: 50 μM and lane 8: 60 μM. For cisplatin it has the value: lane 1: 5 μM, lane 2: 10 μM, lane 3: 15 μM, lane 4: 20 μM, lane 5: 25 μM, lane 6: 30 μM, lane 7: 35 μM, lane 8: 40 μM, and lane 9: 50 μM.

Gel Electrophoresis

Interaction with pBR322 Plasmid DNA

Fig. (**5**) gives the electrophoretograms applying to interaction of pBR322 plasmid DNA with increasing concentrations of TH8 and cisplatin for a period of 4 h at 37ºC.

Generally two bands corresponding to the forms I and II were observed in untreated pBR322 plasmid DNA. As the plasmid DNA was interacted with the increasing concentrations of TH8, two bands corresponding to forms I and II were observed for concentrations ranging 6.25 μM to 25 μM above which the two bands coalesced to form one band, indicative of changes in DNA conformation as a result of (possibly) interstrand covalent binding of TH8 with G7 centres of guanine. As the plasmid DNA was interacted with the increasing concentrations of cisplatin, two bands corresponding to forms I and II were observed for concentrations less than or equal to 12.5 μM above which the bands appeared essentially as one band, again indicating changes in DNA conformation as a result of (possibly) intrastrand covalent binding of platinum with N7 centres of guanine.

BamH1 Digestion

Fig. (**6**) shows the electrophoretograms applying to incubated mixtures of pBR322 plasmid DNA and varying concentrations of TH8 and cisplatin for a period of 4 h at 37ºC followed by BamH1 digestion for a further period of 1 h at the same temperature.

Untreated and undigested pBR322 plasmid DNA gave one band corresponding to form I or two bands corresponding forms I and II (Lane A). When untreated pBR322 plasmid band was digested with BamH1, only one band corresponding to form III band was observed (band B). When pBR322 plasmid DNA was interacted with increasing concentrations of TH8 followed by BamH1 digestion, three bands corresponding to forms I, II and III were observed for concentrations of TH8 ranging from 6.25 μM to 20 μM above which only form I band was observed. When pBR322 plasmid DNA was interacted with increasing concentrations of cisplatin followed by BamH1 digestion, two bands corresponding to forms I and II were observed for all concentrations of cisplatin ranging from 5 μM to 50 μM. Table **5** lists the bands observed as a function of concentration. Increasing prevention of BamH1 digestion with the increase in concentration of TH8 and cisplatin, indicates changes in DNA conformation brought about by the covalent binding of TH8 and cisplatin with the DNA.

Interaction with Nucleobases (HPLC)

As stated earlier, 1 mM solution of TH8 was mixed with equal volume of 2 mM solution of guanine following which the mixture was incubated for 2 h at 37^{o}C in a shaking water bath. 5-20 μL of solutions of guanine, TH8 and incubated mixture were injected into the HPLC. The retention times of the main peaks with the detector set at λ = 260 nm are shown in Table **6**. Change in retention time (from 12.42 min for guanine to 10.84 min for the incubated mixture of guanine and TH8) supports the idea that TH8 have reacted with guanine forming a covalent adduct. The occurrence of three peaks in the chromatogram of TH8 indicates that the pres-

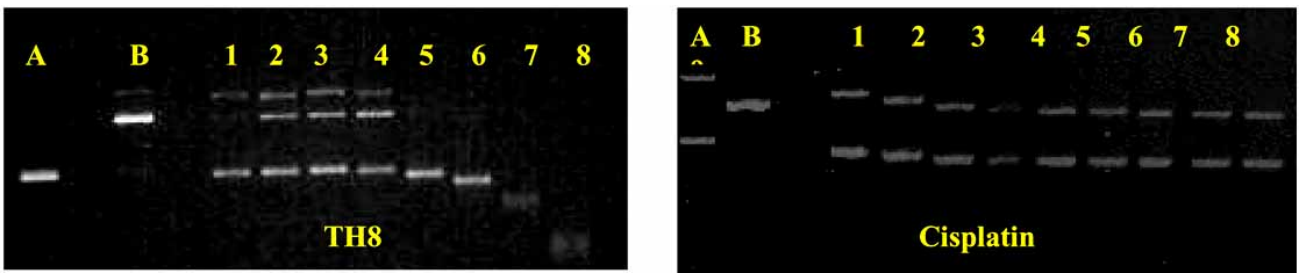

Fig. (6). Electrophoretograms applying to the incubated mixtures of pBR322 plasmid DNA and varying concentrations of TH8 and cisplatin followed by digestion with BamH1. Lane A applies to the untreated pBR322 and undigested plasmid DNA, lane B applies to untreated but digested plasmid DNA. Lanes 1 to 9: apply to pBR322 plasmid DNA interacted with increasing concentrations of the compounds followed by BamH1 digestion. For TH8: lane 1: 6.25 μM, lane 2: 12.5μM, lane 3: 15 μM, lane 4: 20 μM, lane 5: 25 μM, lane 6: 35 μM, lane 7: 40 μM and lane 8: 45 μM. For cisplatin it has the value: lane 1: 5 μM, lane 2: 10 μM, lane 3: 15 μM, lane 4: 20 μM, lane 5: 25 μM, lane 6: 30 μM, lane 7: 35 μM, lane 8: 40 μM, and lane 9: 50 μM.

Table 5. Bands Observed After BamH1 Digestion of Incubated Mixtures of TH8 and Cisplatin with pBR322 Plasmid DNA

Compound	Concentration indicator for the Bands #								
	1	2	3	4	5	6	7	8	9
TH8	I,II,III	I,II,III	I,II,III	I,II,III	I	I	I	I	-
Cisplatin	I,II	I,II	I,II	I,II	I,II	I,II	I,II	I,II	I,II

[#] For cisplatin,1: 5 μM, 2: 10 uM, 3: 15 uM, 4: 20 uM, 5: 25 uM, 6: 30 uM, 7: 35 uM, 8: 40 uM, and 9: 50 uM; 5: 25 uM, 6: 30 uM, 7: 35 uM and 8: 40 uM; for TH8: 1: 6.25 uM, 2: 12.5 uM, 3: 15 uM, 4: 20 uM, 5: 25 uM, 6: 35 uM, 7: 40 uM and 8: 45 uM.

ence of three species in solution formed possibly due to hydrolysis of TH8.

The (G:Pt) binding ratio calculated from the determination of Pt and guanine contents in the major peak fraction applying to the incubated mixture of TH8 and guanine was found to be 1.6 instead of the expected value of 1:1 for the trinuclear Pt-Pd-Pt cation [The molar extinction coefficient (ε) for guanine at 273 nm was measured to be 10.9 x 10^3 mol^{-1} cm^2]. The observed higher binding ratio indicates that TH8 might have undergone partial decomposition within and/or outside the cell. Because of the *trans*-labilizing effect of chloride ion, some decomposition of Pt-Pt-Pt and Pt-Pd-Pt complexes associated with the breakdown of the bonds between the terminal platinum ions and linking diamines is always expected. The presence of Pd^{2+} would make TH8 more susceptible to decomposition, as Pd^{2+} is much more labile than Pt^{2+}. It may be noted that based on cell uptakes and levels of Pt and Pd binding with DNA also, it was concluded earlier that TH8 could break down (at least partially) within/out of the cell.

CONCLUSION

A trinuclear Pt-Pd-Pt complex code named TH8 containing two 4-hydroxypyridine ligands bound to the central metal ion has been synthesized and investigated for antitumour activity against human ovarian cancer cell lines A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$. Although TH8 is found to be less active than cisplatin against the parent cell line A2780, it is more active against cisplatin-resistant cell line $A2780^{cisR}$, indicating that TH8 has been better able to overcome the resistance operating in $A2780^{cisR}$ cell line. Ratios of platinum and palladium cell uptakes, Pt-DNA and Pd-DNA binding and the HPLC results related to the interaction of TH8 with guanine suggest that TH8 may undergo significant decomposition within the cell.

AUTHOR'S CONTRIBUTIONS

HT carried out all experiments described. JQ assisted in cell culture studies and AAS and HPLC measurements. KF recorded mass spectrum. PB also assisted in cell culture study. FH conceived the project and participated in the design and coordination of the study.

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ABBREVIATIONS

TH7	=	[*trans*-$PdCl_2$(4-hydroxypyridine)$_2$]
Cisplatin	=	*cis*-dichlorodiamminplatinum(II)
EDTA	=	Ethylene diamine tetraacetic acid
Tris	=	Tris-hydroxymethylaminomethane
Tris-HCl	=	Tris-hydroxymethylaminomethane hydrochloride
Hepes	=	(N-[2-Hydroxymethyl]piperazine-*N'*-[2-ethanesulfonic acd]
1xTAE buffer	=	0.05 M Tris base + 0.05 M glacial acetic acid + 1 mM EDTA, pH = 8.0
MTT	=	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
DMSO	=	Dimethyl sulfoxide
PBS	=	Phosphate buffered saline
AAS	=	Atomic absorption spectrophotometry
DMF	=	*N, N'*-Dimethylformamide
FCS	=	Foetal calf serum

Table 6. The Retention Times of the Main Peaks with the Detector Set at λ = 260 nm for Guanine (G), TH8 and their Incubated Mixture

Reactant A	Reactant B	Retention Time (min) (Major Peak 1)	Retention Time (min) (Major Peak 2)	Retention Time (min) (Minor Peak 3)	Pt:G Binding Ratio
Guanine	None	12.42			
TH8	None	4.89	8.15	9.82	
TH8	Guanine	10.84			1.6:1

PBS	=	Phosphate-buffered saline
AAS	=	Atomic absorption spectrophotometry
HPLC	=	High pressure liquid chromatography
RF	=	Resistance factor
G	=	Guanine

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