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Synthesis and Activity of a Trinuclear Platinum Complex: [{trans-PtCl(NH_3)$_2$}$_2$μ-{trans-Pt(3-hydroxypyridine)$_2$($H_2N(CH_2)_6NH_2$)$_2$}]Cl_4 in Ovarian Cancer Cell Lines

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This paper describes the synthesis, characterisation, and cytotoxicity of a novel trinuclear platinum complex code named TH1. In addition to its activity against human ovarian cancer cell lines: A2780, A2780^{cisR}, and A2780^{ZD0473R}, cell uptake, DNA-binding, and the nature of the compound interaction with pBR322 plasmid DNA have been determined. TH1 is found to be significantly more cytotoxic than cisplatin - two times more active than cisplatin against the parent cell line A2780, thirteen times more active against the cisplatin-resistant cell line A2780^{cisR} and 11.5 times more active against the cell line A2780^{ZD0473R}. Whereas the resistance factors for cisplatin as applied to the cell lines A2780 and A2780^{cisR}, and A2780 and A2780^{ZD0473R} are 12.9 and 3.0 respectively, the corresponding values for TH1 are 1.98 and 0.5. The results suggest that TH1 has been able to significantly overcome resistance in A2780^{cisR} and A2780^{ZD0473R} cell lines. Whereas cisplatin binds with DNA forming mainly intrastrand GG adduct that causes local bending of a DNA strand, TH1 should bind with DNA forming mainly interstrand GG adducts that would cause more of a global change in DNA conformation. Provided it has favourable toxicity profile, TH1 has the potential to be developed into a highly active anticancer drug with a wider spectrum of activity than cisplatin.

Introduction

Widespread use in the clinic and increasing volume of sale of cisplatin and carboplatin indicate that even in postgenomic age there is a great demand for shotgun chemotherapy to kill cancerous cells. Although highly effective against testicular and ovarian cancers, cisplatin and its analogues such as carboplatin and oxaliplatin have a limited spectrum of activity, numerous side effects, and the problem of intrinsic and/or acquired resistance. Following Farrell's suggestion that platinum compounds that bind with DNA differently than cisplatin may have an altered spectrum of activity,[1] increasing research effort has been directed at platinum compounds that violate classical structure–activity relationships in one way or another. Two classes of rule-breaker compounds are *trans*-planaramineplatinum(II) complexes and compounds containing two or more platinum centres.[1–3] In this laboratory we have also designed a number of mononuclear *trans*-planaramineplatinum(II) complexes of the forms: *trans*-[Pt(NH_3)LCl_2] and *trans*-[PtL_2Cl_2 where L stands for a planaramine ligand[4,5] and multicentred rule-breaker platinum compounds. One complex of the form: *trans*-[Pt(NH_3)LCl_2] where L is (1,2-α)pyridine (code named YH12) is found to have significantly greater activity than cisplatin against ovarian cancer cell line A2780^{cisR}.[6] A number of di- and trinuclear Pt-Pt-Pt and Pt-Pd-Pt complexes some containing one or two planaramine ligands bound to the central metal ion have also been synthesised.[7–10] Four of them code named DH6Cl, DH7Cl, CH9, and CH25 have been found to be significantly more active than cisplatin in a number of ovarian cancer cell lines. In this paper, we report on the synthesis, characterisation, and activity of a new highly active trinuclear complex: [{*trans*-PtCl(NH_3)$_2$}$_2$μ-{*trans*-Pt(3-hydroxypyridine)$_2$($H_2N(CH_2)_6NH_2$)$_2$}]Cl_4 [code named TH1], and also on its binding with DNA and its constituents. TH1 that has two 3-hydroxypyridine ligands is moderately soluble in water (12 mg per 10 mL of mQ water). Figure 1 shows the structure of TH1.

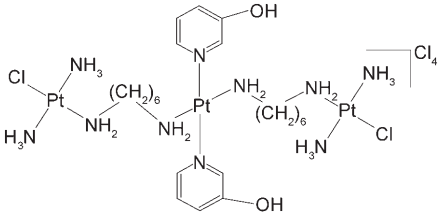

Figure 1. Structure of TH1.

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Results and Discussion

Activity against ovarian cancer cell lines

Table 1 lists the IC_{50} values and resistance factors (RF) of TH1 and cisplatin against human ovarian cancer cell lines A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$.

Table 1. IC_{50} values and resistance factors of TH1 and cisplatin.[a]

	IC_{50} (μM) ± s.d. and resistant factors				
Compound	A2780	$A2780^{cisR}$	RF	$A2780^{ZD0473R}$	RF
TH1	0.53 ± 0.2	0.99 ± 0.2	1.98	0.27 ± 0.1	0.5
Cisplatin	1.0 ± 0.4	12.9 ± 0.4	12.9	3.1 ± 1.2	3.0

[a] As applied to the human ovarian cancer cell lines: A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$. The results are averages of those obtained from four identical wells with 4000 cells per well.

TTH1 is found to be significantly more active than cisplatin against the three human ovarian cancer cell lines A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$—two times more active against A2780, 13 times more active against $A2780^{cisR}$ and 11 times more active against $A2780^{ZD0473R}$. Whereas the resistance factor for cisplatin as applied to the cell lines A2780 and $A2780^{cisR}$ is 12.9 that for TH1 is 1.98. Also, TH1 has a much lower resistance factor than cisplatin (0.5 versus 3.0) as applied to the cell lines A2780 and $A2780^{ZD0473R}$. The results indicate that TH1 has been better able to overcome resistances operating in both $A2780^{cisR}$ and $A2780^{ZD0473R}$ cell lines.

Cell uptake

Table 2 gives the total intracellular platinum levels (expressed as nanomoles Pt per 2×10^6 cells) found in the cell lines A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$ after exposure to 50 μM concentrations of TH1 and cisplatin for 2, 4, 24, and 72 h.

When the cell uptakes of Pt applying to TH1 and cisplatin at 2 h, 4 h, 24 h, and 72 h are compared, it is found that for both TH1 and cisplatin, intracellular platinum levels in all three cell lines A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$ increase with time during the period 0 to 24 h. At 72 h, there is a sharp drop in Pt uptake for TH1 and on face value, there is also a drop in the case of cisplatin that remains uncertain because of large errors of measurement. Platinum uptake from TH1 are found to be much higher than those from cisplatin in all the three ovarian cancer cell lines A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$ even though cisplatin is neutral and TH1 cation would be positively charged. However, this is in line with the higher activity of TH1. It should also be noted that much higher cellular uptake has been observed for other polynuclear platinum complexes.[8,9]

DNA binding

As anticancer activity of platinum drugs is believed to be associated with their binding with cellular DNA, the level of binding with cellular DNA is considered to provide more meaningful information (related to activity) than total cellular uptake especially as platinum drugs may be deactivated by complexation with cellular platinophiles such as glutathione and metallothionein so that only a very small fraction of the drugs actually binds with DNA.[11] Table 3 gives the levels of platinum DNA binding expressed as nanomol of Pt per milligram of DNA in A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$ cells in 2, 4, 24, and 72 h for TH1 and cisplatin.

It can be seen that levels of Pt binding with cellular DNA are higher for TH1 than cisplatin at 4 h, 24 h, and 72 h for the three ovarian cell lines A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$ generally in line with the greater activity of TH1. At 2 h, the values are higher for TH1 than cisplatin for the resistant cell lines $A2780^{cisR}$ and $A2780^{ZD0473R}$; however, for the parent cell line A2780, the value is higher for cisplatin. At 72 h, there is a sharp decrease in the level of binding of both TH1 and cisplatin with nuclear DNA of all the three ovarian cancer cell lines

Table 2. Total intracellular platinum levels found in the cell lines A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$.[a]

Drug	A2789				$A2789^{cisR}$				$A2780^{ZD0473R}$			
	2 h	4 h	24 h	72 h	2 h	4 h	24 h	72 h	2 h	4 h	24 h	72 h
TH1	3.51 ± 0.36	3.72 ± 0.35	13.61 ± 0.13	3.42 ± 0.02	2.56 ± 0.73	6.42 ± 0.25	8.25 ± 1.21	1.91 ± 0.06	2.71 ± 0.07	8.83 ± 0.14	13.55 ± 0.18	1.68 ± 0.15
Cisplatin	0.21 ± 0.26	0.26 ± 0.26	0.50 ± 0.34	0.36 ± 0.07	0.09 ± 0.12	0.03 ± 0.02	0.21 ± 0.19	0.29 ± 0.03	0.24 ± 0.26	0.20 ± 0.09	1.30 ± 0.26	0.11 ± 0.08

[a] Expressed as nanomol Pt per 2×10^6 cells, after exposure to 50 μM concentrations of TH1 and cisplatin for 2, 4, 24, and 72 h.

Table 3. Levels of Pt binding in A2780, $A2780^{cisR}$, and $A2780^{ZD0473R}$ cells.[a]

Drug	A2780				$A2780^{cisR}$				$A2780^{ZD0473R}$			
	2 h	4 h	24 h	72 h	2 h	4 h	24 h	72 h	2 h	4 h	24 h	72 h
TH1	3.5 ± 0.3	32.5 ± 0.1	33.2 ± 3.7	17.9 ± 0.0	99.1 ± 2.1	36.4 ± 1.1	34.1 ± 1.6	2.8 ± 0.1	7.2 ± 1.7	2.1 ± 2.1	8.6 ± 1.0	2.1 ± 0.2
Cisplatin	13.8 ± 4.3	0.26 ± 0.26	3.2 ± 0.2	25.4 ± 3.4	62.5 ± 6.4	15.7 ± 2.9	1.3 ± 0.5	1.0 ± 0.0	0.5 ± 0.0	1.3 ± 0.4	1.7 ± 0.1	1.4 ± 0.0

[a] Expressed as nanomol Pt per mg of DNA, after exposure to 50 μM concentrations of TH1 and cisplatin for 2, 4, 24, and 72 h.

A2780, A2780^{cisR}, and A2780^{ZD0473R}, possibly indicating the occurrence of increased DNA repair over the period 24 to 72 h. The ratios of Pt-DNA binding for TH1 and cisplatin at 2 h, 4 h, 24 h, and 72 h for the cell lines: A2780, A2780^{cisR}, and A2780^{ZD0473R} are: at 2 h—A2780: (1:4), A2780^{cisR} (1:1.6), A2780^{ZD0473R} (14.4:1); at 4 h—A2780: (10.2:1), A2780^{cisR} (2.3:1), A2780^{ZD0473R} (27.8:1); at 24 h—A2780: (10.2:1), A2780^{cisR} (2.3:1), A2780^{ZD0473R} (27.8:1); at 72 h—A2780: (6.4:1), A2780^{cisR} (2.8:1), A2780^{ZD0473R} (1.5:1). Whereas TH1 has three Pt centres, cisplatin has only one, the corresponding molar ratios will be one third of the Pt ratios.

It was noted earlier that for TH1, the level of Pt binding with nuclear DNA increases with time during the period 0–24 h followed by a sharp decrease in the cell lines A2780 and A2780^{ZD047R}. For the cell line A2780^{cisR}, the level of Pt–DNA binding is found to be lowest at 72 h. The decrease in the level of Pt–DNA binding with increased time (especially during the period 24 to 72 h) observed for TH1, indicates that increased DNA repair may be a dominant mechanism of resistance operating in all the resistant cell lines. It may be noted that the three main mechanisms of resistance applying to platinum drugs in ovarian cancers are 1) reduced cell uptake, 2) increased deactivation within the cell, and 3) increased DNA repair.[12] For the trinuclear compound BBR3464, increased DNA repair was found to be a dominant mechanism of resistance operating in A2780/BBR3464 resistant cell line.[13] As TH1, like other trinuclear platinum complexes,[8,9,14] can vary its effective length (because of the presence of flexible linker chains), it would bind with DNA forming a range of interstrand GG adducts causing a global change in its conformation from B- to Z- and A-forms.[3]

TH1, being a trinuclear complex, only the two terminal platinum centres would bind covalently with DNA forming a range of interstrand Pt(GG)Pt adducts, causing a global change in DNA conformation. In contrast, cisplatin is known to form mainly intrastrand Pt(GG) adduct that causes a local bending of a DNA strand. The central platinum ion in TH1 would undergo only noncovalent interactions including electrostatic interaction and hydrogen bonding. The two 3-hydroxypyridine ligands bound to the central platinum ion may also undergo other types of noncovalent interactions such as stacking interactions. It is believed that the higher activity of TH1 against all the three cell lines as compared to cisplatin, is due to differences in both the nature and the actual level of binding with DNA. When platinum–DNA binding levels at 72 h are compared it is found that TH1 has a greater level of binding with DNA in the A2780 cell line than in the A2780^{ZD0473R} cell line, even though TH1 is found to be more active against the A2780^{ZD0473R} cell line than the parent cell line A2780. In fact, the level of DNA binding in the A2780^{ZD0473R} cell line is less than that in the other resistant cell line A2780^{cisR} in which TH1 has the lowest activity. The results show that even the differences in the level of binding with DNA in different cancer cells may not correlate with differences in activity. It is possible that the three different ovarian cancers may differ in gene and protein expression that may be playing key roles in the activity of the drug. Further studies would be required to provide more insight into the matter.

It is believed that the presence of two 3-hydroxypyridine ligands bound to the central platinum ion makes TH1 soluble in water. The above results suggest that TH1 has the potential for development as a highly potent anticancer drug provided it meets the toxicity requirement. It may be noted that the novel trinuclear compound BBR3464 that has shown activity in both cisplatin-sensitive and cisplatin-resistant human and murine cancer cell lines was in the phase II stage of clinical development before it was stopped because of toxicity. Studies using suitable animal models would show whether TH1 has a better toxicity profile than BBR3464 or not. If it were indeed found that TH1 was less toxic than BBR3464, it would lead to the development of a highly active anticancer drug with a spectrum of activity different from that of cisplatin

Interaction with pBR322 plasmid DNA

As platinum drugs bind with DNA, changes in DNA conformation and DNA damage may occur, that may be reflected as changes in mobility and intensity of DNA bands. As stated earlier, the pBR322 plasmid DNA can exist in three forms: supercoiled form I, singly nicked relaxed circular form II, and doubly nicked linear form III. DNA being negatively charged because of the phosphate backbone will migrate through the gel from the negative to positive electrode. The supercoiled form I migrates at the fastest rate, 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 the trinuclear compound TH1, a prominent form I band was observed for concentrations ranging from 0.25 to 4.5 μM. In addition, a faint band corresponding to form II was observed for the concentrations of TH1 ranging from 0.25 and 1 μM (Figure 2). Although a part of the decrease in intensity observed in the case of TH1, is believed to be an artifact of the experiment, as indicated by difference in intensity of the blanks on the two sides of the gel, it is also clear that increasing concentrations of TH1 caused increasing damage to DNA that resulted in a decrease in intensity of the bands. As stated earlier, like BBR3464, TH1 is a trinuclear platinum complex in which the three platinum centres are linked together by two 1,6-diaminohexane chains. However, in TH1 the central platinum ion is bound to two 3-hydroxypyridine ligands whereas that in BBR3464 is bound to ammonia ligands so that TH1 can undergo additional types of noncovalent interactions (such as stacking interactions) with DNA. As a result of the *trans*-labilizing effect of chloride, the Pt–N bonds between terminal Pts and the linking diamine chains are expected to be weakened so that the trinuclear cations in TH1 may also undergo some dissociation producing multiple mononuclear species.[15]

BamH1 digestion

Drug–DNA incubation followed by BamH1 digestion was used to provide further insight into changes in DNA conformation.

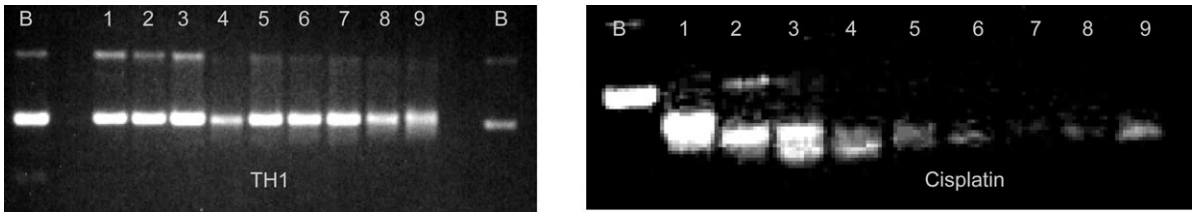


Figure 2. Electrophoretograms relating to the interaction of pBR322 plasmid DNA with increasing concentrations of TH1 and cisplatin. Lane B in both electrophoretograms contains untreated pBR322 plasmid DNA to serve as a control; lanes 1 to 9 relate to plasmid DNA interacted with increasing concentrations of TH1 and cisplatin. For TH1, lane 1: 0.25 μM, lane 2: 0.5 μM, lane 3: 1 μM, lane 4: 1.5 μM, lane 5: 2 μM, lane 6: 2.5 μM, lane 7: 3 μM, lane 8: 3.5 μM, and lane 9: 4.5 μM. For cisplatin, lane 1: 5 μM, lane 2: 10 μM, lane 3: 15 μM, lane 4: 20 μM, lane 5: 25 μM, lane 6: 30 μM, lane 7: 35 μM, lane 8: 40 μM, and lane 9: 50 μM.

When unreacted pBR322 plasmid DNA was digested with BamH1, only one band corresponding to the form III band was observed, whereas in the untreated and undigested pBR322 plasmid DNA, generally two bands corresponding to forms I and II were observed. When the DNA was interacted with increasing concentrations of TH1 followed by BamH1 digestion, at low concentration mainly form III was observed whereas at higher concentrations three bands corresponding to forms I, II, and III were observed. In the case of cisplatin, two bands corresponding to forms I and II were observed for all concentrations of the compound ranging from 5 μM to 9: 50 μM (Figure 3). Table 4 gives a more detailed list of the bands as a function of concentration. Prevention of BamH1 digestion with increasing concentration of TH1, gives support to the idea that interstrand covalent binding of TH1 with guanine bases in DNA induces global change in its conformation.

Table 4. Bands observed after BamH1 digestion of incubated mixtures of TH1 and cisplatin with pBR322 plasmid DNA.

Compound	1	2	3	4	5	6	7	8	9
Cisplatin	I, II	I, II	I, II	I, II	I, II	I, II	I, II	I, II	I, II
TH1	I, III	I, III	I, II, III	I, II, III	I, II, III	I, II, III	I, II, III	I, II, III	I, II, III

[a] For cisplatin, 1: 5 μM, 2: 10 μM, 3: 15 μM, 4: 20 μM, 5: 25 μM, 6: 30 μM, 7: 35 μM, 8: 40 μM, and 9: 50 μM; for TH1, 1: 0.25 μM, 2: 0.5 μM, 3: 1 μM, 4: 1.5 μM, 5: 2 μM, 6: 2.5 μM, 7: 3 μM, 8: 3.5 μM, and 9: 4.5 μM.

Table 5. Retention times of the main peaks for guanine and the mixture of TH1 and guanine.[a]

Reactant A	Reactant B	Major peak	
		Retention time (min)	Guanine:Pt ratio
Guanine	None	12.42	N/A
Guanine	TH1	13.69	1.2 ± 0.4

[a] With the detector set at $\lambda = 260$ nm.

Binding of TH1 with guanine

HPLC combined with UV-spectrophotometry and graphite furnace atomic absorption spectroscopy was used to investigate the binding of TH1 with guanine. The major peak in the chromatogram (Table 5) was found to have the G:Pt binding ratio of 1.2:1, indicating that TH1 would form essentially 1:2 complex with guanine, in agreement with the fact that TH1 has two labile chloride ligands bound to the two terminal Pt centres. It is believed that in the 1:2 adduct between TH1 and guanine, the terminal platinum ions are bound to N7 positions. As noted earlier, because of the *trans*-labilizing effect of the chloride ion, some decomposition of TH1 associated with the breakdown of the bonds between the terminal platinum ions and linking diamines cannot be ignored and this may be a reason for the observed higher binding ratio between platinum and guanine.

Conclusions

A novel trinuclear platinum complex code named TH1 has been synthesised and characterised based on elemental analy-

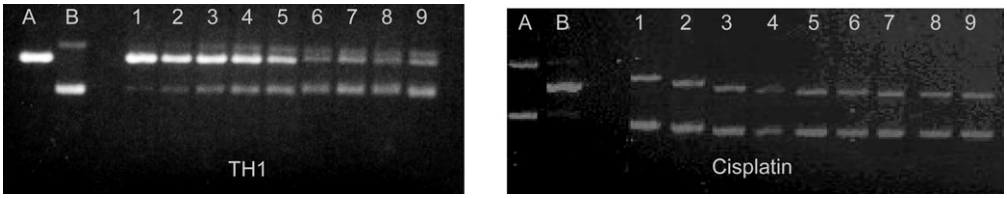


Figure 3. Electrophoretograms relating to the incubated mixtures of pBR322 plasmid DNA and varying concentrations of TH1 and cisplatin followed by BamH1 digestion: For both TH1 and cisplatin, lane A is untreated pBR322 plasmid DNA but digested with BamH1, lane B is untreated and undigested with BamH1 and lanes 1 to 9 are pBR322 plasmid DNA interacted with increasing concentrations of the compounds followed by BamH1 digestion. For TH1, lane 1: 0.25 μM, lane 2: 0.5 μM, lane 3: 1 μM, lane 4: 1.5 μM, lane 5: 2 μM, lane 6: 2.5 μM, lane 7: 3 μM, lane 8: 3.5 μM, and lane 9: 4.5 μM. For cisplatin, lane 1: 5 μM, lane 2: 10 μM, lane 3: 15 μM, lane 4: 20 μM, lane 5: 25 μM, lane 6: 30 μM, lane 7: 35 μM, lane 8: 40 μM, and lane 9: 50 μM.

ses and a number of spectral studies. The activity of the compound against three human ovarian cancer cell lines A2780, A2780^{cisR}, and A2780^{ZD0473R} has been determined based on a MTT reduction assay. The compound is soluble in water and much more active than cisplatin against all the three cell lines.

Experimental Section

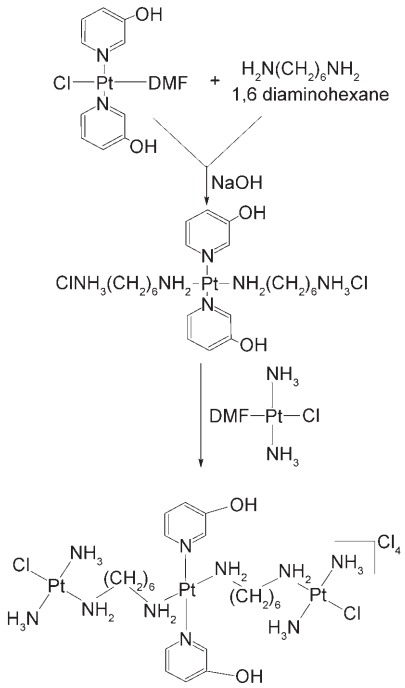
Materials: Potassium tetrachloroplatinate $K_2[PtCl_4]$, *N*,*N*-dimethylformamide [DMF] [$(CH_3)_2NCHO$], 3-hydroxypyridine, and 1,6-diaminohexane were obtained from Sigma Chemical Company St. Louise USA; acetone [$(CH_3)_2CO$] and silver nitrate ($AgNO_3$) were obtained from Ajax Chemicals Auburn NSW Australia; methanol [CH_3OH], ethanol [C_2H_5OH], dichloromethane [CH_2Cl_2] were obtained from Merck Pty. Limited Kilsyth VIC Australia. pBR322 plasmid DNA was purchased from ICN Biomedicals, Ohio, USA. Foetal calf serum, 5×RPMI 1640, 200 mM L-glutamine, and 5.6% sodium bicarbonate were obtained from Trace Biosciences Pty Ltd, Australia. Other reagents were obtained from Sigma-Aldrich Pty Ltd, NSW, Australia. Commercially available JETQUICK Blood DNA Spin Kit/50 used to isolate high molecular weight DNA from cell pellet was obtained from Astral Scientific, Australia.

Synthesis:

CH1: CH1 denoting [*trans*-$PtCl_2$(3-hydroxypyridine)$_2$, required for the synthesis of TH1 was prepared according to previously published method.[5] Yellow-orange solid; Formula: $C_5H_8N_2Cl_2OPt$; purity: 99.8%; Yield: 75.0%

Composition: Calc.%: C, 26.3; H, 2.2; N, 6.2; Cl, 15.3; Pt, 42.8 Obs.%: C, 26.4±0.4; H, 2.0±0.4; N, 6.2±0.4; Cl, 15.3±0.4; Pt, 43.1±1.2.

TH1: A step-up method of synthesis, branching out from the central unit was used for the synthesis of TH, following a method similar to that used for the synthesis of DH6Cl.[16] Briefly, the method was as follows: Transplatin (0.300 g, 1 mmol) was dissolved in 20 mL of DMF to which 0.1698 g of silver nitrate (0.990 mmol) was added. The mixture was stirred at room temperature for 24 h in the dark followed by centrifugation at 4800 rpm for 30 min. The supernatant was kept at −16 °C. A suspension of CH1 (0.228 g, 0.5 mmol in 10 mL of DMF) was gently heated with stirring at 70 °C for about 20 min to which 1,6-diaminohexane (0.116 g, 1 mmol) dissolved in 4 mL of DMF and acidified with 1 mL of 1 M HCl was added dropwise with stirring. Stirring was continued for 1 h at 70 °C followed by 4 h at 50 °C. Then, 200 μL of 1 M NaOH was added with stirring. Stirring was continued for a further 20 min resulting in a clear light brown solution and some brown precipitate. The transplatin filtrate (0.5 mmol) was then added to the light brown solution with stirring at 50 °C for 25 min. Then 150 μL of 1 M NaOH was added dropwise with stirring to the solution. Stirring was continued for further 5 min at the same temperature. Then 0.5 mmol of transplatin was added to the mixture. Stirring was continued for 50 min at 55 °C then for 15 days at room temperature. The volume of the filtrate was reduced to about 4 mL using a vacuum concentrator consisting of Javac DD150 double stage High Vacuum Pump, Savant RVT 4104 Refrigerated Vapor Trap and Savant Speed Vac 110. About 10 mL of dichloromethane was added to the concentrated solution. The mixture was left standing at 5 °C for 6 h. The light yellow precipitate produced was collected by filtration at the pump, washed in succession with mQ water, methanol, and ethanol. It was then air dried. The crude product of TH1 was purified by repeated dissolution in 1:1 mixture of DMF and mQ water and precipitation by the addition of dichloromethane. The identity of the compound was confirmed by microanalyses and spectral studies. The steps in the synthesis are shown in Scheme 1.



Scheme 1. Synthesis of TH1

Characterisation

C, H, N, and Cl were determined using the facility at the Australian National University. Platinum was determined by graphite furnace atomic absorption spectroscopy (AAS) using the Varian Spectra-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. Spectra were recorded at a resolution of 4 cm^{-1}, with the addition of 128 scans and a Blackman-Harris 3-Term apodisation function was applied.

Molar Conductivity. The molar conductivity for TH1 was determined using a PW9506 digital conductivity meter. The compound was first dissolved in a 1:1 mixture of DMF and water to obtain 1 mM solution, which was then progressively diluted with mQ water to obtain solutions at concentrations ranging from 0.5 mM to 0.0625 mM. Molar conductivity values were then plotted against concentration to obtain the limiting value, that is, the value at zero concentration or infinite dilution.[17]

TH1; Formula: $C_{22}H_{54}Cl_6N_{10}O_2Pt_3$; Molar mass: 1288.681 g mol^{-1} yield = 63%

Composition: C: Calc.%: C, 20.9; H, 4.2; N, 10.9; Cl, 16.5; Pt, 45.4 Obs.%: C, 21.1±0.4; H, 4.6±0.4; N, 11.2±0.4; Cl, 16.3±0.4; Pt, 44.3±1.2

The limiting molar conductivity of TH1 at zero concentration was 384 $ohm^{-1}cm^2mol^{-1}$.

The achieved purity of TH1 is estimated to be about 95% even after repeated purification by dissolution and precipitation. This was found to be the case with other trinuclear complexes.[3,9,16] One likely reason for the low purity is the formation of some dinuclear complexes along with trinuclear complex as was observed in

the synthesis of BBR3464 and related compounds (Farrell et al., 1999).

IR: The bands at 3765 and 3530 cm^{-1} are believed to be due to O–H stretching vibrations whereas that at 3341 cm^{-1} is believed to be due to the N–H stretching vibration. The bands at 3192, 3016 2936, 2810, 2684, and 2386 cm^{-1} are due to N–H and C–H stretching vibrations. The band at 1600 cm^{-1} is due to the ring stretching vibration, that at 1072 cm^{-1} is due to the C–N stretching vibration and that at 700 cm^{-1} is due to the C–H bending vibration. Finally the band at 383 cm^{-1} is due to the Pt–Cl stretching vibration.

Mass spectrum: ESI-MS (DMF)(*m/z*: *M*=1288.681). No peak was observed corresponding to M indicating that the molecule broke down in solution or in the spectrometer. The peak at *m/z*=751 corresponds to $[M-Pt-(NH_3)_4Cl_2(NH_2(CH_2)_6]$, that at *m/z*=713 corresponds to $[M-Pt(NH_3)_2NH_2(CH_2)_6NHCl_2]$, that at *m/z*=656 corresponds to $[M-Pt(NH_2(CH_2)_6NH_2)(NH_3)_4Cl_2]$, that at *m/z*=595 corresponds to $[M-Pt_2(NH_3)_4Cl_2]$ and that at *m/z*=536 corresponds to $[M-Pt_2(NH_3)_4Cl(NH_2(CH_2)_6]$. The peak at *m/z*=479 corresponds to $[M-Pt_2(NH_3)_4(NH_2(CH_2)_6NH_2]$, that at *m/z*=460 corresponds to $[M-Pt_2(NH_3)_4Cl_2(NH_2(CH_2)_6NH_2]$ and that at *m/z*=381 corresponds to $[M-(PtNH_2(CH_2)_6NH_2)_2O_2ClN_2]$.

^{1}H NMR: The resonance at $\delta=8.2$ ppm (doublet) is believed to be due to CH *ortho* and that at $\delta=7.4$ ppm (quartet) is believed to be due to CH *meta*. The resonance at $\delta=7.3$ ppm (doublet) is believed to be due to CH *para* and that at $\delta=4.7$ ppm (singlet) is believed to be due to NH_2. The resonance at $\delta=4.68$ ppm (singlet) is believed to be due to CH_2. The resonance at $\delta=2.6$ ppm (singlet) is believed to be due to DMSO and that at $\delta=1.58$ ppm (broad) is believed to be due to CH_2 meta. Finally, the resonance at $\delta=$ 1.3 ppm (broad) is believed to be due to CH_2.

Cytotoxicity assays

Cytotoxicity of the compounds against human ovarian cancer cell lines: A2780, A2780^{cisR}, and A2780^{ZD0473R} was determined using a MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) reduction assay.[18,19] Between 8000 to 12000 cells (maintained in logarithmic growth phase in complete medium consisting of RPMI 1640, 10% heat-inactivated fetal calf serum, 20 mM hepes, 0.112% bicarbonate, and 2 mM glutamine without antibiotics), depending on the growth characteristics of the cell line, were seeded into the wells of the flat-bottomed 96-well culture plate in 10% FCS/RPMI 1640 culture medium. The plate was then incubated for 24 h at 37 °C in a humidified atmosphere to allow them to attach. TH1 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 sterilise. Serial fivefold dilutions of the drugs (ranging from 0.004 μM to 40 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. Four hours after the addition of MTT solution (50 μL per well of 1 mg mL^{-1} MTT solution), the yellow formazan crystals produced from the reduction of MTT were dissolved in 150 μL of DMSO and read with a 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.

Drug uptake and binding with DNA

The method used for cell subculture was a modification of that described by Freshney[13] and the method used for cell treatment as applied to the determination of drug uptake and DNA was a modification of that described by Di Blasi et al.[20] The platinum complexes (at 50 μM final concentration) were added to culture plates containing exponentially growing A2780, A2780^{cisR}, and A2780^{ZD0473R} cells in 10 mL 10% FCS/RPMI 1640 culture medium (cell density = 1×10^6 cells mL^{-1}). The cells containing the drugs were incubated for 2, 4, 24, and 72 h at the end of which cell monolayers were trypsinised 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 TH1 and cisplatin. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and the pellets were stored at −20 °C until assayed. At least three independent experiments were performed.

Drug accumulation in cells: Following drug incubation, the cell pellets were suspended in 0.5 mL 1% triton-X, held on ice then sonicated. Total intracellular platinum contents were determined by graphite furnace atomic absorption spectrophotometry.

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.[21] 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. 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. 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 10600 rpm through the silica membrane using the 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 10600 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 10600 rpm. To further clear the silica membrane from residual liquid, the sample columns were centrifuged again for 2 min at full speed (13000 rpm). The column receivers were changed and the purified DNA in the column was eluted from the membrane with 200 μL of 10 mM Tris-HCl buffer (pH 8.5). Platinum contents were determined by graphite furnace AAS. A_{260}/A_{280} ratios were found to be between 1.75 and 1.8 for all samples indicating high purity of the DNA.[22]

Interaction with pBR322 plasmid DNA

Interaction of TH1 and cisplatin with pBR322 plasmid DNA was studied by agarose gel electrophoresis based on a method described by Stellwagen.[23] In the study, the amount of DNA was kept constant while the concentrations of compounds were varied. Exactly 1.5 μL of supplied pBR322 plasmid DNA in solution was added to varied amounts of solutions of the compounds at different concentrations ranging from: 0.25 μM to 4.5 μM. The total volume was made up to 20 μL by adding mQ water. The DNA blank was prepared by adding 18.5 μL mQ water to 1.5 μL of pBR322 plasmid DNA.

The samples including the DNA blank were incubated for 4 h on a shaking water bath at 37 °C in the dark. At the end of incubation, the reaction was quenched by rapid cooling to 0 °C for 20 min. The samples were thawed then mixed with 4 μL of marker dye (0.25% bromophenol blue and 40% of sucrose). 17 μL of each sample was loaded onto 1% agarose gel made in TAE buffer that contained ethidium bromide (1 mg mL^{-1}). The gel was stained in the same buffer.[24] Electrophoresis was carried out also in TAE buffer contain-

ing ethidium bromide at 80 V for 3 h at room temperature. The bands of the plasmid DNA were viewed under short wave UV light using the BIO-RAD Trans illuminator IEC1010 and photographed with a Polaroid camera (orange filter) using Polaroid black and white print film, type 667.

BamH1 digestion

BamH1 is known to recognise the sequence G/GATCC and hydrolyse the phosphodiester bond between adjacent guanine sites.[25] pBR322 contains a single restriction site for BamH1 which converts pBR322 plasmid DNA from supercoiled form I and singly nicked circular form II to linear form III. In this experiment, a same set of drug–DNA mixtures as that described previously, was first incubated for 4 h in a shaking water bath at 37 °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.

HPLC

High pressure liquid chromatography (HPLC) combined with UV-visible spectrophotometry and graphite furnace spectroscopy was used to investigate the binding between TH1 and guanine. Equal volumes of 1 mM solution of TH1 and 2 mM solution of guanine were mixed together and incubated at 37 °C in a shaking water bath for 2 h. To dissolve guanine in mQ water, pH was increased to about ten by adding a tiny drop of 0.1 M NaOH. After incubation, 5–20 μL of each of the mixtures and appropriate components was injected separately into a Waters HPLC system, consisting of a Waters 600 controller, a Waters 600 pump, a Waters 746 data module, a Waters Dual λ absorbance detector, and a Waters Nova-Pak C18 column consisting of Waters RCM 8×10 Module and Resolve Cartridge set at a pressure of 17 Mpa. The wavelength was set at 260 nm. The mobile phase consisted of 5% methanol and 95% of ammonium acetate (0.1 M at pH 5.5) with a flow rate of 1 mL min^{-1}. The retention times of the peaks applying to the mixtures and the components were recorded and the peak fractions were collected and analysed for platinum and guanine contents to obtain binding ratio between platinum and guanine.

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Keywords: cisplatin · DNA binding · ovarian cancer · platinum complexes

[1] N. Farrell, L. R. Kelland, J. D. Roberts, M. Van Beusichem, *Cancer Res.* **1992**, *52*, 5065–5072.
[2] A. Gelasco, S. Lippard, *Metallopharmaceuticals I: DNA interactions* (Eds.: M. J. Clarke and P. J. Sadler), Springer, Berlin, London **1999**, pp. 1–43.
[3] N. Farrell, *Platinum-Based Drugs in Cancer Therapy* (Eds.: L. R. Kelland and N. P. Farrell), Humana, New Jersey, **2000**, pp. 321–338.
[4] F. Huq, H. Daghriri, J. Q. Yu, P. Beale and K. Fisher, *Eur. J. Med. Chem.* **2004**, *39*, 691–697.
[5] A. Chowdhury, F. Huq, A. Abdullah, P. Beale, K. Fisher, *J. Inorg. Biochem.* **2005**, *99*, 1098–1112.
[6] F. Huq F, J. Q. Yu, H. Daghriri, P. Beale, *J. Inorg. Biochem.* **2004**, *98*, 1261–1270.
[7] F. Huq, H. Daghriri, J. Q. Yu, H. Tayyem, P. Beale, M. Zhang, *Eur. J. Med. Chem.* **2004**, *39*, 947–958.
[8] H. Daghriri, F. Huq, P. Beale, *J. Inorg. Biochem.* **2004**, *98*, 1722–1733.
[9] H. Cheng, F. Huq, P. Beale, K. Fisher, *Eur. J. Med. Chem.* **2005**, *40*, 772–781.
[10] H. Cheng, F. Huq, P. Beale, K. Fisher, *Eur. J. Med. Chem.* **2006**, *41*, 896–903.
[11] T. Boulikas, M. Vougiouka, *Oncol. Rep.* **2003**, 1663–1682.
[12] M. Ohmichia, J. Hayakawa, K. Tasaka, H. Kurachi, Y. Murata, *Trends Pharmacol. Sci.* **2005**, *26*, 113–115.
[13] P. Perego, L. Gatti, C. Caserini, R. Supino, D. Colangelo, R. Leone, S. Spinelli, N. Farrell, F. Zunino, *Cancer Res.* **1996**, *56*, 556–562.
[14] C. Manzotti, G. Pratesi, E. Menta, R. Di Domenico, E. Cavalletti, H. H. Fiebig, L. R. Kelland, N. Farrell, D. Polizzi, R. Supino, G. Pezzoni, F. Zunino, *Clin. Cancer Res.* **2000**, *6*, 2626–2634.
[15] Y.-P. Ho, S. C. F. Au-Yeung, K. K. W. To, *Med. Res. Rev.* **2003**, *23*, 633–655.
[16] F. Huq, H. Daghriri, J. Q. Yu, P. Beale, K. Fisher, *Pure Appl. Chem.* **2006**, *1*, 493–507.
[17] P. W. Atkins, *Physical chemistry*, Oxford University, Oxford UK, **1998**, pp. 737–738.
[18] R. I. Freshney, *Culture of animal cells: a manual of basic technique*, Wiley-Liss, New York.
[19] T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55–63.
[20] P. Di Blasi, A. Bernareggi, G. Beggiolin, L. Piazzoni, E. Menta, M. L. Formento, *Anticancer Res.* **1998**, *18*, 3113–3118.
[21] D. D. Bowtell, *Anal. Biochem.* **1987**, *162*, 463–465.
[22] J. Holford, P. J. Beale, F. E. Boxall, S. Y. Sharp, L. R. Kelland, *Eur. J. Cancer* **2000**, *36*, 1984–1990.
[23] N. Stellwagen, *DNA gel electrophoresis. Nucleic acid electrophoresis*, Springer, Berlin, New York, pp. 1–53.
[24] G. B. Onoa, V. Moreno, *Int. J. Pharm.* **2002**, *245*, 55–65.
[25] L. A. Smith, J. G. Chirikjian, *Biol. Chem.* **1979**, *254*, 1003–1006.

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