Studies on Synthesis, Characterization, DNA Binding and Biological Activities of Two Trans-Planaramineplatinum(II) Complexes of the form PtL_2R_2 where L = 3-hydroxy Pyridine and R= Acetate Ligand

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Abstract: The present study deals with the synthesis and characterization of *trans*-platinum(II) complexes of general formula *trans*-[$PtL_2(R)_2$], where L is 3-hydroxy pyridine and R is acetate ligand. The new complexes contain acetate ligands *trans* to the planaramine group 3-hydroxypyridine. The complexes were characterised by a combination of elemental analysis, IR, mass spectrum and ^{1}H NMR spectroscopy. The increasing prevention of BamH1 digestion of form I and form II pBR322 plasmid DNA with the increase in concentration of the compound is believed to be due to interstrand binding that brings about global changes in DNA conformation. Acetate complexes' activity against human ovarian cancer cell lines: A2780, A2780^{cisR} have also been determined. The complexes show a reduced reactivity, and decrease in cytotoxic activity compared to their chloro-counterparts. Further, the complexes are better able to overcome cisplatin resistance; they therefore present an interesting class of antitumour active *trans*-platinum acetate complexes.

Keywords: *trans*-platinums, drug design, steric hindrance, DNA binding, apoptosis.

1. INTRODUCTION

Cisplatin is one of the most potent chemotherapeutic agents currently in use, exerting its cytotoxic action through the formation of intra-strand DNA crosslink adducts [1]. For its toxicity and drug resistance, there has been a widespread search for related complexes with similar or improved activity. Thus there remains an urgent need to discover new platinum drugs with a broader spectrum of activity, reduced toxicities and an improved clinical effectiveness. In more recent years, a number of *trans* compounds with high antitumour activities in both cisplatin-sensitive and cisplatin-resistant cell lines have been discovered [2-4].

We believe that one of the promising approaches to the development of highly effective low-toxicity antitumor *trans*-platinum complexes is synthesis of platinum complexes with planaramine ligands which activates the *trans* geometry for anticancer activity by slowing down the reactivity of the compounds and enhancing the chance of formation of interstrand bifunctional adducts. Furthermore, recently reported that trans-dicarboxylate complexes can have greatly increased aqueous solubility compared to their dichloro counterparts [4, 5]. In fact, several second-generation platinum drugs that have entered clinical trials such as carboplatin, nedaplatin and oxaliplatin have all exchanged the Cl- leaving groups of cisplatin for kinetically less labile chelating dicarboxylate or glycolate ligands [6, 7] The primary objective in the present study, the synthesis, characterization, nature of binding with DNA, cytotoxicity and cellular accumulation of new planaramineplatinum (II) complexes of the form *trans*-PtR_2L_2 (L = 3-hydroxypyridine) with acetate ligands.

CHEMISTRY

Trans-bis(3-hydroxypyridine) diacetateoplatinum (II) [code named FH1], *trans*-bis(3-hydroxypyridine) ditrifluroacetateoplatinum (II) [code named FH4] have been synthesized by replacement of chloride leaving groups in CH1 prepared according to published method [8] with acetate ligand as shown in Figure **1**.

2. MATERIALS AND METHODS

2.1. Materials

Cisplatin and CH1 were prepared according to previously described methods [8]. Potassium tetrachloroplatinate (K_2PtCl_4), N,Ndimethylformamide (DMF) (C_3H_7NO), 3-hydroxypyridine was obtained from Sigma Chemical Company, St. Louise, USA; hydrochloric acid (HCl) was obtained from Asia Pacific

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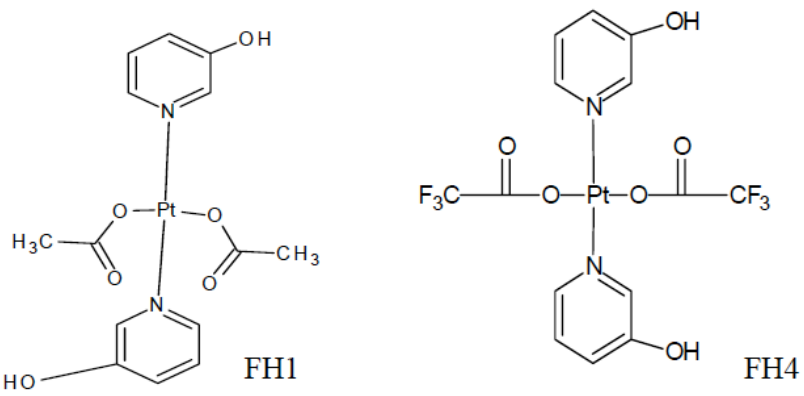

Figure 1: Structures of FH1 and FH4 *trans*-planaramine platinum(II) complexes.

Specialty Chemicals Ltd., NSW, Australia. 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. Silver acetate ($C_2H_3O_2Ag$) (Sigma Chemical Company, Germany) and charcoal (Sigma Aldrich Chemical Company Milwaukee USA). 200 mM L-glutamine, 5.6% sodium bicarbonate and foetal calf serum (FCS), RPMI-1640 were obtained from Trace Biosciences Pty Ltd Australia. Other chemicals were mostly purchased from Sigma-Aldrich, Sydney, Australia. A2780, A2780^{cisR} and A2780^{ZD0473R} ovarian cancer cell lines were gifts from Ms. Mei Zhang, Royal Prince Alfred Hospital, Sydney, Australia. Stock solutions of platinum compounds (1mM) were prepared in 1:1 (DMF)-mQ water mixture. Synthesis of the drugs carried out according to published method [8] starting from K_2PtCl_4 using the *trans*-effect of chloride. Chloro compound (CH1) is used as starting material in the synthesis of the carboxylate acetate target compounds (FH1 and FH4).

2.2. Synthesis

2.2.1. FH1

FH1 was prepared by replacement of chloride leaving groups in CH1 with acetate ligand. CH1 which is [*trans*-$PtCl_2$(3-hydroxy pyridine)$_2$ was prepared according to published method [8]. CH1 (1 mmol, 0.457g) dissolved in 5 mL of DMF. Silver acetate (2 mmol, 0.332 g) mixed with 3 mL of 1:1 mixture of acetone and water was slowly added to the solution while it was stirred. Stirring was continued for 24 h at room temperature in dark. After 24 h, the mixture was centrifuged at 5500 rpm for 10 min to remove precipitate of silver chloride. The supernatant was collected. The volume was reduced to about 5 mL by using the vacuum concentrator (described earlier) to give crude product of FH1. The crude product was dissolved in methanol and stirred with charcoal for 5 to 10 min. FH1 in solution was collected by filtration. The volume of the filtrate was reduced to about 5 mL by using the vacuum concentrator. Addition of 2 mL of diethyl ether caused precipitation of FH1. The product was left in the fridge at 0^{o}C overnight for FH1 (which is white in colour) to precipitate. The precipitate was collected by filtration at the pump. The precipitate which was collected by filtration at the pump, washed with ice-cold water, ethanol, and air-dried.

2.2.2. FH4

FH4, was prepared by replacement of chloride leaving groups in CH1 with trifluroacetate ligand. 1 mmol of CH1 was dissolved in 12 mL acetone. 2 mmol of silver trifluroacetate mixed with 10 mL of water was slowly added to the solution of CH1. Stirring was continued for 24 h at room temperature in dark. After 24 h, the mixture was centrifuged at 5500 rpm for 10 min to remove precipitate of silver chloride. The supernatant was collected. The volume was reduced to about 5 mL by using a vacuum concentrator (described earlier) to give crude product of FH4. The crude product was dissolved in methanol and stirred with charcoal for 5 to 10 min. FH4 in solution was collected by filtration. The volume of the filtrate was reduced to about 5 mL followed by the addition of 2 mL of di ethyl ether to cause FH4 to precipitate. The mixture was left in the fridge at 4^{o}C for FH4 to precipitate, which was collected by filtration at the pump. The precipitate was collected by filtration at the pump, washed with ice-cold water and ethanol, and air-dried.

2.3. Characterization

2.3.1. Microanalyses

Carbon, hydrogen and nitrogen were determined using the Carlo Erba 1106 automatic analyzer available

at the Research School of Chemistry, Australian National University. Platinum was determined by graphite furnace atomic absorption spectrophotometry (AAS) using the Varian SpectrAA-20 plus Atomic Absorption Spectrophotometer with Varian GTA-96 Graphite Furnace Tube Atomiser available at the Bioinorganic Research Laboratory in the School of Biomedical Sciences, University of Sydney.

2.4. *In Vitro* DNA Studies

Interaction of FH1, FH4 with pBR322 plasmid DNA was studied by agarose gel electrophoresis. The method used was a modification of that described by Stellwagen [18]. pBR322 plasmid DNA aliquots (0.05 mg/mL) were incubated in the presence of the increasing concentrations of compounds ranging from 0.5μM to to 64 μM. Incubation was carried out in a shaking water bath at 37°C for 4 h. Drug–DNA mixtures containing 2 μL of pBR322 plasmid DNA were loaded onto the 1% agarose 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) and visualised under UV light.

2.4.1. Enzymatic Digestion

An identical set of drug–DNA mixtures was first incubated for 4 h in a shaking water bath at 37°C and then subjected to BamH1 (10 units/ μL) digestion. To each 20 μL of incubated drug-DNA mixtures, 2 μL of 10x digestion buffer SB first and then 0.1 μL BamH1 (1 unit) were added. The mixtures were left in a shaking water bath for 1 h, at 37°C at the end by rapid cooling, the reaction was terminated. The gel was stained by ethidium bromide and visualised under UV light and photographed

2.5. Cytotoxicity

Cytotoxicity of the compounds against human ovarian cancer cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ was determined using MTT (3-(4,5-Dimethyl- 2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) reduction assay [19]. Each cell line plated at a density of 4000 to 6000 cells/ well, in the carbon dioxide incubator, left overnight. Following this, the cells in the 96 well plates (10% FCS/RPMI 1640 culture medium) were treated with different concentrations of compounds. Platinum complexes were first dissolved in a minimum volume of DMF, then diluted to the required concentrations by adding mQ water and finally filtered to sterilize. A serial fourfold dilutions of the drugs ranging from 2.50 to 160.0 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 assay [19]. Each treatment was done in triplicate.

2.6. Pt Cellular Accumulation and Pt-DNA Binding Studies

As the action of platinum containing drugs is associated with their binding with DNA, cellular accumulation of platinum and platinum-DNA binding levels in A2780 and $A2780^{cisR}$ cell lines were determined for *cis* and *trans*-platinums as reported earlier [20, 21].

2.6.1. Cellular Accumulation

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.

2.6.2. Drug–DNA Binding

DNA isolated from the cell pellet using H440050 JETQUICK Blood DNA Spin Kit/50 Austral Scientific Pty Ltd and the modified protocol of Bowtell [20] were determined by graphite furnace AAS. A260/A280 nm ratios were found to be between 1.75 and 1.8 for all samples indicating high purity of the DNA.

3. RESULTS AND DISCUSSION

3.1. Synthesis and Characterisation

The known, earlier reported active *trans*-platinum complex CH1 served as a starting point for the current study. Synthesis was based on literature procedures [8]. The compound CH1, containing (3-hydroxy pyridine)$_2$ ligands *trans* to one another, and have shown high *in vitro* activities in both cisplatin sensitive and cisplatin resistant cell lines.[8] This activity may be related to the fact that these complexes react with DNA in *trans* geometry, quite unlike platinum complexes with *cis* geometry. Using these chloro compounds as starting materials in the synthesis of the target compounds FH1 and FH4, the Cl− ligands to be exchanged for OAc− (see Figure **2** for the reactions). Treating the chloro complexes CH1 with silver acetate or trifluroacetate yielded the desired complexes FH1 and FH4 in a simple overnight reaction. Table **1** shows

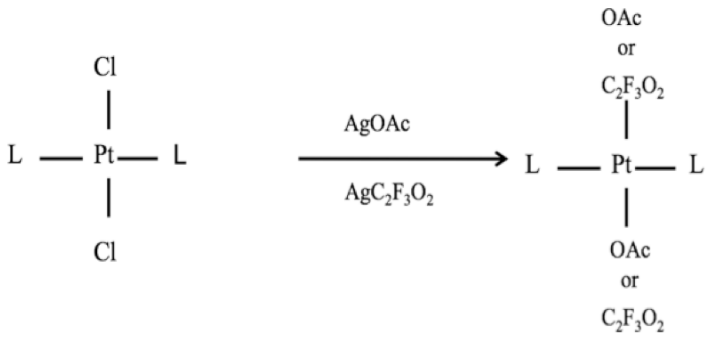

Figure 2: Synthesis of *trans* carboxylate complex *trans*-[Pt(OAc)$_2$-(3 hydroxy pyridine)$_2$ starting from the chloride precursor CH1. a) $CH_3COOAg/C_2AgF_3O_2$ (1:1) in H_2O and acetone.

Table 1: Elemental Composition of FH1and FH4

	FH1 ($C_{14}H_{16}N_2O_6Pt$)		FH4 ($C_{14}H_{10}F_6N_2O_6Pt$)	
	Calculated (%)	**Observed (%)**	**Calculated (%)**	**Observed (%)**
C	33.2	32.0 ± 0.4	27.5	26.2 ± 0.4
H	3.2	3.4 ± 0.5	1.7	1.9 ± 0.5
N	5.6	5.5 ± 0.4	4.6	4.9 ± 0.4
Pt	38.8	38.1±0.6	31.9	30.5 ± 0.5

the theoretical and observed percentage values for carbon, hydrogen, nitrogen and platinum.

3.2. Biological Evaluation

To analyse the compounds potential as antitumour agents the IC_{50} values were determined in three human ovarian cancer cell lines, *i.e.* one sensitive (A2780) and two resistant to cisplatin treatment ($A2780^{cisR}$ and $A2780^{ZD473R}$). The results are illustrated in Table **2**. The compounds are moderately active, less active than cisplatin, the change in activity of the compound in going from cisplatin-responsive cell line: A2780 to the resistant cell lines: $A2780^{cisR}$ and $A2780^{ZD0473R}$ is less marked than that for cisplatin. The results suggest that at the level of their activity the compounds have been able to overcome mechanisms of resistance operating in $A2780^{cisR}$ cell line, they have much lower resistance factor than cisplatin. As stated earlier, unlike cisplatin FH1 and FH4 are expected to form interstrand adducts with DNA. It is possible that the presence of a bulky planaramine ligand makes FH1 and FH4 much less reactive than cisplatin. It is of interest that changing the leaving groups to acetate appears to alter the cytotoxic properties of the parent compound CH1.

The nature of interaction of the designed platinum compounds FH1, FH4 and cisplatin with pBR322 plasmid DNA followed by BamH1 digestion was studied using agarose gel electrophoresis in order to obtain information on DNA damage and change in DNA conformation. As pBR322 plasmid DNA was interacted with increasing concentrations of compounds, broadening or streaking of the bands was observed in the case of FH1 and significant decrease in intensity of the bands. The broadening of the band as well as decrease in intensity of the bands indicated the occurrence of DNA damage. As both FH1 and FH4 can bind with DNA forming mainly interstrand bifunctional

Table 2: IC_{50} Values and Resistance Factors (RF) for Cisplatin and *trans*-platinums as Applied Cell Lines A278, $A2780^{cisR}$ and $A2780^{ZD0473R}$

A2780 IC_{50} (μM)		$A2780^{cisR}$ IC_{50} (μM)	Resistant Factor	$A2780^{ZD0473R}$ IC_{50} (μM)	Resistant Factor
FH1	6.73 ± 0.65	24.3 ± 0.56	3.61	53.82 ± 0.97	8.00
FH4	12.10 ± 0.95	23.10 ± 0.72	1.91	16.77 ± 0.21	1.4
Cisplatin	0.52 ± 0.09	4.73 ± 0.67	9.14	3.04 ± 1.79	7.36

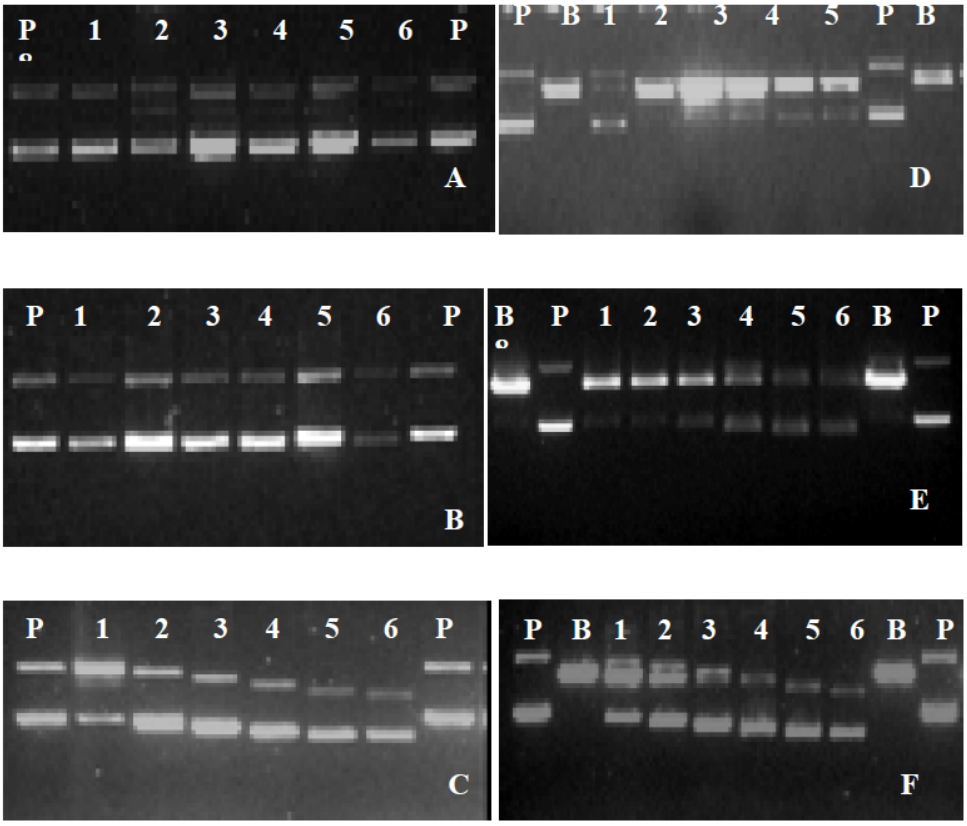

Figure 3: Electrophoretograms applying to the interaction of pBR322 plasmid DNA (**A**, **B**, **C**) with increasing concentrations of FH1, FH4 and cisplatin (**D**, **E**, **F**) followed by their digestion with BamH1. (P: untreated pBR322 plasmid DNA, B: untreated and undigested pBR322 plasmid DNA, 1: 0.5 μM, lane 2: 2 μM, lane 3: 8 μM, lane 4: 16 μM, lane 5: 32 μM, lane 6: 64 μM and lane 7).

Pt(GG) adducts that will cause more of a global change in DNA conformation. The difference in ability to cause DNA damage can be seen to illustrate structure-activity relationships. Besides damage to DNA, binding of platinum drugs with DNA can bring about conformational change in the DNA that in turn brings about changes in mobility of the DNA bands. The change in mobility of the DNA bands was more prominent in the case of cisplatin. It may be noted that the interaction of platinum drugs (that can bind covalently with DNA) involves a number of stages. These include: aquation of the complex to produce positively charged species, pre-association of the resulting positive ions with DNA, formation of monofunctional platinum-DNA adducts that may evolve into bifunctional platinum-DNA adducts such as Pt(GG) that activates several signal transduction pathways including those associated with pro-apoptotic machinery [10]. It is likely that FH1and FH4 form monofunctional adducts with G followed by the formation of bifunctional interstrand GC adducts that may evolve into interstrand Pt(GG) adducts. It has been suggested that when bifunctional interstrand adducts are formed, planaramine ligand (3-hydroxypyridine) will be positioned along the helix axis so that they will push apart adjacent base pairs [8, 11]. In the interaction of pBR322 plasmid DNA with cisplatin, two bands were observed for all concentrations of cisplatin ranging from 0.5 μM to 64 μM. However, the separation between the bands decreased with the increase in concentration of the compound, this is believed to be due to change in DNA conformation. Whereas cisplatin forms mainly intrastrand bifunctional GG and AG adducts with duplex DNA [12] (and hence induces a local kink in a DNA strand), FH1 and FH4, are more likely to form monofunctional adducts with guanine and bifunctional intrastrand adducts such as Pt(GG) [13]. BamH1 digestion was also carried out to get further insight into the nature of conformational change in pBR322 plasmid DNA. As pBR322 plasmid DNA exists in three forms: super coiled form I, singly nicked relaxed circular form II and doubly nicked liner form III. The super coiled form I migrates at the fastest rate through the gel; the singly nicked circular form II has the lowest migration rate whereas the doubly nicked linear form III DNA has the intermediate migration rate.When pBR322 plasmid DNA was interacted with increasing

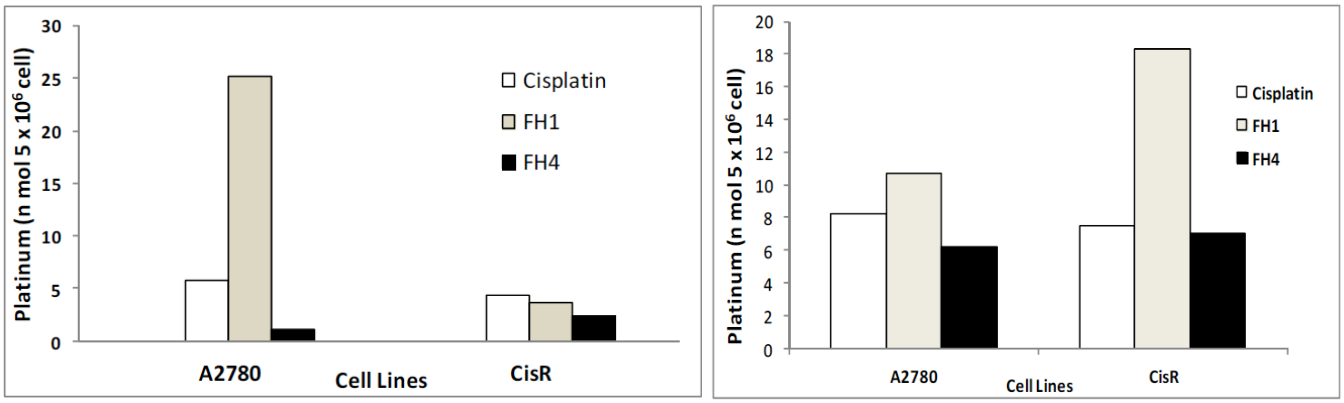

Figure 4: a: Total intracellular platinum accumulation levels (expressed as nanomoles Pt per 5 x10^6 cells) found in the cell lines: A2780 and A2780^{cisR} after exposure to 50 μM concentrations of FH1, FH4 and cisplatin for 24 h.
b: Platinum–DNA binding levels in A2780 and A2780^{cisR} cell lines from FH1, FH4 and cisplatin after 24 h time gap.

concentrations of FH1 followed by BamH1 digestion, generally three bands corresponding to forms I, II and III were observed at lower concentrations (0.5 μM to 16 μM) with the form III band being most prominent whilst forms I and II bands were weaker, and two bands corresponding to forms I and II were observed at higher concentrations (32 μM to 64 μM). The results indicate that binding of FH1 with pBR322 plasmid DNA has been able to partially prevent BamH1 digestion at lower concentrations and fully so at higher concentrations. This is believed to be due to changes in DNA conformation brought about by interstrand covalent binding of FH1 with nucleobases in the DNA. In the case of FH4 a weak frontal band followed by a more intense band were observed at lower concentrations (ranging from 0.5 μM to 8 μM). At the next higher concentration (16 μM) three bands corresponding to forms I, II and III could be seen and at still higher concentrations two bands corresponding to forms I and II were observed. One again, the results indicate changes in DNA conformation believed to be brought about by covalent binding of FH4 with the DNA. As the antitumour activity of platinum drugs is believed to be associated with their binding with DNA and as platinum drugs must enter the cell before they can bind with DNA, it is logical to think that accumulation of platinum drugs in the cell and the level of platinum–DNA binding may provide important information on activity of the compounds. The cellular accumulation of platinum was used as a measure of cellular accumulation for cisplatin and *trans*-planaramineplatinum(II) compounds whereas the level of platinum binding with nuclear DNA was used as a measure of level of drug-DNA binding. Figure **4a** gave a graphical representation of platinum accumulation in A2780 and A2780^{cisR} cell lines as applied to cisplatin, FH1 and FH4 in 24 h. It can be seen that in the A2780 and A2780^{cisR} cell lines, the observed accumulation of FH1 was greater than that for FH4. In the parent A2780 cell line the compound FH1 was found to have higher cellular accumulation of platinum than cisplatin, although FH1 was found to be much less active than cisplatin and in the resistant A2780^{cisR} cell line nearly same as that for cisplatin. The results illustrate that cellular accumulation of platinum do not necessarily correlate with cell killing effect as platinum compounds may be deactivated due to binding with cellular platinophiles such as glutathione and metallothionein before binding with DNA [14]. It has been reported in a number of platinum drugs only about 1% of the compound entering the cell actually binds with DNA [15]. The above result is in line with the reported observations that the uptake of pyridine complexes by L1210 cells was greater for the *trans*-isomer over the *cis*-isomer by a factor of about 4 [16]. Figure **4b** gave the levels of platinum-DNA binding for cisplatin, FH1 and FH4 in 24 h as applied to the ovarian A2780 and A2780^{cisR} cancer cell lines. Once again FH1 was found to have the highest level of Pt-DNA binding after 24 h in both A2780 and A2780^{cisR} cells, in line with the highest cellular accumulation of the compound (except that for cisplatin in A2780^{cisR} cell line). Planaramineplatinum(II) complexes are also found to have high level of cellular accumulation drug and drug-DNA binding even though their activity might be lower than that of cisplatin. One *cis*-planaramineplatinum(II) has been reported [17] to have very high cellular accumulation of platinum and platinum–DNA binding even though the compound was found to be totally lacking in activity. From the present study it was observed that the Pt-DNA binding levels from *trans*-platinums are much higher than that from cisplatin in A2780^{cisR} cells and this difference appears

to be related to the difference in the nature of the adducts formed. Cisplatin ultimately forms mainly intrastrand bifunctional adducts, whereas FH1 and FH4 can be expected to form mainly interstrand bifunctional adducts moreover it appears that *trans*-planaramineplatinum(II) complexes at the level of their activity are able to overcome cisplatin resistance in $A2780^{cisR}$ cells (results in line with resistant factor values Table **2**), therefore present an interesting class of antitumour active *trans*-platinum complexes.

4. CONCLUSION

The primary aim of the present study was to enhance water solubility and bioavailability of designed platinums by modulation of their leaving groups. Indeed the designed complexes containing carboxylate-based leaving groups are found to be readily soluble in water although have lower *in vitro* activity than the corresponding complexes in the chloride form. As the compounds interacted with pBR322 plasmid DNA, they were able to induce changes in DNA conformation and also DNA damage. The changes in DNA conformation are believed to be due to binding of the compounds with the DNA. Formation of interstrand adducts induces more of a global change in the conformation of the DNA. The positioning of the planaramine ligands along the helix may cause further distortion by pushing the neighbouring base pairs away. After studying the cellular accumulation of platinum and levels of platinum-DNA binding relating to the designed complexes were compared, perhaps pointing to the fact that the antitumour activity in platinum drugs is simply not a consequence of drug-DNA binding. Although platinum DNA binding may be an essential key event, apoptosis may actually be brought about by downstream processes in the cell cycle in which many proteins are likely to be involved.

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