Synthesis, characterisation, activities, cell uptake and DNA binding of [{trans-PtCl(NH3)(2)}{mu-(H2N(CH2)(6)NH2)}trans-PdCl(NH3)(2)](NO3)Cl

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Original article

Synthesis, characterisation, activities, cell uptake and DNA binding of [{*trans*-$PtCl(NH_3)_2$} {μ-$(H_2N(CH_2)_6NH_2)$} {*trans*-$PdCl(NH_3)_2$](NO$_3$)Cl

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Abstract

The dinuclear complex: [{*trans*-$PtCl(NH_3)_2$}{μ-$(H_2N(CH_2)_6NH_2)$}{*trans*-$PdCl(NH_3)_2$](NO$_3$)Cl (code named DHD) has been synthesized and characterized. The activity against human cancer cell lines including ovarian: A2780, A2780^{cisR}, cell up take, level of binding with DNA and nature of interaction of the compound with pBR322 plasmid and salmon sperm DNAs have been determined. The compound is found to exhibit significant anticancer activity against ovarian cancer cell lines: A2780, A2780^{cisR} and A2780^{ZD0473R}—about two times as active as cisplatin against A2780 cell line, about five times as active as cisplatin against A2780^{cisR} and A2780^{ZD0473R} cell lines. The higher activity of DHD suggests that the compound is able to overcome multiple mechanisms of resistance operating in A2780^{cisR} and A2780^{ZD0473R} cell lines. DHD is believed to form a range of interstrand GG adducts with duplex DNA that induces global changes in the DNA conformation, unlike cisplatin and ZD0473 that form mainly intrastrand adducts that induces 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 DHD provides support to the idea that the interstrand binding of DHD with pBR322 plasmid DNA brings about global changes in DNA conformation.

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

1. Introduction

Although cisplatin is a widely used anticancer drug [1,2], its clinical utility has been limited due to the frequent development of drug resistance [3,4] and severe side effects [5,6]. During the last 30 years, thousands of cisplatin analogues have been prepared by varying the nature of the leaving groups and the carrier ligands. However, all cisplatin analogues generally form similar adducts with DNA that often translate into a similar spectrum of activity. Currently, attention is given to platinum compounds with structures distinctly different from that of cisplatin with the idea that their different nature of interaction with DNA would translate into different spectrum of activity and toxicity profile [7,8]. One such class of compounds are the polynuclear platinum complexes [9] that contain two or more platinum units linked together by diaminoalkane chains [10]. A notable example is BBR3464 that consists of three *trans*-platinum units joined together by 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 [11–13]. BBR3464 was in phase II stage of clinical trial [14] before it was stopped due to significant toxicity namely neutropenias, diarrhoea and nausea. The two terminal platinum units in BBR3464 undergo covalent binding (mainly interstrand) with DNA whereas the central platinum unit undergoes only non-covalent interactions such as

Abbreviations: DHD: [{*trans*-$PtCl(NH_3)_2$}{μ-$(H_2N(CH_2)_6NH_2)$}{*trans*-$PdCl(NH_3)_2$](NO$_3$)Cl; ssDNA: salmon sperm DNA; cisplatin: *cis*-dichlorodiammineplatinum(II); 1 × TAE 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; DMF: *N*, *N*-dimethylformamide; DMSO: dimethyl sulfoxide; PBS: phosphate buffered saline; DMF: *N*, *N*-dimethylformamide; AAS: atomic absorption spectrophotometry; EDTA: ethylene diamine tetraacetic acid.

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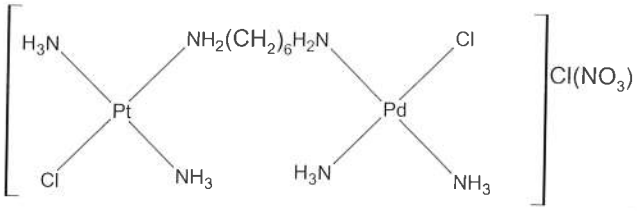


Fig. 1. Structure of DHD.

hydrogen bonding and electrostatic interactions [15–17]. 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 leading to a different spectrum of activity, recently we have prepared a number of Pt-Pd-Pt trinuclear complexes having the general formula: [18]. All of the compounds were found to display significant activity against human cancer cell lines (one being about 20 times as active as cisplatin against $A2780^{cisR}$ cell line) [19]. Having found the trinuclear Pt-Pd-Pt complexes to be tumour active, we were interested to find out whether dinuclear Pt-Pd complexes would also display any anticancer activity. In this paper we report on the synthesis, activities, the nature of interaction with DNA, cell uptake and extent of binding with DNA of [{*trans*-$PtCl(NH_3)_2$}{μ-($H_2N(CH_2)_6NH_2$)}{*trans*-$PdCl(NH_3)_2$]$Cl(NO_3)$ (code named DHD). DHD is a dinuclear complex made by joining together of a transplatin unit and a transpalladin unit by a molecule of 1,6-diaminohexane and in which the balancing negative ions are Cl^- and NO_3^-. The structure is given in Fig. 1. The compound is indeed found to possess significant antitumour activity against human cancer cell lines and is believed to overcome multiple mechanisms of resistance operating in $A2780^{cisR}$ and $A2780^{ZD0473R}$ cell lines.

2. Materials and methods

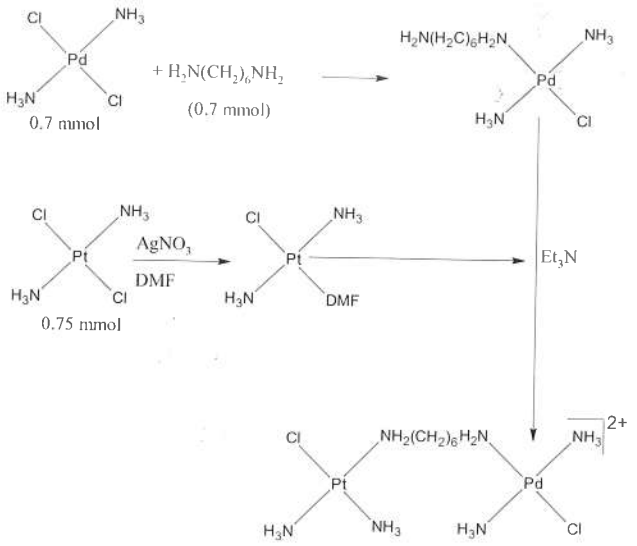
2.1. Materials

pBR322 plasmid DNA was purchased from ICN Biomedicals, Ohio, USA. Acetone [$(CH_3)_2CO$] and silver nitrate ($AgNO_3$) were obtained from Ajax Chemicals, Auburn, NSW, Australia. 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. Methods

2.2.1. Synthesis

As stated earlier, DHD is a dinuclear compound composed of a *trans*-platinum unit and a *trans*-palladium unit



Scheme 1. Steps in the synthesis of DHD: only the dipositive cation of DHD is shown, the valencing anions are not shown.

connected together by 1,6-diaminohexane. It may be noted that the dinuclear cation: [{*trans*-$PtCl(NH_3)_2$}{μ-($H_2N(CH_2)_6NH_2$)}{*trans*-$PdCl(NH_3)_2$]$^{2+}$ present in DHD is dipositively charged. Scheme 1 depicts the steps in the synthesis of DHD.

A 0.75 mmol of transplatin (0.225 g) was dissolved in 18 ml of DMF to which was added 0.710 mmol of silver nitrate (0.121 g). The mixture was stirred in the dark at room temperature for 24 h following which it was centrifuged at 5500 rpm for 30 min to separate precipitate of AgCl. The supernatant called 'transplatin filtrate' was collected and kept at −16 °C.

A suspension of 0.7 mmol (0.148 g) of transpalladin, made in 10 ml of DMF, was gently heated with stirring at 30–40 °C for about 30 min. A 0.7 mmol of 1,6-diaminohexane dissolved in 3 ml DMF was added dropwise to transpalladin suspension to obtain a clear yellow solution that afterwards turned slightly turbid. The mixture was stirred for 5 h at room temperature. The transplatin filtrate (0.7 mmol) (that was prepared earlier and kept at −16 °C), was added to the mixture followed by the addition of 90 μl of triethyl amine. The mixture was stirred for 3 h at 45 °C to produce a clear yellow solution that had a tiny amount of precipitate. Stirring was continued for a further 45 h at room temperature. The mixture was filtered to remove any undisclosed material. The volume of the filtrate was reduced to 4 ml by using the vacuum concentrator consisting of Javac DD150 Double Stage High Vacuum Pump, Savant RVT4104 Refrigerated Vapor Trap and Savant Speed Vac110 Concentrator. A 40 ml of dichloromethane was added to the concentrated solution. The mixture was left standing at 5 °C for 6 h. The resulting yellow precipitate was collected by filtration at the pump, washed in succession with ice-cold water, methanol and ethanol and air-dried. The crude product of DHD was dissolved in 25 ml of DMF and filtered. The volume of the filtrate was reduced to 4 ml by

using the vacuum concentrator to which 50 ml of dichloromethane was added. The mixture was left standing at room temperature for 3 h. The bright yellow precipitate of DHD produced was collected at the pump, washed in succession with ice-cold water, methanol and ethanol. The mass of the final product was 0.198 g giving a yield of 60.5%.

2.2.2. Characterization

C, H, N and Cl were determined out using the facility at the Australian National University. Palladium and platinum were 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 to 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 apodisation 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 to 50 cm^{-1}. A 180° 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 apodisation function was applied and the spectra were not corrected for instrument response. To obtain mass spectra, solution of DHD, made in 10% DMF and 90% methanol, were sprayed into a Finnigan LCQ ion trap mass spectrometer. ^{1}H NMR spectrum of DHD was recorded in dimethyl sulfoxide-d_6 (DMSO-d_6) solution in a Bruker AVANCE DPX 400 spectrometer. Spectra were referenced to internal solvent residues and were recorded at 300 K (± 1 K).

2.2.3. Interaction with pBR322 plasmid DNA and salmon sperm DNA (ssDNA)

Interaction between DHD and cisplatin with pBR322 plasmid DNA and salmon sperm DNA (ssDNA) was studied by agarose gel electrophoresis. The method used was a modification of that described by Stellwagen [20].

2.3. pBR322 plasmid DNA

Solutions of pBR322 plasmid DNA (at concentration 0.5 mg 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 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 was used).

2.4. ssDNA

To 2 μl of ssDNA (1 mg/ml), was added 2 μl of solutions of DHD and cisplatin at nine different concentrations. The total volume was made up to 20 μl by adding 16 μl of mQ water so that the concentrations of the compounds in the mixtures were: 5, 7.5, 10, 15, 20, 30, 40, 60 and 80 μM. DNA blank was made by adding 18 μl mQ water to 2 μl of ssDNA. The mixtures were first incubated for 4 h on a water bath at 37 °C. The samples were thawed and treated as described previously. Electrophoresis was carried out also in TAE buffer containing ethidium bromide at 85 V for 2.5 h at room temperature and ssDNA bands were viewed under short wave UV light and photographed with Polaroid camera.

2.4.1. BamH1 digestion

BamH1 is known to recognize the sequence G/GATCC and hydrolyse the phosphodiester bond between adjacent guanine sites [21]. pBR322 contains a single restriction site for BamH1 [22] which converts pBR322 plasmid DNA from supercoiled form I and singly nicked circular form II to linear form III DNA. 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, visualised by UV light then a photograph of the gel was taken as described previously.

2.5. Cytotoxicity assays

The human ovarian cancer cell lines: A2780, A2780^{cisR}, A2780^{ZD0473R}, the melanoma cell line: Me10538 and the non-small lung cancer cell line: NCI-H460 cell line 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 [23].

Cytotoxicity was determined using MTT growth inhibition assay [24]. Between 5000 and 9000 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 incu-

bated for 24 h at 37 °C in a humidified atmosphere to allow them to attach. Compounds (DHD and cisplatin) were first dissolved in a minimum amount of DMF, then diluted to the required concentrations by adding mQ water and finally filtered to sterilize. A serial fivefold dilutions of the drugs ranging from 0.02 to 62.5 μM 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 [23]. A 4 h after the addition of MTT of 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.

2.6. Drug uptake and binding with DNA

The method used for cell subculture was a modification of that described by Freshney [23] 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 mM final concentration) were added to culture plates containing exponentially growing A2780 and $A2780^{cisR}$ 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 4 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 DHD 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.

2.7. 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 atomic absorption spectrophotometry using a variant of standard addition technique [25].

2.8. 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 [26]. 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. A 25-μl 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. A 200 μl 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). DNA content was measured using Varian Cary 1E UV-visible spectrophotometer set at 260 nm and 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 [27].

3. Results and discussion

3.1. Characterisation

3.1.1. Elemental composition

DHD: Formula: $C_6H_{28}N_7Cl_3O_3PtPd$.

Molar mass: 654.19 g mol^{-1}.

Table 1 gives the composition of DHD.

Repeated dissolution and precipitation were carried out to purify the compound. It can be seen that for hydrogen and carbon the differences between calculated and observed contents lie within the limits of measurements. The observed value for chlorine is significantly higher than the expected value and that for nitrogen is significantly lower. For hydrogen, carbon, palladium and platinum the differences between observed and calculated contents are less than the errors of the measurements. It appears that some impurities are present in the mixture. Increase in activity with the increase in purity suggests that the antitumour activity is due to the complex rather than any impurity.

3.1.2. IR, Raman, mass and ^1H NMR spectral analyses

The bands observed in IR, Raman, mass and ^1H NMR spectra of DHD are given in Table 2. The interpretation of the bands has been based on published spectra [28–32].

Table 1
Composition of DHD

Element	Calculated (%)	Found (%)
C	11.0	11.4±0.4
H	4.3	4.1±0.4
N	15.0	14.4±0.4
Cl	16.3	16.8±0.3
Pt	29.8	29.7±1.2
Pd	16.3	15.6±1.4

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

IR (cm^{-1})	3290s, 3213s br, 3140s, 2924s, 2852m, 2353w, 1743m, 1576s, 1288s br, 1194m, 1068m d, 993s, 823s d, 725m, 575w, 501w d, 420w
Raman (cm^{-1})	3213w d, 2897m, 2856m, 1595w, 1439w, 1192w, 1044m, 708w, 589w d, 533s, 405w, 322s, 311w, 211s, 83s
Mass (*m/z*)	EIS-MS (DMF) (*m/z*: $(M–NO_3) = 590$ (0.64 [a]); $Cl(NH_2)_2Pt$-μ-$\{NH_2(CH_2)_6NH_2\}PdCl(NH_3)(NH_2) = 553$ (1.00); $(Cl(NH_3)_2Pt\{NH_2(CH_2)_6NH_2\}$-H) = 380 (1.00), $Cl(NH_3)Pt\{NH_2(CH_2)_6NH_2\} = 364$ (0.25)
^{1}H NMR	^{1}H NMR DMSO δ ppm: 4.4 (NH_3); 4.0 (NH_2); 3.4 (CH_2); 2.4 (br, CH_2); 1.9 (br, CH_2); 1.6 (br, CH_2)

[a] The number in parentheses after m/z value indicates the intensity.

3.1.2.1. IR. The bands at 3290 and 3213 cm^{-1} are due to N–H stretch whereas those at 2924 and 2852 cm^{-1} are due to CH_2 stretching vibrations. The bands at 1743 and 1576 cm^{-1} are due to N–H bending vibrations whereas that at 1288 cm^{-1} is due to C–H bending vibration. The band at 1068 cm^{-1} is due to CH_2 wagging. The band at 993 cm^{-1} is believed to be due to C–C stretch. The bands at 823 and 725 cm^{-1} are believed due to N–H wagging. The bands at 501 and 420 cm^{-1} are due to Pt–N and Pd–N stretching vibrations.

3.1.2.2. Raman. The band at 3213 cm^{-1} is due N–H stretching vibration whereas those at 2897 and 2856 cm^{-1} are believed to be due to C–H stretching vibrations. The band at 1439 cm^{-1} is due to N–H bending vibration. The band at 1192 cm^{-1} is believed to be due to C–H bending vibration whereas that at 1044 cm^{-1} is due to CH_2 wagging. The band at 708 cm^{-1} is due to N–H wagging. The bands at 589 and 533 cm^{-1} are due to Pt–N and Pd–N stretching vibrations. The band at 322 and 311 cm^{-1} are due to Pt–Cl and Pd–Cl stretching vibrations that are in agreement with previously published value for Pd–Cl stretching vibration ranging from 304 to 319 cm^{-1} [33,34]. The band at 211 cm^{-1} is due to Pt–N and Pd–N bending vibrations. The band at 83 cm^{-1} is believed to be associated with lattice mode

3.1.2.3. Mass. The peak observed in the mass spectrum of DHD with $m/z = 590$ may be due to $(M–NO_3)$, that at 553 corresponds to $Cl(NH_2)_2Pt$-μ-$\{NH_2(CH_2)_6NH_2\}PdCl(NH_3)(NH_2)$, that at 380 may be due to $(Cl(NH_3)_2Pt\{NH_2(CH_2)_6NH_2\}$-H) and that at 364 may be due to $Cl(NH_3)Pt\{NH_2(CH_2)_6NH_2\}$. It can be seen that peaks observed in the mass spectrum of DHD provide support for the suggested structure for the compound. In particular, it clearly indicates the existence of multinuclear cation.

3.1.2.4. ^{1}H NMR. The ^{1}H NMR spectrum of DHD gives a broad proton resonance with chemical shift value of 4.4 ppm. This is believed to be due to NH_3 protons. The peak at $\delta = 4.0$ ppm (Fig. 6) is believed to be due to NH_2 protons. Although there are hydrogen atoms bonded to six different carbon atoms, analyses of COSY spectra (Figs. 7–10 show) that only four proton resonances are observed, due to overlap of some of the resonances. For the NMR spectral analysis, the carbon atoms in the linking diamine are numbered from 1 to 6, starting with 1 at the palladium end. The resonance at $\delta = 3.4$ ppm is due to C_1 protons which give only one cross-peak. The resonance at 2.4 ppm is believed to be due to C_2 and C_6 protons that give two cross-peaks. The resonance at $\delta = 1.9$ ppm is due to C_5 protons. The resonance at 1.6 ppm is believed to be due to C_3 and C_4 protons, both of which lie in the middle of the chain.

3.1.3. Gel electrophoresis

Interaction between pBR322 plasmid DNA and drug (DHD and cisplatin). Fig. 2a,d gives the electrophoretograms applying to the interaction for 4 h at 37 °C of pBR322 plasmid DNA with increasing concentrations of DHD and cisplatin ranging from 1.25 to 60 μM.

As pBR322 plasmid DNA which was initially found to be mixture of mainly of super coiled form I (band at the front) and a small amount of singly-nicked form II, was allowed to interact with DHD and cisplatin, the mobility of both forms I and II plasmid DNA bands increased: at different rates in the case of DHD such that the two bands approached each other but not enough to coalesce and almost at the same rate in the case of cisplatin so that two bands remained essentially parallel at all concentrations of the compound. At high concentrations of DHD, the intensity of both form I and form II bands decreased. There was no observable change in intensity of the bands when pBR322 plasmid DNA was allowed to interact with the increasing concentration of cisplatin.

The change in mobility of plasmid DNA bands as a result of interaction with DHD is believed to be due interstrand ladder-like binding of the dinuclear cation primarily to the GG sites of DNA. The high flexibility of the DHD means that a plethora of different interstrand adducts of varying lengths dictated by the sequence of nucleobases could be formed. Recently, it has been reported that the trinuclear complex BBR3464 forms 1,4-interstrand cross-link with the self-complementary DNA octamer 5′-d(ATG*TACAT)$_2$-3′, with the two terminal platinum atoms coordinated in the major groove at the N7 positions of guanine [28]. Since the length of BBR3464 is long enough to cover a distance of over eight nucleotides, the results suggest that depending on the sequence of nucleobases in DNA, BBR3464 can potentially form a number of GG interstrand adducts, made possible by the flexibility of the molecule. In contrast, the change in mobility of plasmid DNA bands as a result of interaction with cisplatin is believed to be due intrastrand bifunctional GG binding of cisplatin with DNA causing a local distortion of DNA.

The initial sharp increase in the intensity of the form II band due to the presence of a small amount of compounds (more so in the case of DHD than cisplatin) points to DNA damage in which form I DNA is changed to form II DNA. The decrease in intensity at higher concentrations of com-

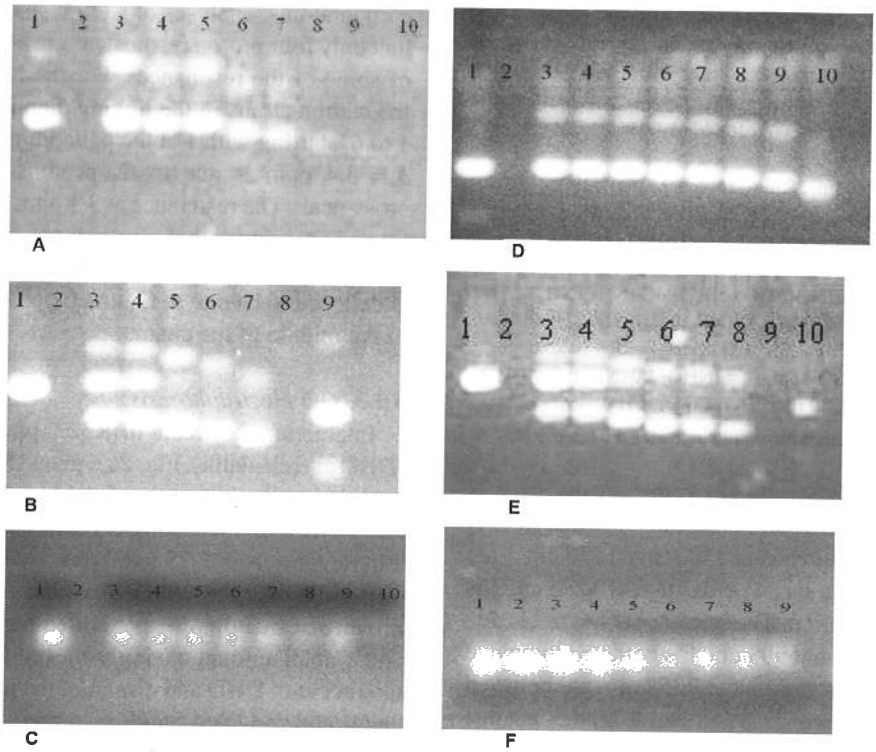


Fig. 2. (a) Interaction between pBR322 plasmid DNA and increasing concentrations of DHD, (b) interaction between pBR322 plasmid DNA and increasing concentrations of DHD followed by BamH1 digestion, (c) interaction between ssDNA and increasing concentrations of DHD, (d) interaction between pBR322 plasmid DNA and increasing concentrations of cisplatin, (e) interaction between pBR322 plasmid DNA and increasing concentrations of cisplatin followed by BamH1 digestion, (f) interaction between ssDNA DNA and increasing concentrations of cisplatin. (a) and (b): Lane 1: untreated pBR322 plasmid DNA, lane 2: blank, lane 3: 1.25 μM, lane 4: 1.88 μM, lane 5: 2.5 μM, lane 6: 3.75 μM, lane 7: 5 μM, lane 8: 7.5 μM, lane 9: 10 μM, lane 10: 15 μM. (b) and (e): Lane 1: untreated but BamH1 digested pBR322 plasmid DNA, lane 2: blank, lane 3: 1.88 μM, lane 4: 2.5 μM, lane 5: 5 μM, lane 6: 10 μM, lane 7: 15 μM, lane 8: blank in (b) and 20 μM in (d). (c) and (f): Lane 1: untreated ssDNA, lane 2: blank, lane 3: 5 μM, lane 4: 7.5 μM, lane 5: 10 μM, lane 6: 15 μM, lane 7: 20 μM, lane 8: 30 μM, lane 9: 40 μM, lane 10: 60 μM.

pounds (more in the case of DHD than cisplatin) is due to a partial damage of the DNA caused by the covalent binding of the compounds.

3.2. BamH1 digestion

Fig. 2b,e gives the electrophoretograms applying to the BamH1 digested incubated mixtures of pBR322 plasmid DNA and varying concentrations of DHD and cisplatin ranging from 1.25 to 15 μM. Lane 1 applies to the untreated pBR322 plasmid DNA that has been digested with BamH1, lane 2: blank, lanes 3–7: apply to pBR322 plasmid DNA interacted with increasing concentrations of compounds (1.87, 2.5, 5, 10, 15 μM, respectively) followed by their digestion with BamH1, lane 8: blank and lane 9: applies to untreated and undigested pBR322 plasmid DNA.

When untreated pBR322 plasmid DNA was digested with BamH1, only one band corresponding to form III band was observed. In the unreacted and undigested pBR322 plasmid DNA, generally 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 DHD (Fig. 2b), three bands corresponding to form I, II, III are observed at concentrations of DHD ranging from 1.87 to 2.5 μM, and two bands corresponding to forms I and II are observed for concentrations of DHD ranging from 5 to 15 μM. In the case of cisplatin (Fig. 2e), three bands corresponding to forms I, II, III are observed for concentrations of cisplatin ranging from 1.87 to 5 μM, two bands corresponding to forms I and II are observed for concentrations of cisplatin ranging from 10 to 20 μM. Table 3 summarises 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 the compounds, there is a corresponding increase in

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

Drug	(Drug) in μM						
	0	1.87	2.5	5	10	15	20
DHD	III	I, II, III	I, II, III	I, II	I, II	I, II	–
Cisplatin	III	I, II, III	I, II, III	I, II, III	I, II	I, II	I, II

prevention of BamH1 digestion at the specific GG site. It is also seen that DHD is better able to prevent BamH1 digestion than cisplatin. As stated earlier, DHD is expected to form a plethora of interstrand GG adducts with 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 in the conformations of the intervening not directly involved in the cross-link are also altered [35].

3.3. Interaction between DHD and cisplatin and ssDNA

A single band was observed in both untreated and reacted salmon sperm DNA. In general, as the concentration of the compounds was increased the intensity of the band was found to decrease. The mobility of the band also decreased slightly with the increase in concentration of the compounds.

3.4. Anticancer activity

Tables 4,5 give the percentage cell survival in A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H640 and Me-10538 cells when the cells were treated with increasing concentrations of DHD and cisplatin. Fig. 3 gives the corresponding cell survival curves. Table 6 gives the IC_{50} values of DHD and cisplatin for the cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H640 and Me-10538.

3.5. Cell uptake and binding with DNA

3.5.1. Cell uptake

Generally, the cellular accumulation of platinum has been used as a measure of the cell uptake of compounds. Since

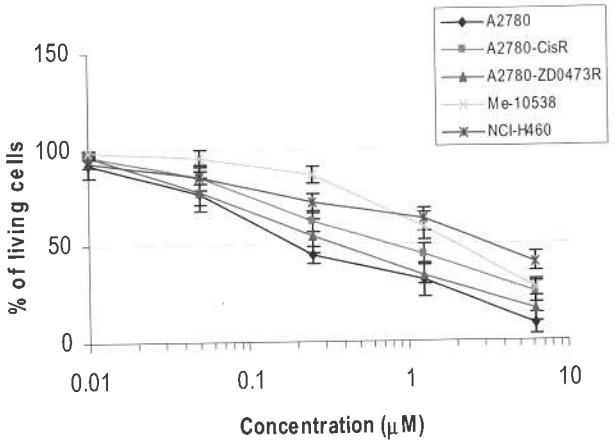


Fig. 3. Survival curve for the cell lines, A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, NCI-H640 and Me-10538 treated with increasing concentrations of DHD.

DHD contains a platinum unit and a palladium unit, the determination of both platinum and palladium levels should provide an equivalent measure of the drug uptake provided the compound (more exactly the dinuclear positive ion) remains essentially intact before entry into the cell. A departure from the expected value for 1:1 molar ratio of Pt and Pd uptake can be taken as a measure of the extent of decomposition of the compound. Thus in this study, cell uptake of palladium in 4 h was also determined to provide a measure of the extent of decomposition of the compound. Table 7 gives the uptake in 4 h of DHD (in terms of both platinum and palladium) and that of cisplatin (in terms of platinum) in the human ovarian cell lines: A2780 and $A2780^{cisR}$ cell lines. Cellular uptakes of platinum and palladium were calculated as nmol Pt per 2×10^6 cells and nmol Pd per 2×10^6 cells, respectively. Fig. 7 gives the uptake in 4 h of DHD and

Table 4
The cell survival rate as a percentage of the control for the ovary cell lines A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$, when the cells were treated with DHD and cisplatin

Concentration of compound (μM)	% Cell survival rate					
	A2780		$A2780^{cisR}$		$A2780^{ZD0473R}$	
	Cisplatin	DHD	Cisplatin	DHD	Cisplatin	DHD
0.01	101.1 ± 8.4	91.8 ± 6.0	102.5 ± 8.9	95.9 ± 1.0	101.8 ± 6.9	96.9 ± 1.9
0.05	93.8 ± 8.1	76.6 ± 6.9	98.5 ± 6.0	95.9 ± 1.0	92.8 ± 7.9	78.2 ± 7.2
0.25	56.4 ± 4.8	44.8 ± 4.7	85.6 ± 6.2	62.2 ± 5.5	78.0 ± 7.3	54.9 ± 8.7
1.25	20.8 ± 3.2	31.2 ± 8.7	75.5 ± 4.1	45.4 ± 5.0	40.8 ± 6.2	34.3 ± 4.9
6.25	8.1 ± 1.0	9.0 ± 5.3	33.8 ± 5.0	25.4 ± 5.4	17.1 ± 3.9	16.0 ± 6.5
IC_{50}	0.44 ± 0.08	0.25 ± 0.05	4.4 ± 0.2	0.96 ± 0.09	1.02 ± 0.1	0.47 ± 0.08

Table 5
The cell survival rate as a percentage of the control for the non-small lung cell line NCI-H640 and melanoma Me-10538, when the cells were treated with DHD and cisplatin

Concentration of compound (μM)	% Cell survival rate			
	Me-10538		NCI-H460	
	Cisplatin	DHD	Cisplatin	DHD
0.01	101.3 ± 6.5	98.1 ± 1.3	102.4 ± 4.8	92.9 ± 2.0
0.05	98.1 ± 9.4	93.7 ± 4.0	92.8 ± 7.1	85.5 ± 3.5
0.25	85.2 ± 5.4	82.6 ± 4.4	75.1 ± 6.8	71.9 ± 5.3
1.25	74.5 ± 7.4	59.6 ± 6.9	34.1 ± 3.8	63.4 ± 6.0
6.25	44.7 ± 5.7	27.5 ± 4.6	17.9 ± 4.1	40.9 ± 6.5
IC_{50}	4.96 ± 0.7	2.70 ± 0.6	0.93 ± 0.07	4.20 ± 0.9

Table 6
IC_{50} values of DHD and cisplatin

	IC_{50} (μM) ± SD and resistant factors					
Compound	A2780	$A2780^{cisR}$	RF	$A2780^{ZD0473R}$	Me-10538	NCI-H460
DHD	0.25 ± 0.05	0.96 ±0.09	3.84	0.47 ± 0.08	2.70 ± 0.6	4.2 ± 0.9
Cisplatin	0.44 ± 0.08	4.39 ± 0.2	9.98	1.02 ± 0.1	4.96 ± 0.7	0.93 ± 0.07

Table 7
Cell uptake in 4 h and Pt:Pd ratio

Compound	A2780 nmol Pt per 2×10^6 cells	$A2780^{cisR}$ nmol Pt per 2×10^6 cells
DHD (Pt)	0.16 ± 0.04	0.272 ± 0.010
DHD (Pd)	0.12 ± 0.01	0.219 ± 0.041
Pt:Pd ratio	1.3	1.2
Cisplatin (Pt)	0.048 ± 0.004	0.028 ± 0.005

cisplatin (in terms of platinum) in the human ovarian cell lines: A2780 and $A2780^{cisR}$.

It is found that the higher cellular uptake of DHD than cisplatin is in line with the higher activity of DHD against A2780 and $A2780^{cisR}$ cell lines. It should however be noted that the cell uptake per se may not necessarily give an indication of the level of the antitumour activity of the compounds as it is known that within the cell platinum compounds may be deactivated by a number of means (eg due to binding with sulfur containing ligands such as glutathione present in the cell) [32] before they have a chance to bind with DNA. Thus, it is more appropriate to consider the level of binding with DNA.

It is found that for the dinuclear compound DHD, the platinum cell uptake in both A2780 and $A2780^{cisR}$ cell lines is much greater than that for cisplatin.

It should be noted that for DHD, platinum cell uptake in the cisplatin-resistant cell line $A2780^{cisR}$ is found to be greater than that in the cisplatin–responsive cell line A2780. It is found that for both DHD and cisplatin, the level of platinum-DNA binding is less in the cisplatin-resistant cell line $A2780^{cisR}$ than in the cisplatin-responsive cell line A2780.

The departure of the Pt:Pd ratio from the expected value of 1:1 means that there has been some decomposition of the compound so that the antitumour activity due to fragments containing a dangling diamine cannot be ruled out. Other possible fragments such transpalladin and transplatin are not known to be tumour active. Because of *trans*-geometry, the fragments like DHD are likely to form interstrand adducts (considered later in the paper) (Fig. 4).

3.5.2. Platinum binding with DNA

Fig. 5 gives the level of platinum binding (in nanomoles per milligram of DNA) in 4 h in A2780 and $A2780^{cisR}$ cells as applied to the DHD and cisplatin.

As stated before, whereas cisplatin is expected to form mainly intrastrand GG, AG and GNG adducts, DHD is expected to form mainly long range interstrand GG adducts. Whereas intrastrand 1,2-bifunctional binding of cisplatin

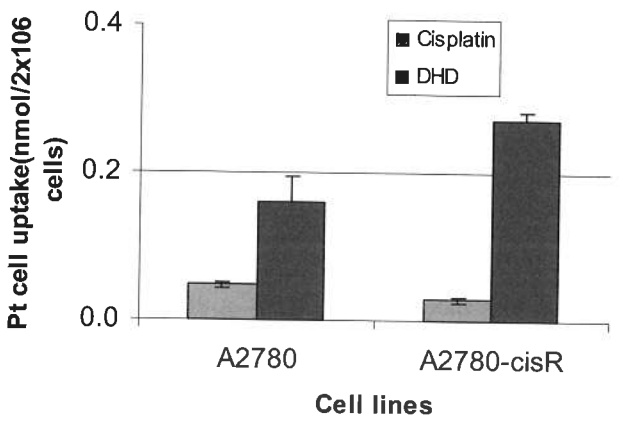


Fig. 4. Uptake of DHD and cisplatin in 4 h in A2780 and $A2780^{cisR}$ cell lines.

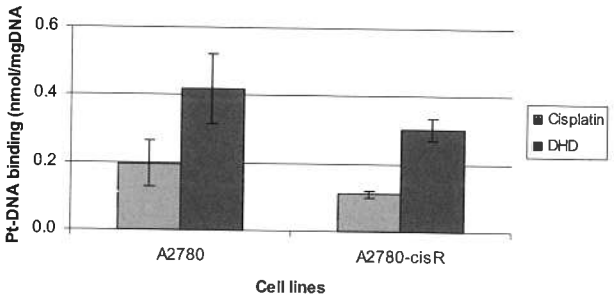


Fig. 5. Level of binding with DNA of DHD and cisplatin in 4 h in A2780 and $A2780^{cisR}$ cell lines.

would cause mainly local bending of DNA, the long range interstrand adducts formed by DHD 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 [35,36]. 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 [37].

The bending induced in DNA by binding of cisplatin is recognized by high-mobility group domain (HMG) proteins and this recognition is believed to be the pathway for processing and differential repair of cellular cisplatin-DNA ad-

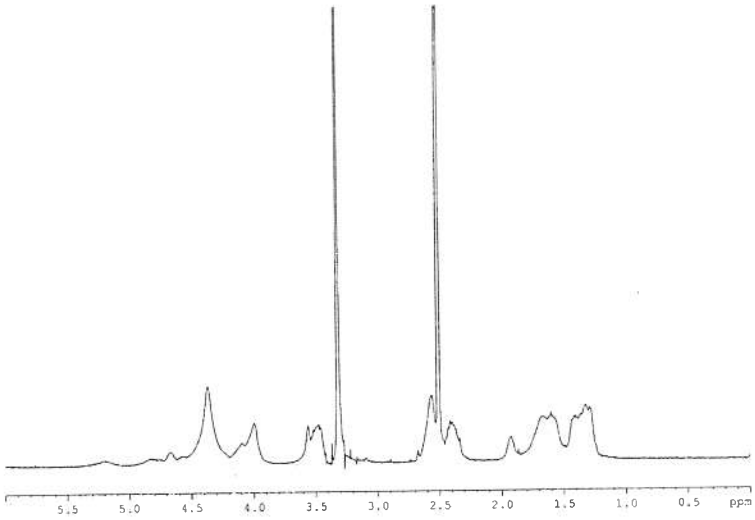


Fig. 6. ^{1}H NMR spectrum of DHD.

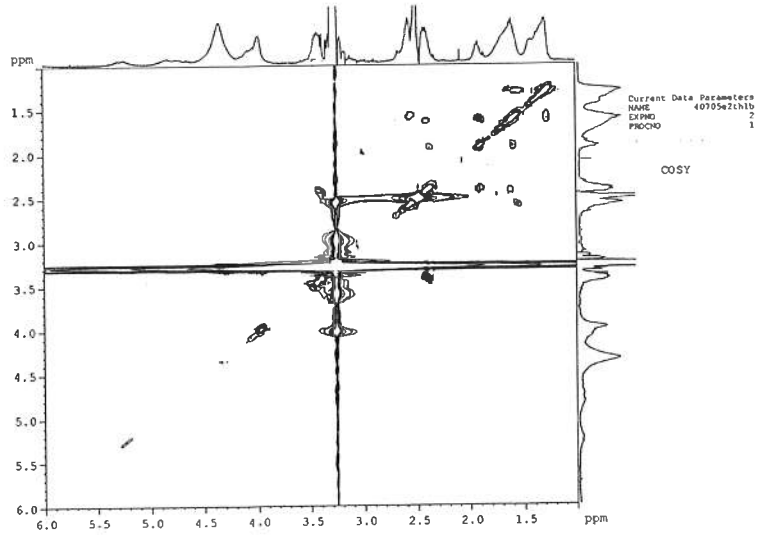


Fig. 7. Cosy.

ducts [38]. However, it has been found that in the formation of long range interstrand adducts, DNA is not sufficiently bent to be recognized by HMG1 proteins [39]. Instead, the long range cross-links are very effective in inducing B to A or B to Z transformations. Cellular alkaline elution studies have shown that interstrand cross-links formed by BBR3464 persist over time, suggesting a lack of DNA repair [40,41]. 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 stated earlier, Qu et al. [35] 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 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. They found that Watson–Crick pairing was 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 [42] that in canonical poly(dGdC).poly(dGdC), polynuclear platinum complexes can form a plethora of intra- and interstrand cross-links. Molecular mechanics calculations using HyperChem 7 show that when fully stretched, the length of DHD but with the chlorides replaced by nitrogens is 0.161 nm. It is found that in the double stranded B DNA, the interstrand N7(guanine) to N7(guanine) distances are: G(1) to G(2): 0.76 nm, G(1) to G(3): 0.85 nm, G(1) to G(4): 1.03 nm, G(1) to G(5): 1.28 nm, G(1) to G(6): 1.59 and G(1) to G(7): 1.93 nm. It can be seen that when fully stretched the

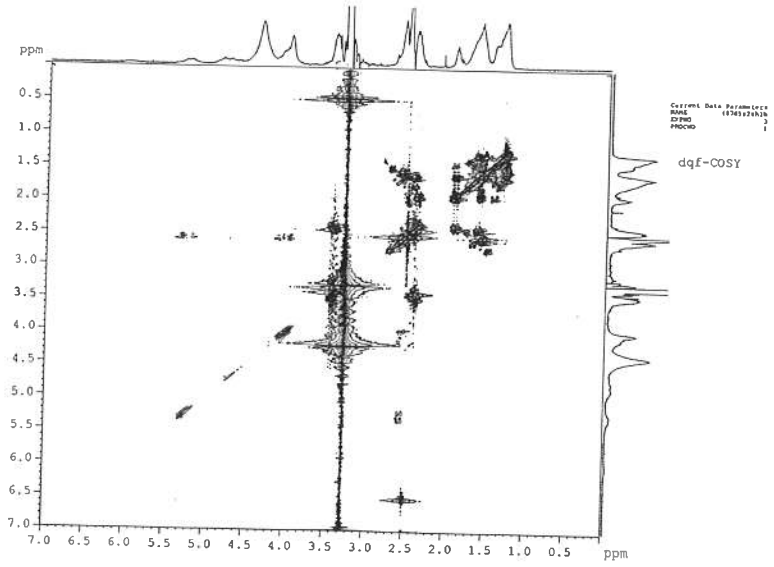


Fig. 8. dqf–cosy.

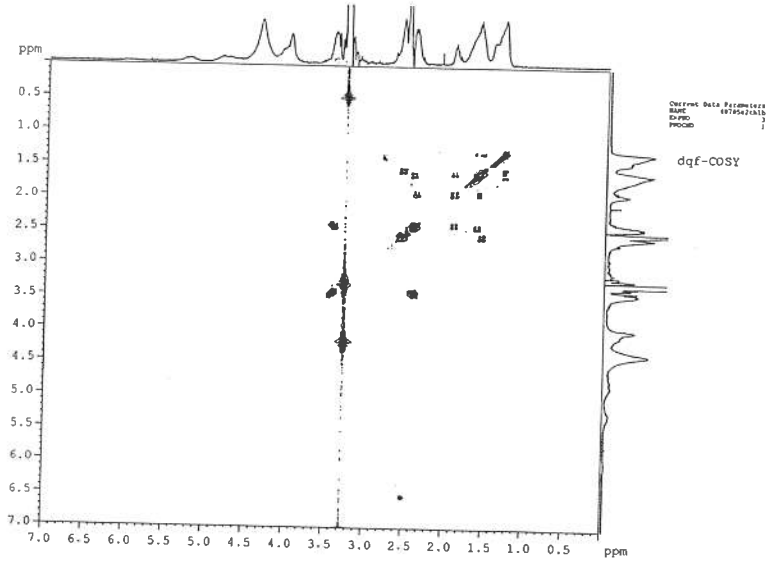


Fig. 9. dqf–cosy.

DHD would form G(1) to G(6) interstrand adduct. It should however be noted that, because of the flexibility of the diamine linkers, a plethora of interstrand adducts of varying lengths can be formed by DHD.

Although the molar conductivity value (16.0 ohm^{-1} cm^2 mol^{-1}), measured in solution in 1:1 mixture of DMF and water, indicate that DHD remains largely as undissociated ionic aggregate in the solution. However, it is expected that in biological fluids the compound (being essentially in solution in water), would dissociate to produce multicentred cation and free anions. The high charge on the multicentred cation produced from DHD 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 [43,44] before binding to specific nucleobases in DNA. Wheate at al. point out that since pre-association is stabilized largely by electrostatic forces, the pre-association of cationic multicentred 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

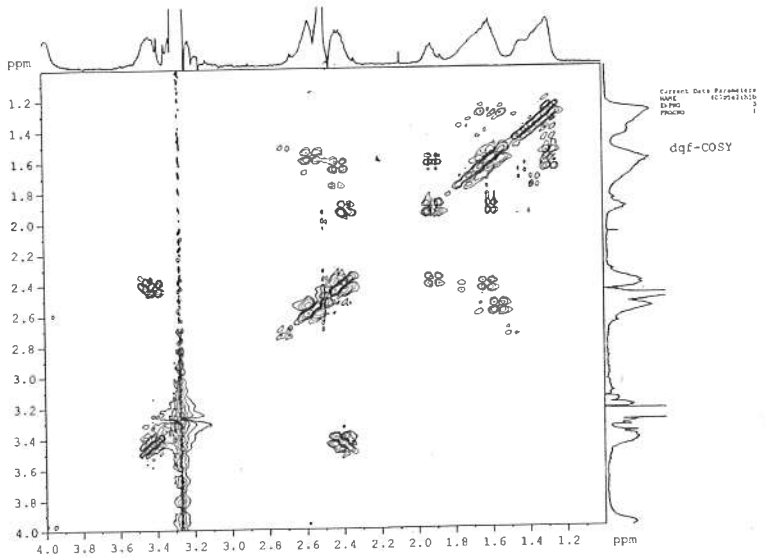


Fig. 10. dqf–cosy.

specific site. Even though (unlike BBR3464) DHD is dinuclear, the above idea may still be applicable to the interaction of DHD with DNA.

The high activity of DHD in $A2780^{ZD0473R}$ cell as compared to that for cisplatin also indicates that the compound has been able to overcome multiple mechanisms ZD0473 resistance operating in the cell. The determination of cell uptake and platinum-DNA binding in $A2780^{ZD0473R}$ cell for the compounds DHD and ZD0473 would provide useful information.

4. Conclusion

The anticancer activity against ovarian cancer cell lines: A2780, $A2780^{cisR}$, $A2780^{ZD0473R}$, the melanoma cell line: Me10538 of the dinuclear complex [{*trans*-$PtCl(NH_3)_2$}{μ-$(H_2N(CH_2)_6NH_2)$}{*trans*-$PdCl(NH_3)_2$}$(NO_3)Cl$ have been determined. The cell uptake and the extent of binding with DNA have also been determined. The nature of interaction with salmon sperm and pBR322 plasmid DNAs have also been determined. The compound displays significant anticancer activity against ovarian cancer cell lines much greater than that for cisplatin. DHD is believed to form long-range interstrand adducts with DNA, causing long-range deformation of the DNA (Figs. 6–10).

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