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Studies on Oligonuclear Palladium Complexes

FAZLUL HUQ*, MOHAMMAD FARHAD, AHMED ABDULLAH,
PHILIP BEALE† and MEI ZHANG‡
School of Biomedical Sciences, Cumberland Campus, C42
The University of Sydney, East Street, PO Box 170, Lidcombe, NSW 1825, Australia
E-mail: f.huq@fhs.usyd.edu.au

Two polynuclear palladium complexes, [{*trans*-$PdCl(NH_3)_2$}$_2$ μ-{*trans*-$Pd(NH_3)_2$-$(H_2N(CH_2)_6NH_2)_2$}]Cl_4 (MH1) and [{*trans*-$PdCl(NH_3)_2$} μ-{*trans*-$Pd(NH_3)_2(H_2N(CH_2)_5NH_2)_2$}]$Cl_4$ (MH2) have been prepared and evaluated for their activity against A2780 and $A2780^{cis\text{-}R}$ ovary cell lines using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) reduction assay. The interaction of the compounds with pBR322 plasmid DNA and salmon sperm DNA has also been studied using gel electrophoresis. Both MH1 and MH2 display a lower activity than cisplatin, MH1 being marginally more active than MH2. The compounds are believed to bind to DNA forming interstrand bifunctional adducts and at higher concentrations may also form interhelical bifunctional adducts. The lower anticancer activity of the compounds as compared to cisplatin is believed to be due to their higher reactivity. The Pd-Cl bonds are broken much more readily than Pt-Cl bonds so that unlike BBR3464 or cisplatin, MH1 and MH2 are aquated more easily. The resulting aquated species are deactivated due to binding with other biomolecules such as glutathione and metallothionein before they have a chance to bind with DNA.

Key Words: Polynuclear, BBR3464, Palladium, Anticancer drugs, Salmon sperm DNA, Plasmid DNA, Gel electrophoresis, Cell culture.

INTRODUCTION

Cis-diamminedichloroplatinum(II) (cisplatin) is a well established anticancer drug[1] that is highly effective against testicular and ovarian cancers[2,3]. Before the introduction of cisplatin, the cure rate for patients with advanced testicular cancer was only 5-10% and after the introduction of cisplatin more than 80% of patients can expect to survive long term, free of disease[4]. It is also used alone or in combination with other drugs such as bleomycin and doxorubicin for the treatment of tumours of head and neck, lung, ovaries, cervix and bladder[5,6]. When

†RPAH, Missenden Road, Camperdown NSW, Australia
‡School of Chemistry, F11, University of Sydney, NSW 2006, Australia.

used in combination with other antitumour drugs, synergistic effects have been achieved[6]. But the clinical utility of cisplatin has been limited due to the frequent development of drug resistance[7] and severe side effects such as neurotoxicity, nephrotoxicity, ototoxicity, myelosuppression, nausea and vomiting[8]. Although high response rates can be achieved in ovarian cancer, the long-term results are disappointing due to the development of drug resistance leading to recurrence and subsequent death of most of these patients[9].

Hence vigorous research effects are applied all over the world to develop new anticancer active compounds that would have a reduced toxicity, a wider spectrum of activity and would circumvent cross-resistance.

During the last thirty years, thousands of cisplatin analogues have been prepared (and tested) by varying the nature of the leaving and the non-leaving groups. However, all the cisplatin analogues generally form similar adducts with DNA that translate often into a similar spectrum of activity. Thus it has been suggested that to have a spectrum of activity markedly different from that of cisplatin we need to develop compounds that would have novel chemical structures and biological properties[10]. One such class of compounds are the polynuclear platinum complexes[11, 12] that contain two or more platinum units linked together by diaminoalkane chains[13]. A notable example is BBR3464 which consists of three *trans*-platinum units joined together to two 1,6-diaminohexane chains. BBR3464 has been found to circumvent the inherent or acquired cisplatin-resistance *in vitro* and *in vivo* in a panel of human adult tumour models[13, 14]. The high level of antitumour activity in cisplatin-resistant cancer cell lines suggests that BBR3464 is able to overcome the multiple mechanism of cisplatin resistance[15]. But the cellular determinants responsible for the activity of BBR3464 are largely unknown[16]. BBR3464 was in phase II stage of clinical trial[17] before it was stopped because of toxicity. The aim of this study is to prepare palladium analogues of BBR3464, determine the nature of their interaction with DNA and assess their activity against cancer cell lines. Generally, palladium complexes are found to be toxic rather than anticancer active because of their high reactivity (palladium compounds are generally 105 times more reactive than the corresponding platinum complexes)[18]. However, this criterion may not apply to multi-centred complexes since it is found that BBR3464 is highly reactive and yet it is found to be anticancer active. It is thus possible that even the multi-centred palladium complexes could display significant anticancer activity even though they are expected to be highly reactive. Even if the compounds are found to be inactive, it is thought that the study would provide valuable information on the nature of their interaction with DNA. Thus, the compounds [{*trans*-$PdCl(NH_3)_2$}$_2$ μ-{trans-$Pd(NH_3)_2$-$(H_2N(CH_2)_6NH_2)_2$}]Cl_4 (MH1) and [{*trans*-$PdCl(NH_3)_2$} {μ-{*trans*-$PdNH_3)_2(H_2N(CH_2)_5NH_2)_2$}]$Cl_4$ (MH2) were prepared (Fig. 1) and their activity against cancer cell lines and nature of interaction with DNA determined.

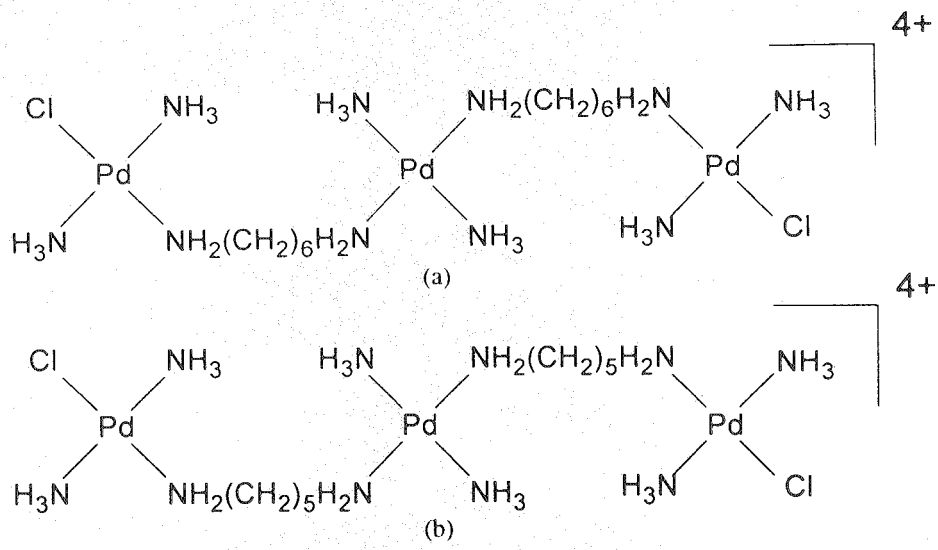


Fig. 1. Structures of (a) MH1 and (b) MH2; only the tetrapositive cations are shown in the structures

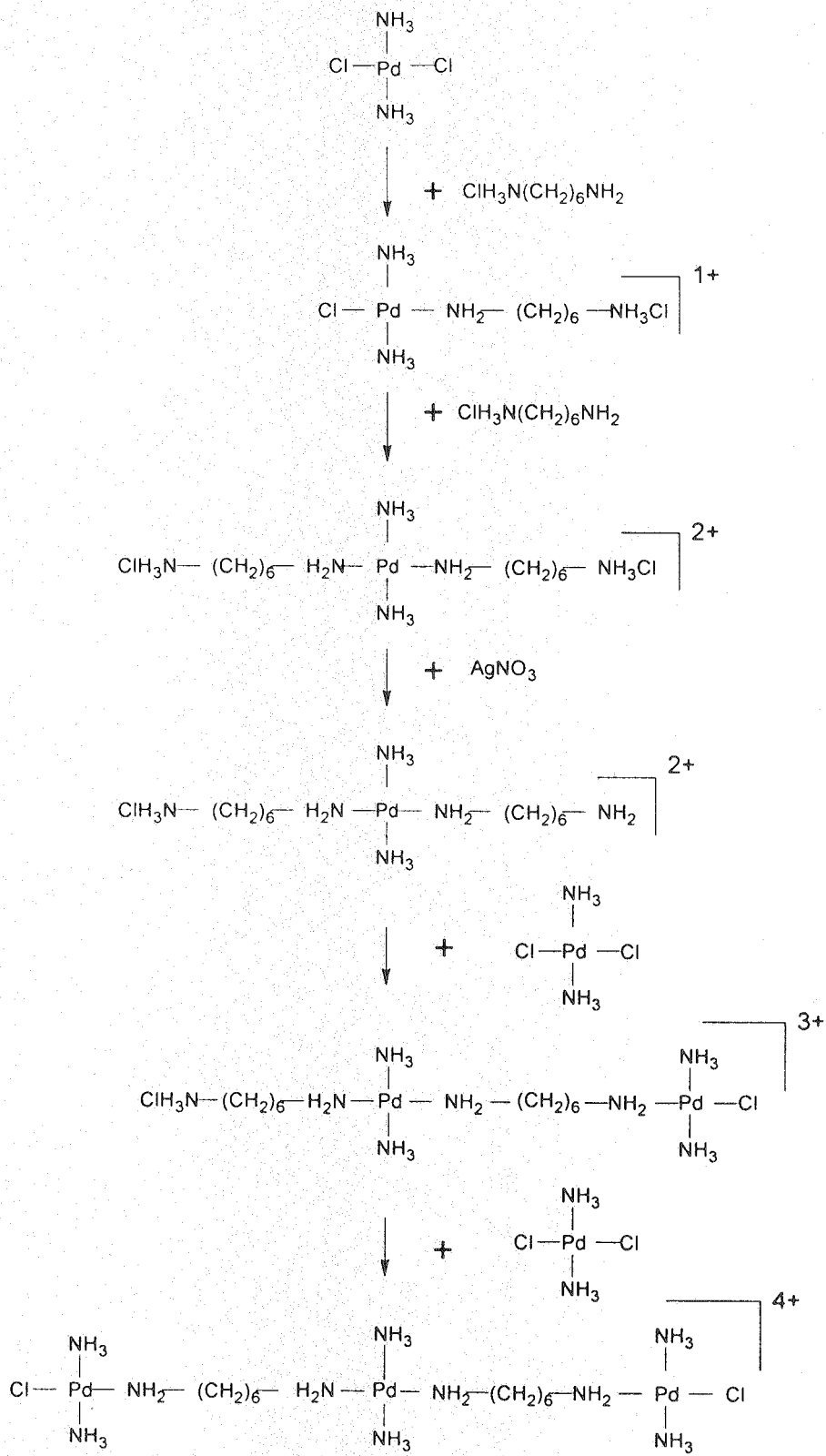
EXPERIMENTAL

A step-up method branching out from the central metal unit has been employed to prepare the trinuclear palladium compounds MH1 and MH2. The steps in the synthesis of MH1 are given in **Scheme-1**. The steps in the synthesis of MH2 would be similar except that the linking diamine was 1,5-diaminopentane.

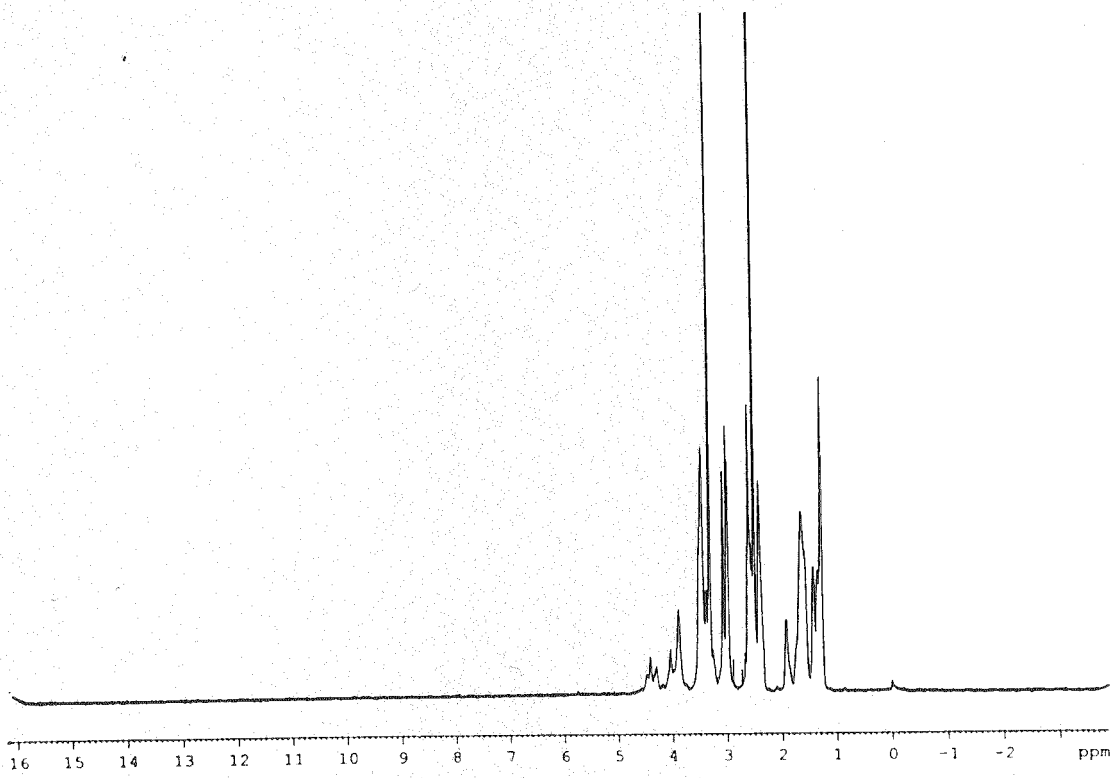
Cisplatin, potassium tetrachloropalladate [K_2PdCl_4], 1,6-diaminohexane [$C_6H_{16}N_2$], 1,5-diaminopentane dihydrochloride (Cadverine) [$C_5H_{14}N_2{\cdot}2HCl$] and N,N-dimethylformamide (DMF) were obtained from Sigma Chemical Company, St. Louise, USA; dichloromethane and potassium tetrachloroplatinate [K_2PtCl_4] were obtained from Aldrich Chemical Company, Milwaukee, USA; acetone and silver nitrate were obtained from Ajax Chemicals, Auburn, NSW, Australia; methanol and ethanol were obtained from Merck Pvt. Ltd., Kilsyth, VIC, Australia. Salmon sperm DNA was purchased from Sigma-Aldrich, NSW, Australia and 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 Pvt. Ltd. Trypsin, Hepes and Dulbecco's phosphate buffered saline powder were obtained from Sigma-Aldrich Pvt. Ltd., NSW, Australia. 96 well culture cluster (flat bottom with lid) was obtained from Edward Keller and 25 cm^2 culture flask was obtained from Crown Scientific. 3-[4,5-Dimethylthiazol-2-yl]-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide were obtained from Sigma-Aldrich Pvt. Ltd, NSW, Australia. Microplate reader BIO-RAD Model 3550 was used to read the optical density of each well.

trans-[$PdCl_2(NH_3)_2$]

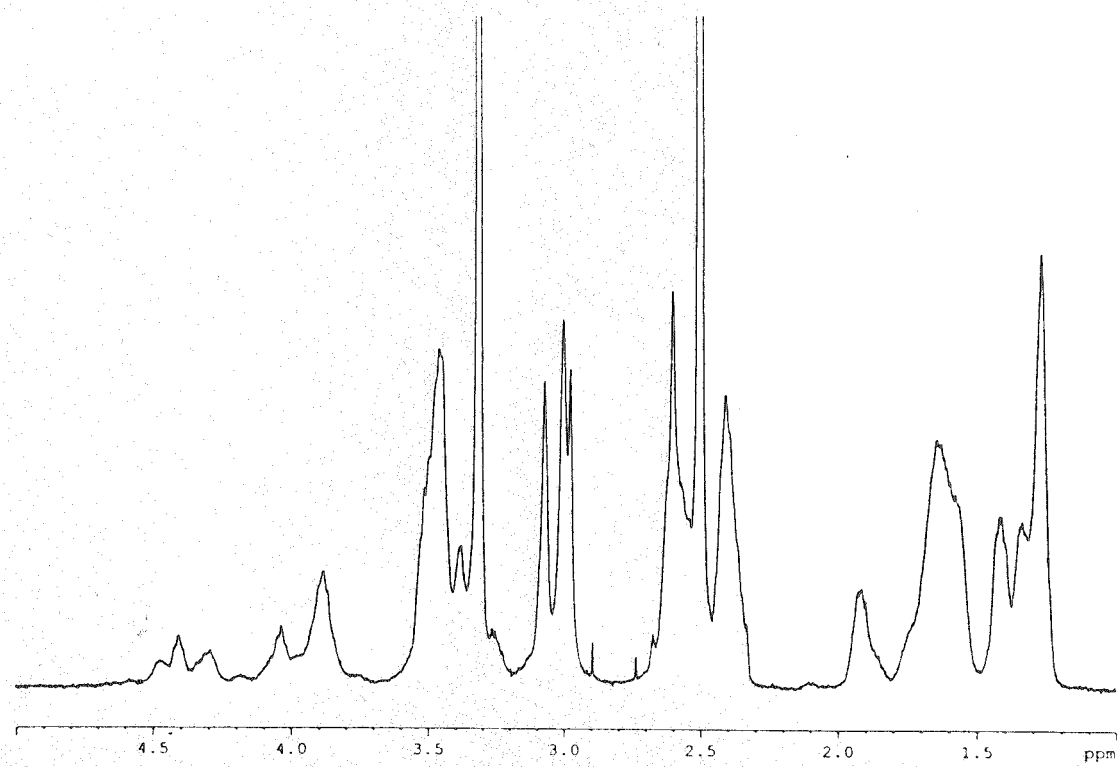
Transpalladin used as the starting material for the synthesis of MH1 and MH2 was prepared using the general method of Dhara[19]. Briefly, 326.5 mg (1 mmol) of potassium tetrachloropalladate(II) [K_2PdCl_4] was dissolved in 7.5 mL of mQ water to produce a dark brown solution. 0.25 mL of concentrated HCl was added



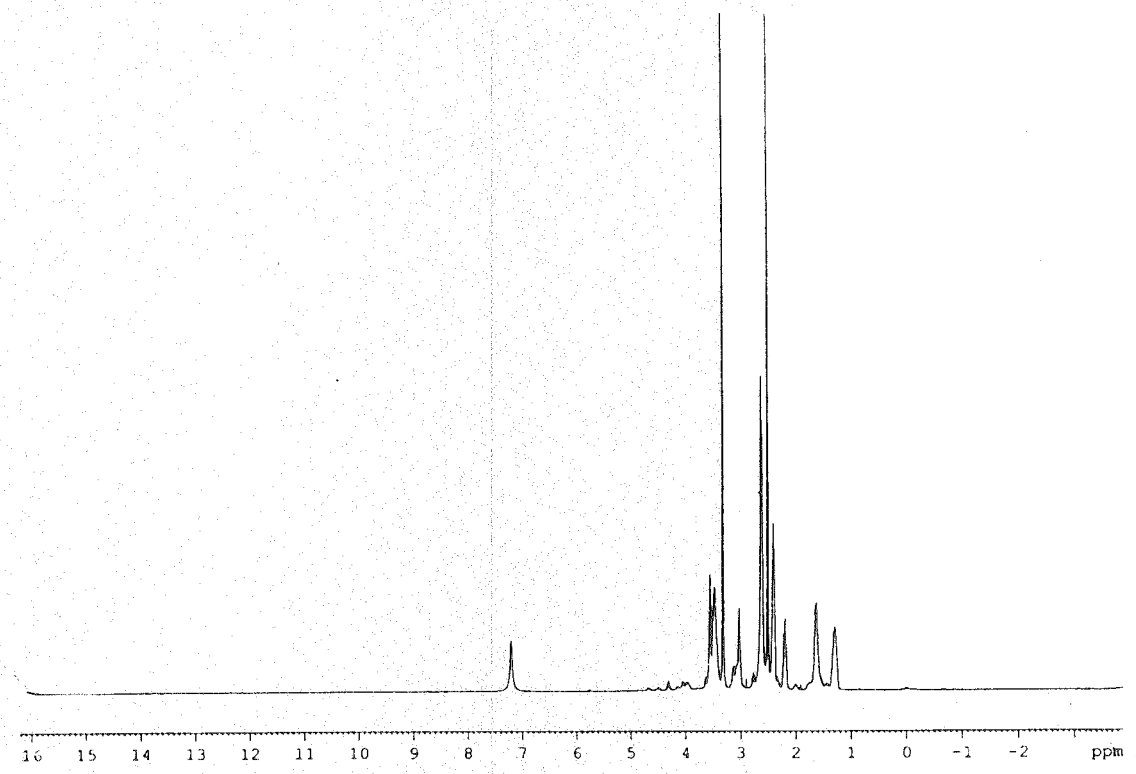
Scheme-1. The steps in the synthesis of MH1: One amino group of 1,6-diaminohexane has been changed to hydrochloride form to prevent chelation with one metal centre



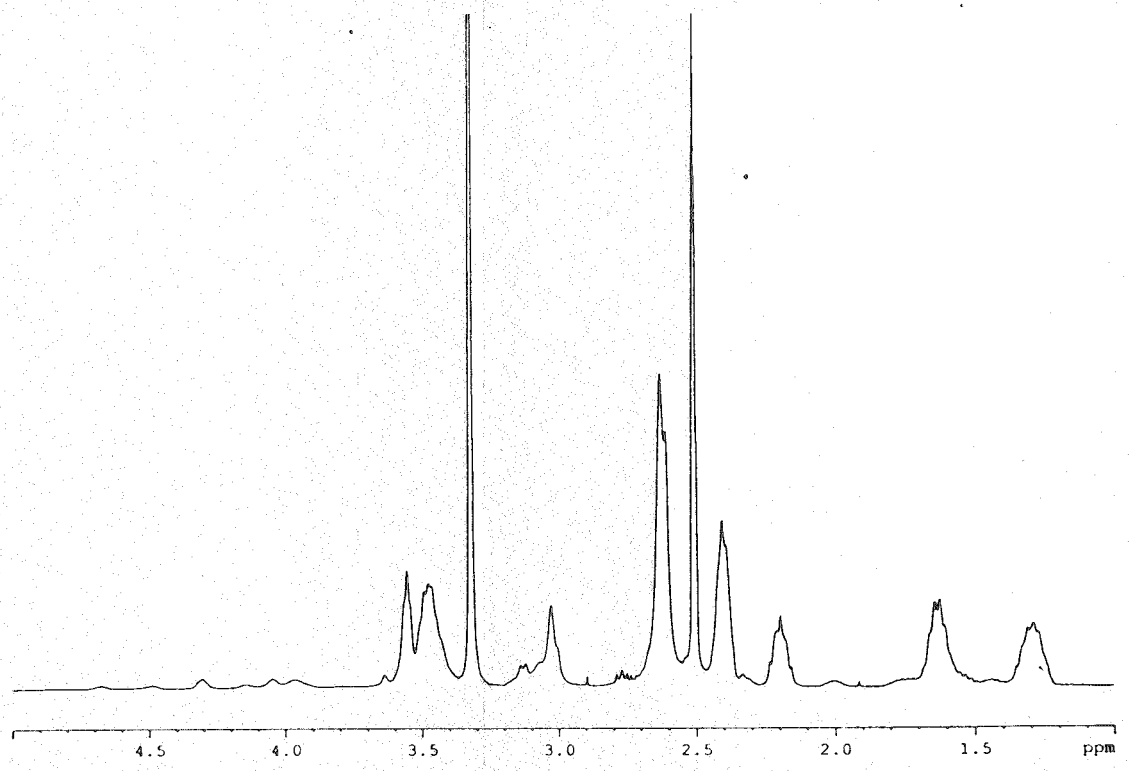
MH1



MH1



MH2



MH2

(with stirring) to the solution; the temperature was increased to about 60°C. 1.2 mL of concentrated aqueous ammonia was slowly added (with stirring) to the solution. The resulting solution was kept at 50–60°C until the colour changed through dark pink to light pink. 40 mL of 6 M HCl was then added to the solution. The turbid mixture produced was stirred for 2 h at 40–50°C. The volume of the solution was reduced to about 3 mL keeping the temperature within 40–50°C, while the solution was constantly stirred in a fume hood. It was then cooled in an ice-bath at 0°C for about 40 min. The precipitate of *trans*-$[PdCl_2(NH_3)_2]$ was collected at the pump, washed with acetone and cold water. The filtrate was evaporated to reduce the volume and cooled in an ice bath to obtain a further crop of *trans*-$[PdCl_2(NH_3)_2]$. The impure *trans*-$[PdCl_2(NH_3)_2]$ was dissolved in a minimum volume of hot 0.01 M HCl and cooled in an ice-bath for 2 h for crystals to be formed. The crystals of *trans*-$[PdCl_2(NH_3)_2]$ (light brown in colour) were collected at the pump. Yield = 60.0%.

MH1

A solution of 0.5 mmol transpalladin (0.106 g) in 10 mL of DMF was made and stirred at 30–40°C for 30 min to produce solution **A**. 1 mmol of 1,6-diaminohexane (0.1162 g) was dissolved in 3 mL of DMF to produce solution **B**. Solution **B** was added to solution **A**. A white precipitate was immediately formed. The mixture was stirred for 15 min and was labelled as solution **C**. 1.0 mmol of transpalladin (0.212 g) was dissolved in 16 mL of DMF. 0.98 mmol of silver nitrate (0.1664 g), dissolved in 4 mL of mQ water, was added to the transpalladin solution. The mixture was stirred in the dark for 20 h at room temperature. It was then centrifuged at 5500 rpm for 10 min. The supernatant (yellow-orange in colour) was collected and filtered to remove any particles of AgCl. The filtrate was added to solution **C**. The resulting mixture was stirred for 3 h at 40°C. First the colour changed to yellow green and then to lighter yellow when most of the precipitate dissolved. The mixture was stirred for a further 20 h at 40°C at the end of which it was filtered. The volume of the filtrate was reduced to about 5 mL by using a vacuum concentrator consisting of Javac DD150 double stage high vacuum pump, Savant RVT4104 refrigerated vapor trap and Savant Speed Vac110 concentrator. 40 mL of dichloromethane was added to the solution to reduce the solubility of the complex. An orange-yellow precipitate was quickly formed. The precipitate was collected and washed with CH_2Cl_2. Finally the precipitate was washed with methanol and ethanol (yield 66%).

MH2

MH2 was prepared following a procedure similar to that for MH1. Briefly a solution of 0.5 mmol transpalladin (0.106 g) in 10 mL of DMF was made and stirred at 30-40°C for 30 min to produce solution **A**. 1 mmol of 1,5-diaminopentane dihydrochloride (0.1751 g) was dissolved in 3 mL of DMF to produce solution ***B***. Solution **B** was added to solution **A**. The mixture was stirred for about 2 h at 40°C to produce a clear yellow solution termed as solution C. 1.0 mmol of transpalladin (0.212 g) was dissolved in 16 mL of DMF. 0.98 mmol of silver nitrate (0.1664 g) dissolved in 4 mL of mQ water was added to transpalladin

solution. The mixture was stirred in dark for 20 h at room temperature. It was then centrifuged at 5500 rpm for 10 min. The supernatant was collected and filtered to remove any particles of AgCl. This was then added to the solution C and stirred for 3 h at 40°C. The colour changed through yellow-green to yellow. The mixture was stirred at 40°C for a further 20 h at the end of which some precipitate was formed. This was then filtered and the volume of the filtrate was reduced to about 5 mL by using the vacuum concentrator. 40 mL dichloromethane was added to the solution to produce yellow-orange precipitate. The precipitate was collected and washed first with dichloromethane and then with methanol and ethanol (yield 65%).

Characterization

C, H, N and Cl analyses were carried out using the facility at the Australian National University and palladium content was determined by graphite furnace atomic absorption spectroscopy (AAS) using the Varian Spectra-20 atomic absorption spectrophotometer available in the School of Biomedical Sciences, The University of Sydney. 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 to 400 cm^{-1}. The instrument was run under vacuum during spectral acquisition. To obtain mass spectra, solutions of MH1 and MH2 in 10% DMF and 90% methanol were sprayed into a Finnigan LCQ ion trap mass spectrometer available in the School of Chemistry, The University of Sydney. The flow rate was 0.2 mL/min consisting of 50% methanol and 50% water. ^1H NMR spectra of MH1 and MH2 were recorded in dimethylsulfoxide-d_6 (DMSO-d_6) solution in a Bruker DPX400 spectrometer using 5 mm high precision Wilmad NMR tube. Spectra were referenced to internal solvent residues and recorded at 300 K (±1 K).

The molar conductivity values of MH1 and MH2 in solution in 1 : 1 mixture of DMF and water at 298 K were determined using PW9506 digital conductivity meter. The conductivity values were measured at the concentrations: 0.5 mM, 0.25 mM, 0.1 mM and 0.01 mM. The molar conductivity (Λ) was calculated as $\Lambda = k/c$ where k is the specific conductivity and c is the concentration. The molar conductivity values obtained were then plotted against concentration to determine the limiting values.

Interaction with DNA

The interaction between cisplatin, transpalladin, MH1 and MH2 and pBR322 plasmid DNA and salmon sperm DNA (ssDNA) was followed by gel electrophoresis. Solutions of cisplatin, transpalladin, MH1 and MH2 in 0.01 M sodium perchlorate were prepared and sterilized by passing through 0.22-μm Millipore filter. A small amount of N,N′-dimethylformamide was added to dissolve MH1 and MH2 (500 μL for 10 mL of 600 μM solution) and a small amount of dimethyl sulfoxide (300 μL for 10.0 mL of 600 μM solution) was added to dissolve cisplatin. Solution of pBR322 plasmid DNA (0.5 mg/mL) in the buffer consisting of 1 mM Tris-HCl at pH 7.6, 1 mM NaCl and 1 mM EDTA (as supplied) was used. Solution of ssDNA (1 mg/mL) was also prepared in the Tris-HCl buffer at

pH 7.6. Appropriate volume of cisplatin, transpalladin, MH1 or MH2 was added to 1 μL of pBR322 plasmid DNA or ssDNA and the total volume was made up to 20 μL so that the concentration of the drug varied from 2.5 μM to 60.0 μM while that of DNA remained unchanged at 60.5 μM in terms of phosphate in the case of plasmid DNA and 70.0 μM in terms of phosphate in the case of ssDNA. The mixtures were incubated at 37°C for 5 h at the end of which the reaction was quenched by rapid cooling to 0°C. Agarose gel (1%, w/v) in TAB buffer (40 mM Tris base, 40 mM acetic acid, 1 mM EDTA, pH 7.6) containing 0.6 μg/mL of ethidium bromide was prepared. Then 16 μL of each of the incubated drug-DNA mixtures, containing the tracking dye (0.25% bromophenol blue and 40% sucrose in water) was loaded on to the gel and electrophoresis was carried under TAB buffer system at 80 V for 2.5 h. At the end of electrophoresis the gel was visualised under UV light using the Bio-Rad Trans illuminator IEC 1010. The illuminated gel was photographed with a Polaroid camera (a red filter and Polaroid type of film were used).

Cytotoxicity studies

The cytotoxicity[20] of MH1 and MH2 on human ovary cell lines: A2780, A2780$^{cis\text{-}R}$, as compared to that for cisplatin and transpalladin was evaluated using MTT assay[21]. The cisplatin-resistant cell line A2780$^{cis\text{-}R}$ has been developed by chronic exposure of parent cisplatin-sensitive A2780 cell line to increasing concentrations of cisplatin[22]. All manipulations were carried out in a Class II recirculating hood (InterMed Microflow Pathfinder) using standard *in vitro* culture techniques[23]. The cell lines were recovered from liquid nitrogen, grown as monolayer cultures at 37°C in RPMI 1640 medium containing 10% (v/v) fetal calf serum, 20 mM Hepes and 1% of L-glutamine. The cells grown in the culture flasks were trypsinized and 100 μL of medium containing 1×10^4 cells/well were seeded into 96 well microplate. Cells were incubated for 24 h to allow the cell to attach. Growing cultures were maintained in humidified atmosphere of 5% CO_2 and 95% air at 37°C. Regular checks were carried out for mycoplasma by DNA fluorescent staining method using fluorochrome DAPI. The cells were sub-cultured two times every week. The concentrations of the drugs in the incubated mixtures were 0.01, 0.05, 0.25, 1.25 and 6.25 μM.

Solutions of compounds at different concentrations were added to the wells (100 μL/well) in quadruplicate. The plates were incubated for 3 d at the end of which the medium was discarded and 50 μL of freshly prepared MTT was added to each well. The plates were incubated for 4–5 h at 37°C. Then MTT was removed and 150 μL of DMSO was added to each well. Within 1 h of addition of DMSO, the absorbance was measured at 540 nm. The data were plotted as cell % of living cells against drug concentration and the IC_{50} values were determined.

RESULTS AND DISCUSSION

Composition

MH1: Anal. (%) Found: C, 18.6 ± 0.4; H, 5.5 ± 0.4; N, 15.2 ± 0.4; Cl, 23.9 ± 0.3; Pd, 35.6 ± 1.2; Calcd. for $C_{12}H_{50}N_{10}Cl_6Pd_3$: C, 16.6; H, 5.8 ; N, 16.2;

Cl, 24.6; Pd, 36.8). Molar conductivity in solution in 1 : 1 mixture of DMF and water at 298 K = 40 ohm^{-1} cm^2 mol^{-1}.

MH2: Anal. (%) Found: C, 15.8 ± 0.4; H, 5.9 ± 0.4; N, 14.1 ± 0.4; Cl, 24.6 ± 0.3; Pd, 37.0 ± 1.2; Calcd. for $C_{10}H_{46}N_{10}Cl_6Pd_3$: C, 14.3; H, 5.5; N, 16.7; Cl, 25.4; Pd, 38.1. Molar conductivity in solution in 1 : 1 mixture of DMF and water at 298 K = 35 ohm^{-1} cm^2 mol^{-1}.

The results of elemental analysis show that even after repeated crystallization, the compounds MH1 and MH2 could not be obtained in a highly pure form. ^1H NMR results also indicate the presence of impurity.

Spectral studies

The bands observed in IR, Raman, mass and ^1H NMR spectra of MH1 and MH2 are given in Table-1. The interpretation of the bands observed in IR and Raman spectra has been based on published spectra[24–29].

TABLE-1
PERCENTAGE OF CELL SURVIVAL RATE FOR CISPLATIN-RESPONSIVE AND CISPLATIN RESISTANT OVARY CELL LINES A2780 AND A2780$^{cis\text{-}R}$

Dose (μM)	Cisplatin		Transpalladin		MH1		MH2	
	A2780	A2780$^{cis\text{-}R}$	A2780	A2780$^{cis\text{-}R}$	A2780	A2780$^{cis\text{-}R}$	A2780	A2780$^{cis\text{-}R}$
0.01	58.3±6.5	78.9±12.6	86.8±11.6	93.6±2.4	75.2±5.7	86.7±9.4	86.8±12.5	90.7±7.5
0.05	42.1±2.6	70.7±9.4	76.3±13.7	87.9± 2.4	58.7±1.3	81.2±5.8	80.5±17.2	86.5±9.9
0.25	24.1±4.3	68.1±11.6	72.8±12.1	82.6±2.7	53.0±2.7	79.8±5.7	76.9±15.3	83.0±8.6
1.25	17.1±2.4	53.7±15.7	68.9±9.9	71.2±13.9	40.9±7.6	72.3±14.0	69.0±13.1	81.3±8.4
6.25	9.8±0.9	25.9±7.6	60.3±9.8	64.3±8.7	32.1±5.9	68.2±15.8	64.2±10.7	75.5±11.3

MH1

IR: The bands at 3284 and 3265 are due to N—H stretching vibrations whereas those at 2920 and 2848 are due to asymmetrical and symmetrical CH_2 stretching vibrations. The 16 bands at 1651 and 1356 are due to N—H bending vibrations whereas that at 1171 is CH_2 wagging. The band at 1012 is believed to be due to C—C stretch. The band at 825 is believed due to N—H wagging. The bands at 754 and 675 are due to C—H out-of-plane bending vibrations. The bands at 588 and 488 are due to Pd—N stretching vibrations[23].

Raman: The band at 3209 is due N—H stretching vibration whereas that at 2856 is believed to be due to C—H stretching vibration. The band at 1437 is due to N—H bending vibration. The band at 1188 is believed to be due to C—H wagging vibration. The band at 1046 is due to C—C stretch. The bands at 598 and 488 are due to Pd—N stretching vibrations. The band at 290 is due to Pd—Cl stretching vibration whereas that at 178 is due to Pd—N bending vibration. The band at 83 is believed to be associated with lattice mode.

TABLE-2
PROMINENT IR, RAMAN, MASS AND ^{1}H NMR SPECTRAL BANDS OBSERVED IN MH1 AND MH2 (ν cm^{-1})

	MH1	MH2
IR (cm^{-1})	3284 s, 3265 s, 2920 s, 2848 m, 2515 w, 2336 w br, 2017 w, 1718 w, 1651 m, 1356 s, 1171 s, 1012 m, 825 m, 754 m, 675 m, 588 m, 488 w, 417 w	3321 s, 3238 m, 2925 m, 2852 w, 2457 w, 1971 w br, 1588 m, 1464 w, 1390 w, 1248 s, 1180 w, 1070 w, 756 m, 494 w, 408 w
Raman (cm^{-1})	3209 w br, 2856 w d, 1437 w, 1294 w, 1188 m, 1046 w, 598 w, 488 m, 413 w, 290 s, 178 m d, 83 m	3236 w, 2990 w, 2891 w br, 1584 w br, 1435 w, 1240 w, 1032 w, 681 w, 490 s, 417 w, 294 s, 216 s, 81 s
Mass	ESI-MS (DMF) M = 866.6 (m/z: (M-6Cl-6NH_3 + H) = 553 (0.06); Pd{$NH_2(CH_2)_6NH_2$}Pd = 330 (0.07); $PdCl_2${$NH_2(CH_2)_6(NH_2$}= 294 (0.06); (H_2PdCl_4) = 250 (1.00); (Pd{$NH_2(CH_2)_6NH_2$} + H) = 223)	ESI-MS (DMF) M = 838.5 (m/z: (M-Cl + 2H) = 805 (0.24); (M-Cl-2H) = 801 (0.98); (M-6Cl-6NH_3-H) = 523 (0.50); ($Cl_2Pd(NH_3)${$NH_2(CH_2)_5NH_2$}+ H) = 298 (0.92); Pd(NH_3){$NH_2(CH_2)_5NH_2$} – 2H) = 207 (1.00)
^{1}H NMR	^{1}H NMR DMSO δ ppm: 4.4 (br, 12H, due to NH_3); 4.05 (br, 6H, due to NH_3); 3.9 (br, 8H, due to NH_2); 3.5 (br, 8H, due to CH_2); 2.45 (br, 8H, due to CH_2); 1.3 (br, 8H, due to CH_2); 3.45 (water); 2.65 (DMSO); 2.55 (DMSO); 1.95 (impurity); 1.65 (impurity).	^{1}H NMR DMSO δ ppm: 4.3 (br, 12H, due to NH_3); 4.05 (br, 6H, due to NH_3); 3.6 (br, 8H, due to NH_2); 3.5 (br, 8H, due to CH_2); 2.45 (br, 8H, due to CH_2); 2.2 (br, due to impurity); 1.3 (br, 4H, due to CH_2); 3.35 (water); 2.65 (DMSO); 2.55 (DMSO); 1.65 (impurity)

(The number in parentheses after each m/z value in mass spectra indicates the relative intensity)

Mass: The mass spectrum of MH1 has a peak with m/z = 553 that corresponds to {M-6Cl-6NH_3 + H}. The peak with m/z = 330 is believed to be due to Pd{$NH_2(CH_2)_6NH_2$Pd}. The peak m/z = 294 is believed to be due to H_2PdCl_4 that may be formed *in situ*. The peak with m/z = 223 is due to (Pd{$NH_2(CH_2)_6NH_2$} + H).

^{1}H NMR: ^{1}H NMR spectrum of MH1 gives a broad resonance with a chemical shift value of 4.4 ppm. It is due to NH_3-Pd. The peak at δ = 4.05 ppm may be due to NH_3-Pd. The small peak at δ = 3.9 ppm is believed to be due to NH_2-Pd. The peak at δ = 1.30 ppm is for CH_2 protons that lie in the middle of the chain; whereas that at δ = 3.5 ppm is believed to be due to CH_2 protons adjacent to NH_2 group and that at δ = 2.40 ppm is believed to be due to CH_2 protons second nearest to NH_2 group. The peak at δ = 3.45 ppm is due to water and that at δ = 2.55 ppm is due to DMSO. The peaks at δ = 1.65 and 1.95 ppm are due to the impurities. The results show that the compound has significant amount of impurities.

MH2

IR: The bands at 3321 and 3238 cm^{-1} are believed to be due to N—H

stretching vibrations whereas that at 2925 cm^{-1} is due to C—H stretching vibration. The bands at 1588 and 1464 cm^{-1} are due to N—H bending vibrations whereas that at 1180 cm^{-1} is due to CH_2 wagging. The band at 1070 cm^{-1} is believed to be due to C—C stretch. The band at 756 cm^{-1} is due to C—H out-of-plane bending vibration. The bands at 494 and 408 cm^{-1} are due to (Pd—N) stretching vibrations.

Raman: The band at 3236 cm^{-1} is due N—H stretching vibration whereas that at 2891 cm^{-1} is believed to be due to C—H stretching vibration. The bands at 1584 and 1435 cm^{-1} are due to N—H bending vibrations. The band at 1240 cm^{-1} is believed to be due to C—H wagging vibration. The band at 1032 cm^{-1} is due to C—C stretch. The bands at 490 and 417 cm^{-1} are due to Pd—N stretching vibrations. The band at 216 cm^{-1} is due to (Pd—Cl) stretching vibration whereas that at 81 is believed to be associated with lattice mode.

Mass: The mass spectrum of MH_2 has a peak with m/z = 805 that corresponds to (M-Cl + 2H), that at m/z = 805 corresponds to (M-Cl-2H). The peak with m/z = 523 is believed to be due to ($M-6Cl-6NH_3 - H$). The peak m/z = 298 is believed to be due to ($Cl_2Pd(NH_3)\{NH_2(CH_2)_6NH_2\} + H$). The peak m/z = 207 is believed to be due to ($Pd(NH_3)\{NH_2(CH_2)_6NH_2\} - 2H$).

It is not clear why the mass spectrum of MH_2 is richer in peaks than that of MH_1.

^{1}H NMR: ^{1}H NMR spectrum of MH2 gives a broad resonance with a chemical shift value of 4.3 ppm due to NH_3-Pd. The peak at $\delta = 4.05$ ppm is believed to be due to NH_3-Pd. The peak at $\delta = 3.6$ ppm is believed to be due to NH_2-Pd protons. The peak at $\delta = 1.30$ ppm is due to CH_2 protons that lie in the middle of the chain whereas those at $\delta = 3.5$ and 2.40 ppm are believed to be due to other CH_2 protons. The peak at $\delta = 3.35$ ppm is due to water and that at $\delta = 2.65$ ppm is due to DMSO. The peaks at 2.2 and 1.65 ppm are believed to be due to impurity. The results show that MH2 also contains significant amount of impurity.

Interaction with pBR322 plasmid DNA and ssDNA

Fig. 2 gives the electrophoretograms applying to the interaction of cisplatin, transpalladin, MH1 and MH2 with pBR322 plasmid and salmon sperm DNAs. Initially, pBR322 plasmid DNA was found to contain one intense band corresponding to form I and two other fainter bands corresponding to forms II and III.

Cisplatin

When the pBR322 plasmid DNA was allowed to interact with cisplatin, three bands corresponding to forms I, II and III were observed for the concentrations of cisplatin ranging from 2.5 to 15 μM and only form II band was observed at higher concentrations of cisplatin, namely 30 and 60 μM. The mobility of the form II band decreased slightly and that of the other two bands increased, as the concentration of the drug was increased. Cisplatin binds covalently with DNA, forming mainly intrastrand bifunctional Pt(GG) and Pt(AG) adducts[30] resulting into local distortion and DNA unwinding. The mobility of the form I band increased with the increase in concentration of the drug could be due to unwinding of the super-coiled DNA as a result of its binding with platinum. The increase in

mobility of the form III band is difficult to explain unless we assume that a reduction in size of the doubly nicked DNA occurred as a result of partial cleavage brought about by covalent binding with the compound. The decrease in mobility of the slowest form II with the increase in concentration of the drug may be due

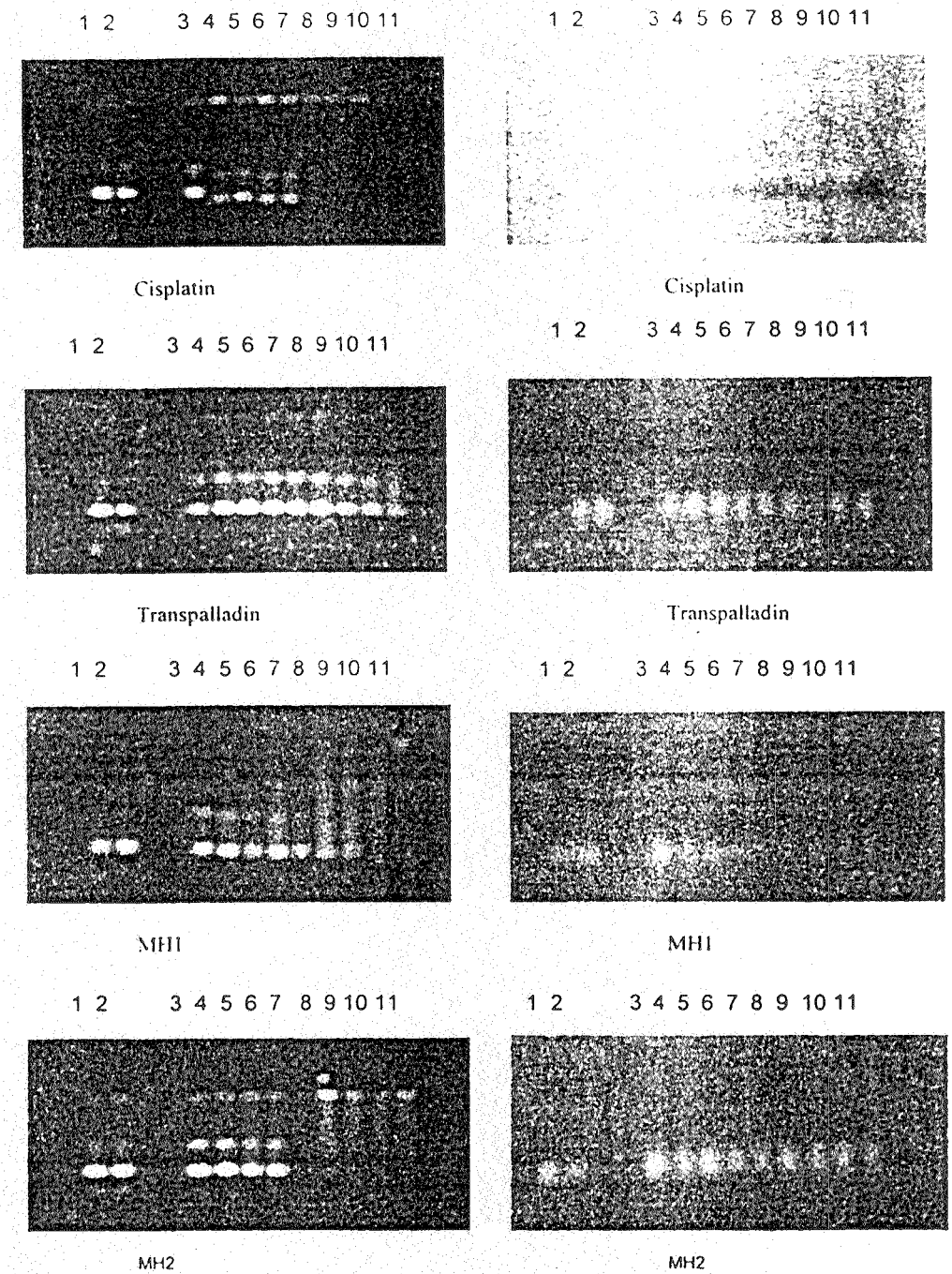


Fig. 2. Interaction between cisplatin, transpalladin, MH1 and MH2 with pBR322 plasmid DNA (column 1) and salmon sperm DNA (ssDNA) (column 2) in TAE buffer at pH 7.6 in air. Lanes 1 and 2: untreated pBR322 plasmid DNA; lanes 3–11: plasmid DNA (at 0.025 μg/uL) varying [compound] (2.5, 5, 7.5, 10, 15, 20, 30, 40 and 60 μM)

to an increase in its molecular mass and a decrease in the net negative charge of the DNA as a result of its binding with the drug. It may be noted that the neutral cisplatin molecule is chemically inert and activated only when chloro ligands are replaced by water molecules. It is well known[31] that in aqueous solutions the chloro ligands of cisplatin are replaced by water molecules in a stepwise manner to form *cis*-$[Pt(Cl)(H_2O)(NH_3)_2]^+$ and *cis*-$[Pt(H_2O)_2(NH_3)_2]^{2+}$. Only a weak form II band is observed at [cisplatin] = 40 and 60 μM and that no form I and form III bands can be seen, perhaps point to the increased degradation of form I and form III at high concentrations of the drug.

A single band was observed in both untreated and treated salmon sperm DNA. As the concentration of the drug was increased, the intensity of the band was found to decrease slightly. The mobility of the band was found to decrease slightly as the concentration of cisplatin was increased from 2.5 to 15 μM. Further increase in [cisplatin] (20 μM or greater) did not cause any noticeable change in the mobility of the band although it became progressively weaker in intensity. The decrease in intensity suggests the occurrence of a partial degradation of DNA due to covalent binding of cisplatin with nucleobases in DNA. The absence of any observable change in mobility would then be due to a balance of the opposing factors such as a decrease in overall negative charge of the DNA due to the binding of cisplatin and a decrease in molecular mass due to its partial degradation. A decrease in DNA fluorescence due to the binding of cisplatin with nucleobases in DNA could also explain why the intensity of the DNA band decreased with increasing concentration of the drug but then in the absence of any DNA degradation we would have observed a decrease in band mobility.

Transpalladin

When the DNA was allowed to interact with transpalladin, again three distinct bands were observed. At [transpalladin] = 60 μM, an elongated streaking band was observed which points to degradation of the DNA. It can be seen that cisplatin and transpalladin bring about distinctly different changes to pBR322 plasmid DNA due to a difference in the nature of binding—predominantly intrastrand in the case of cisplatin and interstrand in the case of transpalladin—and also a difference in the reactivity of the two compounds—transpalladin being much more reactive than cisplatin. When transpalladin was allowed to interact with salmon sperm DNA, there was no significant change in the intensity and mobility of the band as the concentration of the compound was increased. The results suggest that the interstrand binding of transpalladin to salmon sperm DNA did not cause any observable damage to the DNA.

MH1

When pBR322 plasmid DNA was allowed to interact with MH1, three distinct bands were observed for the concentration of MH1 ranging from 0 to 20 μM. For higher concentrations of MH1 (30 to 60 μM), essentially one streaking band was observed, the intensity of which decreased as the concentration of MH1 was increased. Whereas at [MH1] = 30 μM, the intensity of the head of the streaking band was greater, at [MH1] = 60 μM, the intensity of the tail of the band was

greater. At [MH1] = 40 μM, the streaking band had essentially the same intensity throughout. The mobility of the form I band was found to increase slightly as the concentration of MH1 was increased. The increase in mobility of the band is due to a change in unwinding of the super coiled form I brought about by the covalent binding of MH1 with the DNA. Like BBR3464, MH1 is believed to form a number of long-range interstrand adducts with nucleobases in DNA *via* the two terminal metal centres.

As in the case[32] of BBR3464, the high positive charge on the multinuclear cation from MH1 (and also on that from MH2 considered later) (if they are made free in solution in water; more about it will be considered later in the paper) would facilitate rapid binding to DNA. In the case of BBR3464, the $t_{1/2}$ for binding with DNA was reported to be approximately 40 min, significantly less than that for cisplatin. One would expect this value to be low for MH1 (and also for MH2) and it is possible that like BBR3464 both MH1 and MH2 would prefer to bind to GG sites in DNA.

The distance between two terminal palladium centres in MH1 is such that it will span a bit less than one turn of double helix (about nine nucleotides) so that ladder-like interstrand adducts are formed. More of this will be considered later. Although transpalladin also is more likely to form interstrand adducts, because of its much shorter length the two palladium ions will bind directly across. Even then, the two DNA strands need to come closer together resulting in local distortion and unwinding. The pattern of the pBR322 plasmid DNA bands observed in its interaction with MH1 is found to be distinctly different from that observed in its interaction with transpalladin, reflects the difference in the actual nature of interaction of the two compounds with the DNA. A greater damage to the pBR322 plasmid DNA is observed in its interaction with MH1 than with transpalladin, suggests that MH1 binds with the DNA more readily. It should be noted that transpalladin develops two units of positive charge when the two chloro ligands are replaced by water molecules whereas the multinuclear cation of MH1 (which has initially four units of positive charge) develops overall six units of positive charge when the two bound chloro ligands are similarly replaced by water molecules. Hence aquated MH1 would be more strongly attracted towards the DNA than aquated transpalladin.

In the interaction of salmon sperm DNA with MH1, a single band was observed in both unreacted DNA and that interacted with MH1. However, the intensity and mobility of the band was found to decrease slightly as the concentration of MH1 was increased. The results suggest the occurrence of increased covalent binding of MH1 with the ssDNA as the concentration of MH1 was increased.

MH2

When pBR322 plasmid DNA was allowed to interact with MH2, three distinct bands were observed for the concentration of MH2 from 0 to 10 μM. For higher concentrations of MH2 (20–60 μM), essentially one streaking band was observed the intensity of which decreased as the concentration of MH2 was increased. At [MH2] = 20–40 μM, the streaking band was found to be uniform in intensity throughout. The pattern of the pBR322 plasmid DNA bands observed in its

interaction with MH2 was found to be generally similar to that observed in its interaction with MH1. The results suggest that both the compounds formed essentially similar interstrand adducts with DNA although MH2 being shorter length than MH1 would span a smaller distance along the helix axis. Unlike that in the case of MH1, the mobility of the three observed bands remained essentially unchanged as the concentration of MH2 was increased.

A single band was found when MH2 was allowed to interact with salmon sperm DNA. The mobility and the intensity of the band decreased as the concentration of MH2 increased. The results suggest increased covalent binding of MH2 with ssDNA as the concentration of MH2 was increased.

Activity against cancer cell lines

The trinuclear palladium compounds MH1 and MH2 were tested for activity against ovary cancer cell lines A2780 and A2780$^{cis\text{-}R}$ where cisplatin and transpalladin were used as reference. Table-1 shows the activity of MH1 and MH2 against ovary cell lines A2780 and A2780$^{cis\text{-}R}$.

For the A2780 cell line, the IC_{50} for MH1 is found to be about 0.50 μM whereas that for cisplatin is 0.04, showing that the cell line is 12.5 times less sensitive for MH1 than for cisplatin. Transpalladin and MH2 are found to be even less active, the least active being transpalladin. For the A2780$^{cis\text{-}R}$ cell line, the IC_{50} for cisplatin is 1.92 μM. MH1, MH2 and transpalladin were found to be much less active than cisplatin. This means that MH1 is much less active than BBR3464 which is found to be more active than cisplatin against the cell line[15]. Although the IC_{50} for MH2 and transpalladin could not be determined from the results, it is clear that the values are likely to be greater than 10 μM. BBR3464 and MH1 are likely to have identical geometries except that whereas in BBR3464 the metal centres contain Pt^{2+} and those in MH1 contain Pd^{2+}. The difference in activity of the two compounds can be explained in terms of a higher reactivity of MH1 than BBR3464. As stated earlier, palladium compounds are found to be on the average 10^5 times more reactive than the corresponding platinum compounds especially as applied to metal-chloride bond[18]. Low molar conductivity values found for both MH1 and MH2 (40 ohm^{-1} cm^2 mol^{-1} for MH1 and 35 ohm^{-1} cm^2 mol^{-1}) when dissolved in solution in 1 : 1 mixture of DMF and water indicate that both the compounds persist as largely ionic aggregates in the solution. However, when the solutions made in DMF were diluted with water and then allowed to stand at room temperature for about 24 h, the molar conductivity values were found to be much greater (close to about 300 ohm^{-1} cm^2 mol^{-1}) indicating that in the diluted solutions both the compounds dissociated to produce free multicentred cations and chloride anions. Further dissociation into mono-centred cations did not appear to take place. It is suggested that in biological fluids also where there is a large excess of water, free multicentred cations would be produced and it is further suggested that most of the Pd-Cl bonds in MH1 (and also in MH2) would be broken resulting in the replacement of the chloro ligands by water molecules. The aquated multinuclear cations are deactivated by reaction with other biomolecules such as glutathione and metallothionein before they have a chance to bind with DNA. It is however possible that for both MH1 and MH2 some binding with

DNA may occur. It could be due to direct binding that some of the aquated molecules with DNA or due to a reaction between deactivated molecules and nucleobases in DNA form the thermodynamically preferred drug-DNA adducts. Binding with DNA would explain why both MH1 and MH2 display some activity against the cancer cell lines. (Studies on cell uptake and DNA binding would provide further light on the matter.) (It should however be noted that binding with DNA is not an essential requirement for biological activity since reaction with cell thiols, *e.g.*, glutathione, thiol groups in proteins such as ribonucleotide reductase could also cause cytotoxicity.) And if this were so, the difference in activity of MH1 and MH2 against the A2780 cell line would be expected to be associated with the difference in size of the two molecules. To explore the possibility, the structures of MH1 and MH2 were optimized based on molecular mechanics and semiempirical calculations using HyperChem 7[33] and the optimized molecules were positioned so as to form interstrand bifunctional GG adducts with the double stranded DNA fragment d(GGGGGGCCCCCC). When fully stretched, the lengths of MH1 and MH2 but with the chlorides replaced by nitrogens are respectively 2.68 nm and 2.41 nm. It is found that in the double stranded B DNA, the interstrand N7 (guanine) to N7 (guanine) distances are: G(1) to G(6): 1.59 nm, G(1) to G(7): 1.93 nm, G(1) to G(8): 2.28 nm, G(1) to G(9): 2.63 nm, G(1) to G(10): 2.97 nm and G(1) to G(11): 3.29 nm. It can be seen that when fully stretched a better match between the length of the molecule and interstrand N7 (guanine) to N7 (guanine) distance occurs for MH1. However, both MH1 and MH2 being flexible molecules, like BBR3464 expected form a range of GG inerstrand adducts of various ranges dictated by the sequence of bases in the DNA[34]. Qu *et al.*[34] reported that in the formation of 1,4-GG interstrand adduct by BBR3464 with the self-complementary 5′-d(ATG*-TACAT)$_2$-3′ octamer the *syn* conformation was 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. They found that Watson-Crick pairing was essentially maintained and that the central linker was 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 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[35, 36] before binding to specific nucleobases in DNA. Wheatle *et al.*[35] point out that since pre-association is stabilized largely by electrostatic forces, the

pre-association of cationic multi-centred 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 equally applicable to the interaction of MH1 and MH2 with DNA.

Conclusion

Two trinuclear palladium complexes code named MH1 and MH2 have been prepared and studied to assess their activity against the two ovary cell lines A2780 and A2780$^{cis\text{-}R}$ and to determine the nature of their interaction with pBR322 plasmid and salmon sperm DNAs. Both the compounds are found to be significantly less active than cisplatin and of course BBR3464. MH1 in which the linking chain is 1,6-diaminohexane is found to be slightly more active than MH2 in which the linking chain is 1,5-diaminopentane. The lower activities of MH1 and MH2 as compared to that for cisplatin (and BBR3464) are believed due to the higher reactivities of the compounds so that most of the Pd-Cl bonds are deactivated before they have a chance to bind with nucleobases in DNA.

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