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# Studies on the Synthesis and Activity of Three Tripalladium Complexes Containing Planaramine Ligands

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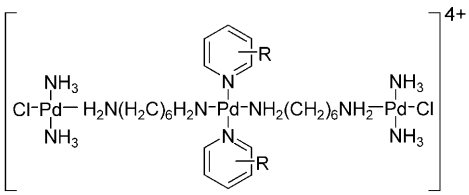
The present study deals with the synthesis, characterization and activity against human cancer cell lines: A2780, A2780$^{cisR}$ and A2780$^{ZD0473R}$ of three tripalladium complexes, MH3, MH4 and MH5, that each have two planaramine ligands bound to the central metal ion. Cellular uptake levels, extent of DNA binding, and nature of interaction with salmon sperm and pBR322 plasmid DNA were determined for each complex. Palladium compounds are much more reactive than their corresponding platinum derivatives, which makes them therapeutically inactive but toxic. However, the results of the present study suggest that significant antitumour activity can be introduced in palladium complexes by lessening their reactivity by the introduction of sterically hindered ligands such as 2-hydroxypyridine, 3-hydroxypyridine and 4-hydroxypyridine. When bound to the central palladium ion, 4-hydroxypyridine appears to be more activating than 2-hydroxypyridine and 3-hydroxypyridine, suggesting that noncovalent interactions, such as hydrogen bonding, may also be key determinants of antitumour activity in addition to the steric effect. While cisplatin binds with DNA to form intrastrand GG adducts that causes local bending of a DNA strand, these planaramine-derived palladium complexes are expected to bind with DNA and form a number of long-range interstrand GG adducts that would cause a global change in DNA conformation, provided the tripalladium cations in MH3, MH4 and MH5 persist under physiological conditions.

## Introduction

Although palladium and platinum belong to the same group in the periodic table, their compounds differ greatly in reactivity and biological activity. Whereas platinum complexes can be active anticancer agents, the corresponding palladium complexes are generally therapeutically inactive but toxic because of higher reactivity. Thus, it is logical to think that antitumour activity could be introduced in palladium compounds if their reactivity could be lowered sufficiently by modulation of the coordination environment, for example, due to the cage effect provided by sterically hindered ligands.

Previously, a number of mononuclear palladium complexes containing sterically hindered ligands,[1] and two trinuclear palladium analogues of BBR3464, MH1 and MH2, were synthesized and tested for activity against human ovarian cancer cell lines.[2] Although MH1 and MH2 showed only slight activity against ovarian cancer cell lines, some of the mononuclear palladium complexes containing planaramine ligands displayed much higher activity, and even more so against resistant cell lines, thus providing support to the hypothesis. A trinuclear Pt–Pd–Pt complex, DH6Cl (analogous to BBR3464), was also found to be much more active than cisplatin against ovarian cancer cell lines, indicating that a significant decrease in reactivity of the central palladium ion has occurred due to the cage effect provided by the 1,6-diaminohexane diamine ligands.[3,4] Another trinuclear Pt–Pd–Pt complex, CH25 (synthesized in this laboratory), in which the central palladium ion is bound to one 2-hydroxypyridine ligand (and one ammonia ligand) was found to be highly active against human ovarian cancer cell lines.[5]

Herein, we describe the synthesis, characterization and activity against human ovarian cancer cell lines of three new trinuclear palladium complexes, MH3, MH4 and MH5, in which the central metal ion is bound to two 4-hydroxypyridine, 3-hydroxypyridine and 2-hydroxypyridine ligands, respectively. In addi-




**MH5**, R = 2-OH
{*trans*-PdCl(NH$_3$)$_2$}$_2${*trans*-Pd(2-hydroxypyridine)}$_2${H$_2$N(CH$_2$)$_6$NH$_2$}$_2$Cl$_4$

**MH3**, R = 4-OH
{*trans*-PdCl(NH$_3$)$_2$}$_2${*trans*-Pd(4-hydroxypyridine)}$_2${H$_2$N)(CH$_2$)$_6$NH$_2$}$_2$Cl$_4$

**MH4**, R = 3-OH
{*trans*-PdCl(NH$_3$)$_2$}$_2${*trans*-Pd(3-hydroxypyridine)}$_2${H$_2$N(CH$_2$)$_6$NH$_2$}$_2$Cl$_4$

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tion to the activity, cellular uptake levels, and extent of DNA binding were determined for each complex. Our working hypothesis is that the cage effect provided by the linker diamine chains and the planaramine ligands would slow down the rates of ligand exchange sufficiently so as to bestow significant antitumour activity on the compounds. The variations in activity of the complexes with the changes in planaramine ligand may provide further information on structure–activity relationships.

## Results and Discussion

### Synthesis

Three new trinuclear palladium complexes, MH3, MH4 and MH5, were synthesized as shown in Scheme 1. The methods used were modifications of those previously described in the literature. Full details of the protocol can be found in the Experimental Section.

### Molar conductivity

The limiting molar conductivity values ($ohm^{-1}cm^2mol^{-1}$) at zero concentration ($\Lambda_0$) for MH3, MH4, MH5 and cisplatin (*cis*-dichlorodiamminoplatinum(II)) were found to be 690, 820, 730 and 270, respectively. The values for MH3, MH4 and MH5 are significantly higher than that for cisplatin.

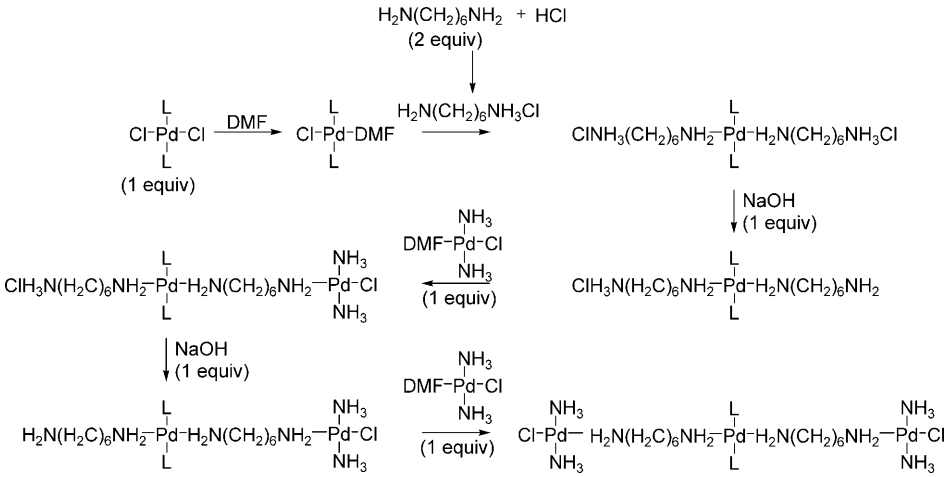
Cisplatin is a neutral molecule that produces a 1:2 electrolyte (composed of a dipositive cation and two chloride ions) only upon hydrolysis, however, MH3, MH4 and MH5 are ionic compounds, each composed of a trinuclear tetrapositive cation and 4 chloride ions. The trinuclear tetrapositive Pd–Pd–Pd cation can further ionize to produce a hexapositive trinuclear cation and two chloride ions. Comparing the limiting molar conductivities of MH3, MH4 and MH5 with those of trinuclear Pt–Pt–Pt compounds such as TH1, reported to have a molar conductivity value of 384 $ohm^{-1}cm^2mol^{-1}$,[6] it is evident that the Pd–Pd–Pd complexes have significantly larger values. The main reason for this difference is believed to be the greater reactivity of the $Pd^{2+}$ ion over the $Pt^{2+}$ ion, causing the Pd–Cl bond to be more labile than the Pt–Cl bond, and making the trinuclear Pd–Pd–Pd cation more likely to breakdown in solution than the corresponding Pt–Pt–Pt cation. Another reason for the difference may be associated with the solvent in which the compounds were dissolved. Whereas TH1 was first dissolved in minimum volumes of DMF and then diluted with mQ water, the designed complexes in this study were first dissolved in minimum volumes of DMSO and then diluted with mQ water. Since cisplatin is given as an intravenous injection, it is expected that the molecule would remain undissociated in blood serum that has a high chloride concentration. This means that cisplatin can cross the cell membrane by passive diffusion. In actual fact, the molecule is known to be transported across the cell membrane by both passive diffusion and carrier-mediated transport.[7] In contrast, MH3, MH4 and MH5 are expected to cross the cell membrane by carrier-mediated transport only as, unlike cisplatin which is expected to remain unionized in blood serum, MH3, MH4 and MH5 as ionic compounds would produce cations of at least four unit charge even in the presence of high chloride concentrations. Recent observations suggest that cisplatin can also cross the cell membrane by pinocytosis.[8] This mechanism may also apply to BBR3464 and tripalladium complexes described here. Studies on cell uptake as a function of change in concentration may provide further insight into this matter.

### Interaction of compounds with DNA

#### *ssDNA*

Figure 1 shows the electrophoretograms applying to the interaction (for 5 h at 37 °C) of salmon sperm DNA (ssDNA) with increasing concentrations of MH3, MH4, MH5 and cisplatin ranging from 2.5 to 60 μM. A single band was observed in both untreated and treated ssDNA assays. As the concentration of the drugs was increased, the intensity of the band decreased. However, there was no observable change in mobility of the band.

The decrease in intensity of the ssDNA band with the increase in concentration of the compounds indicates the occurrence of DNA damage caused by interaction of the compounds with DNA. All the tripalladium complexes are believed to bind covalently with ssDNA via the terminal metal centers forming a number of interstrand GG adducts (dictated by the sequence of nucleobases) that cause a global change in DNA conformation.[9,10] The planaramaine ligands are likely to be involved in noncovalent interactions such as hydrogen bonding




**Scheme 1.** Steps in the synthesis of MH3, MH4 and MH5 (L = planaramine ligand).

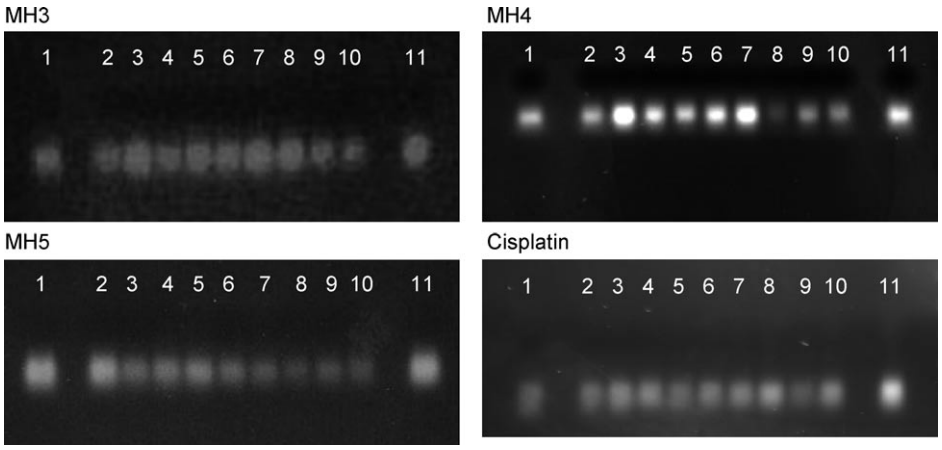



**Figure 1.** Electrophoretograms applying to the interaction for 5 h at 37 °C of ssDNA with increasing concentrations of MH3, MH4, MH5 and cisplatin. Lane 1: untreated ssDNA; lane 2: 2.5 μM; lane 3: 5 μM; lane 4: 7.5 μM; lane 5: 10 μM; lane 6: 15 μM; lane 7: 20 μM; lane 8: 30 μM; lane 9: 40 μM; lane 10: 60 μM; lane 11: untreated ssDNA.

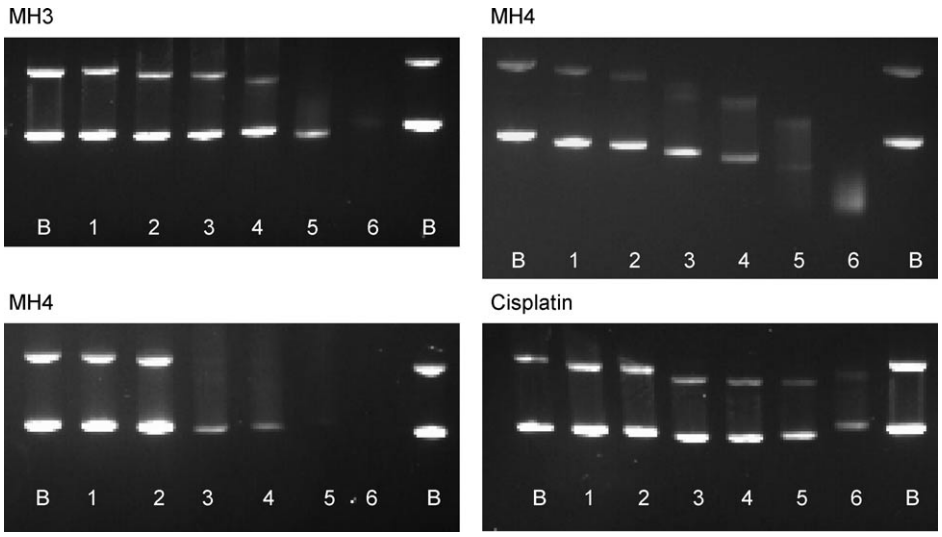



**Figure 2.** Electrophoretograms applying to the interaction of pBR322 plasmid DNA with increasing concentrations of MH3, MH4, MH5 and cisplatin. Lane B: 0 mM; lane 1: 2 mM; lane 2: 4 mM; lane 3: 8 mM; lane 4: 16 mM; lane 5: 32 mM; lane 6: 64 mM.

and stacking interactions with nucleobases in DNA. Among the tripalladium complexes, DNA damage was more significant in the case of MH5 that has two 3-hydroxypyridine ligands bound to the central palladium ion. The differences in DNA damage suggest that noncovalent interactions involving planaramine ligands may be playing a key role in inducing the DNA damage. That 2-hydroxypyridine and 3-hydroxypyridine in a *trans*-geometry are found to be more damaging to DNA than the 4-hydroxypridine ligand indicates that the position of the hydroxy group may be a key determinant of the extent of DNA damage (and also the level of activity that will be considered later), thus illustrating structure–reactivity (and structure–activity) relationships. Based on steric considerations it is reasonable to assume that the trinuclear palladium ion may be better protected from the onslaught of solvent molecules and other palladinophiles when it is bound to 2-hydropyridine and 3-hydroxypyridine than 4-hydroxypyridine in a *trans*-geometry.

***pBR322 plasmid DNA***

Figure 2 shows the electrophoretograms applying to the interaction of pBR322 plasmid DNA (for 5 h at 37 °C) with increasing concentrations of MH3, MH4, MH5 and cisplatin ranging from 0 to 64 μM. When pBR322 plasmid DNA was interacted with increasing concentrations of MH3, two distinct bands corresponding to forms I and II were observed for concentrations of the compound ranging from 2 μM to 16 μM. At the next higher concentration (32 μM), essentially one elongated band was observed with the front of the band being more intense than the tail. At 64 μM concentration, a single faint band could be seen. As the concentration of MH3 was increased, separation between forms I and II bands decreased. In the case of MH4, two bands corresponding to forms I and II could be seen at concentrations ranging from 2 to 16 μM. At the next higher concentration (32 μM), although forms I and II bands could be seen, both became less distinct. At the highest concentration (64 μM), only one coalesced band could be seen. As the concentration of MH4 was increased, the separation between forms I and II bands decreased. In the case of MH5, two distinct bands corresponding to forms I and II could be seen at 2 μM and 4 μM. At the next two higher concentrations (8 μM and 16 μM), essentially one elongated band could be seen with the front of the band being more intense. At still higher concentrations, no band could be found. In the case of cisplatin, two bands corresponding to forms I and II could be seen at all concentrations of the compound. The separation between the bands decreased as the concentration of cisplatin was increased.

Figure 3 shows the electrophoretograms applying to the interaction of pBR322 plasmid DNA with increasing concentrations (from 0 μM to 64 μM) of MH3, MH4, MH5 and cisplatin for a period of 4 h at 37 °C followed by BamH1 digestion for a further period of 1 h at the same temperature.

BamH1 is a restriction endonuclease that recognizes the sequence G/GATCC and hydrolyses the phosphodiester bond between adjacent GG sites. pBR322 plasmid DNA contains a

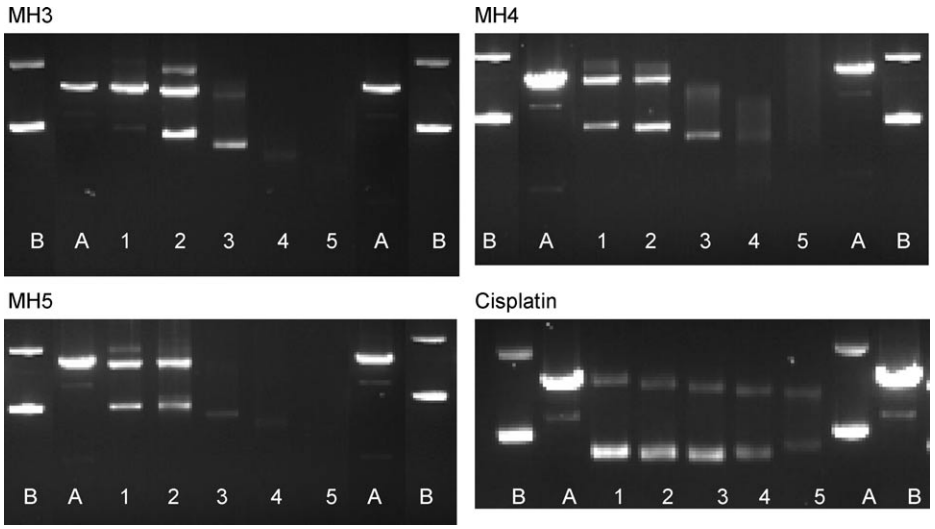



**Figure 3.** Electrophoretograms applying to the interaction of pBR322 plasmid DNA with increasing concentrations of MH3, MH4, MH5 and cisplatin followed by their digestion with BamH1. Lane A: untreated and digested pBR322 plasmid DNA; B: untreated and undigested pBR322 plasmid DNA; lane 1: 4 μM; lane 2: 8 μM; lane 3: 16 μM; lane 4: 32 μM; lane 5: 64 μM.

single restriction site for BamH1 that converts the supercoiled form I and also singly nicked circular form II into linear form III DNA. Thus, when untreated pBR322 plasmid DNA was digested with BamH1, only one band corresponding to form III was observed, whereas in the untreated and undigested pBR322 plasmid DNA, two bands corresponding to forms I and II were generally observed. Table 1 lists the bands observed after BamH1 digestion in the incubated mixtures of pBR322 plasmid DNA with varying concentrations of MH3, MH4, MH5 and cisplatin.

When pBR322 plasmid DNA was interacted with increasing concentrations of MH3 followed by BamH1 digestion, three bands corresponding to forms I, II and III were observed at concentrations 4 to 16 μM. At 32 μM, only one band corresponding to form I was observed. No bands were observed at 64 μM. In the case of MH4, three distinct bands corresponding to forms I, II and III were observed at concentrations 4 μM and 8 μM. At the next two higher concentrations (16 and 32 μM) essentially one elongated band corresponding forms I and III could be seen. No band could be seen at 64 μM. In the case of MH5, three bands corresponding to forms I, II and III were observed at concentrations 4 μM and 8 μM. At higher concentrations bands progressively became less distinct. In the case of cisplatin, two bands corresponding to forms I and II and III could be seen at all concentrations ranging from 4 μM to 64 μM.

The change in mobility of the bands is believed to be primarily due to changes in DNA conformation as a result of its interaction with the compounds. The binding of the polynuclear cations with the DNA will also serve to increase its molecular mass and reduce its overall negative charge, resulting in a decrease in the rate of migration of the DNA bands towards the positive electrode. The decrease in intensity of the DNA bands (and streaking of the bands observed in the case of MH4) indicates the occurrence of DNA damage that is also believed to be caused by the binding of the compound with the DNA. The position of the hydroxy group on the planaramine ligand appears to be a key determinant of the extent of DNA damage; 3-hydroxypyridine and 2-hydroxypyridine were found to cause greater damage than 4-hydroxypyridine. It was noted earlier that 2-hydroxypyridine and 3-hydroxypyridine ligands would provide greater protection to the metal ion from the onslaught of solvent molecules than 4-hydroxypyridine, causing the trinuclear cations of MH4 and MH5 to persist in solution more than those of MH3. However, the extent of DNA damage does not appear to be simply the result of the frequency of the long-range interstrand GG adducts, as no significant damage is observed for MH5, which has two 2-hydroxypyridine ligands bound to central palladium ion. Besides the steric effect, other noncovalent interactions, including hydrogen bonding and stacking interaction involving planaramine ligands and nucleobases in DNA, may be playing key roles in inducing both DNA damage and DNA conformational change.

**Table 1.** The bands observed after BamH1 digestion in the incubated mixtures of varying concentrations of MH3, MH4, MH5 and cisplatin, and pBR322 plasmid DNA.

| Compound | Concentration [μM] | | | | |
|---|---|---|---|---|---|
| | 4 | 8 | 16 | 32 | 64 |
| MH3 | I,II,III | I,II,III | I,II,III | I | – |
| MH4 | I,II,III | I,II,III | I, II | I,II | – |
| MH5 | I,II,III | I,II,III | I | I | – |
| Cisplatin | I,II | I,II | I,II | I,II | I,II |

Supercoiled DNA, form I; singly nicked circular DNA, form II; linear DNA, form III.

## Cytotoxicity

The cytotoxicities of MH3, MH4, MH5 and cisplatin (used as a reference) against human ovarian cancer cell lines, A2780, A2780$^{cisR}$ and A2780$^{ZD0473R}$, were determined using an MTT reduction assay (ZD0473 stands for *cis*-amminedichloro(2-methylpyridine)platinum(II)). The $IC_{50}$ values and resistance factors (RF) of MH3, MH4, MH5 and cisplatin are listed in Table 2. It can be seen that MH3, MH4 and MH5 (especially MH3 and MH5) display significant activity against the ovarian cancer cell lines, although much less than cisplatin. However, the compounds are found to have much lower resistance factors than cisplatin.

MH4 has two 3-hydroxypyridine ligands bound to central palladium ion, and was found to be the least active complex. Conversely, MH3, with two 4-hydroxypyridine ligands bound to

**Table 2.** $IC_{50}$ values and resistance factors (RF) for MH3, MH4, MH5 and cisplatin as applied to the human ovarian cancer cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$.[a]

| Compound | $IC_{50}$[b] [μM] and resistant factors A2780 | $A2780^{cisR}$ | RF | $A2780^{ZD0473R}$ | RF |
|---|---|---|---|---|---|
| MH3 | 16.5 ± 3.0 | 24.7 ± 0.4 | 1.50 | 28.9 ± 1.2 | 1.75 |
| MH4 | 32.1 ± 2.0 | 24.5 ± 2.4 | 0.76 | 24.9 ± 2.1 | 0.77 |
| MH5 | 18.2 ± 1.5 | 21.9 ± 4.5 | 1.20 | 25.1 ± 2.6 | 1.38 |
| Cisplatin | 0.6 ± 0.2 | 3.7 ± 0.4 | 6.1 | 3.6 ± 1.5 | 6.0 |

[a] The results are averages of those obtained from four identical wells with 6000–9000 cells per well. [b] The $IC_{50}$ values were obtained from the results of quadruplicate determinations of at least three independent experiments, and are given ± standard deviation (SD).

**Table 3.** Pd or Pt accumulation in A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ cells over time.[a]

| Cell line | Compounds[b] | Time 2 h | 4 h | 24 h |
|---|---|---|---|---|
| A2780 | Cisplatin (Pt) | 0.57 ± 0.04 | 0.45 ± 0.00 | 0.52 ± 0.03 |
| | MH3 (Pd) | 1.24 ± 0.15 | 1.72 ± 0.20 | 1.17 ± 0.16 |
| | MH4 (Pd) | 0.38 ± 0.02 | 0.83 ± 0.14 | 0.71 ± 0.05 |
| | MH5 (Pd) | 0.98 ± 0.07 | 0.32 ± 0.04 | 0.54 ± 0.01 |
| $A2780^{cisR}$ | Cisplatin (Pt) | 0.16 ± 0.02 | 0.40 ± 0.03 | 0.32 ± 0.03 |
| | MH3 (Pd) | 1.44 ± 0.13 | 1.80 ± 0.17 | 1.59 ± 0.30 |
| | MH4 (Pd) | 0.73 ± 0.06 | 1.13 ± 0.03 | 0.86 ± 0.03 |
| | MH5 (Pd) | 0.72 ± 0.08 | 0.27 ± 0.04 | 1.14 ± 0.10 |
| $A2780^{ZD0473R}$ | Cisplatin (Pt) | 0.19 ± 0.01 | 0.13 ± 0.01 | 0.51 ± 0.02 |
| | MH3 (Pd) | 1.48 ± 0.15 | 1.76 ± 0.14 | 0.38 ± 0.04 |
| | MH4 (Pd) | 0.83 ± 0.04 | 0.72 ± 0.03 | 0.53 ± 0.01 |
| | MH5 (Pd) | 1.45 ± 0.08 | 0.95 ± 0.04 | 1.60 ± 0.07 |

[a] Cells exposed to to 50 μM concentrations of MH3, MH4, MH5 and cisplatin for 2, 4 and 24 h. Total intracellular palladium and platinum levels expressed as the number of nanomoles of Pd or Pt per $2 \times 10^6$cells. [b] The symbol within parentheses indicates the metal to which it applies.

the central palladium ion, was the most active compound against the parent cell line A2780. While, against the resistant cell lines $A2780^{cisR}$ and $A2780^{ZD0473R}$, MH3 was found to be less active than MH5.

Whereas at the terminal positions, the stabilizing effect would be more critical (since the metal ions at the positions are more likely to be exposed to solvent molecules), it is expected to be less so at the central position (because of the stabilization already provided by the linking diamine chains) and also because Pd–N bond would be stronger than Pd–Cl bond. Indeed it has been found that among the tripalladium complexes in which the two terminal metal ions are each bound to two monohydroxypyridine ligands, the one containing 2-hydroxypyridine is most active in line with the greatest steric hindrance provided by the ligand.[11] However, as applied to the central metal ion, other factors besides steric hindrance (e.g. hydrogen bonding) may be a more critical determinant of activity. This means that the activating effect of a planaramine ligand is dependent upon position.

### Cell uptake

The cellular accumulation of palladium was used as a measure of the cell uptake of MH3, MH4 and MH5. Also the cellular accumulation of platinum was used as a measure of the cell uptake for cisplatin, which was used as a reference. Table 3 gives the total intracellular palladium and platinum levels found in the cell lines: A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ after exposure to MH3, MH4, MH5 or cisplatin over time. When the cell uptakes of palladium compounds in the three ovarian cancer cell lines are compared with those of cisplatin, trinuclear palladium compounds were frequently found to have much higher cell uptakes than cisplatin, especially at 2 h. However, the results in cell line $A2780^{ZD0473R}$ appear to be more complicated. Generally, these cells were found to contain smaller amounts of Pt in the case of cisplatin compared with Pd (trinuclear palladium compounds).

The results illustrate that tripalladium compounds like their triplatinum analogues,[12] can cross the cell membrane much faster than cisplatin. Cisplatin crosses the cell membrane by both passive diffusion and carrier-mediated transport,[13] however, positively charged trinuclear palladium ions in MH3, MH4 and MH5 (similar to triplatinum ions in BBR3464) are expected to cross the cell membrane by carrier-mediated transport only.[4,14] Among the trinuclear palladium compounds, MH3 was found to have the highest cell uptake in the ovarian cancer cell lines A2780 and $A2780^{cisR}$, in line with its higher activity, although it is the level DNA binding that is likely to be a more critical determinant of activity. Notably, in a number of platinum drugs only ~1% of the compound entering the cell actually binds with DNA.[11,15] The fraction may be even smaller in the case of palladium complexes, as they are more likely to be deactivated before binding with DNA.

### DNA binding

Table 4 gives the levels of palladium–DNA binding for MH3, MH4, MH5 and platinum–DNA binding for cisplatin in A2780, $A2780^{cisR}$ and $A2780^{ZD0473R}$ cells over time. It can be seen that, for the three cell lines, the lowest level of metal–DNA binding applies to cisplatin at 2, 4 and 24 h. When the levels of Pt–DNA binding of cisplatin are compared with the levels of Pd–DNA binding of tripalladium complexes, it is found that the Pt–DNA binding levels are much lower than the Pd–DNA binding levels even though cisplatin is much more active than all the tripalladium complexes.

Whereas cisplatin is expected to form mainly intrastrand Pt(GG) and Pt(AG) adducts that cause local bending of a DNA strand, trinuclear Pd cations (like their platinum analogues, such as BBR3464 and TH1) are expected to form a plethora of long-range interstrand GG adducts. However, the Pd–DNA adducts, being much more labile than the corresponding Pt–DNA adducts, may not persist long enough in solution to translate into significant antitumour activity. In fact, one of the disadvantages of even the trinuclear platinum drugs, such as BBR3464, is their progressive biotransformation and degradation in solution in the cellular matrix.[16]

**Table 4.** Levels of Pd binding with DNA in A2780, A2780$^{cisR}$ and A2780$^{ZD0473R}$ cells over time.[a]

| Cell line | Compounds[b] | Time | | |
|---|---|---|---|---|
| | | 2 h | 4 h | 24 h |
| A2780 | Cisplatin (Pt) | 16.2 ± 3.0 | 13.3 ± 1.4 | 12.0 ± 3.1 |
| | MH3 (Pd) | 85.3 ± 3.9 | 27.4 ± 1.0 | 32.2 ± 0.8 |
| | MH4 (Pd) | 68.2 ± 2.4 | 46.6 ± 2.9 | 39.3 ± 4.2 |
| | MH5 (Pd) | 44.0 ± 2.4 | 33.5 ± 1.6 | 38.2 ± 2.0 |
| A2780$^{cisR}$ | Cisplatin (Pt) | 15.4 ± 1.4 | 13.1 ± 1.2 | 12.1 ± 4.5 |
| | MH3 (Pd) | 77.9 ± 2.4 | 29.9 ± 2.5 | 79.2 ± 3.8 |
| | MH4 (Pd) | 41.5 ± 7.5 | 120.4 ± 1.2 | 50.8 ± 0.2 |
| | MH5 (Pd) | 36.3 ± 3.2 | 45.7 ± 2.5 | 29.3 ± 0.7 |
| A2780$^{ZD0473R}$ | Cisplatin (Pt) | 12.0 ± 3.1 | 15.0 ± 2.4 | 14.4 ± 0.1 |
| | MH3 (Pd) | 33.7 ± 23 | 47.6 ± 3.5 | 63.4 ± 0.1 |
| | MH4 (Pd) | 60.7 ± 2.0 | 67.9 ± 2.0 | 49.3 ± 0.5 |
| | MH5 (Pd) | 53.2 ± 2.5 | 62.8 ± 2.0 | 73.1 ± 1.9 |

[a] Number of nanomoles of Pd or Pt per mg DNA. [b] The symbol within parentheses indicates the metal to which it applies.

Higher levels of binding do not necessarily result in higher activity, as can be seen for MH6 in A2780, A2780$^{cisR}$ and A2780$^{ZD0473R}$ cell lines at 24 h. The lowest level of Pd–DNA binding for MH6 was observed in the A2780$^{cisR}$ cell line, although it is the most active against this cell line. The above results indicate that no clear conclusions regarding activity of the designed tripalladium complexes can be made—perhaps because of their high reactivity and hence low biological half-life—except to say that the presence of 2-hydroxypyridine ligand at the terminal positions serves to enhance activity by providing protection to the trinuclear cation from the onslaught of solvent molecules. However, as applied to the central position, 4-hydroxypyridine is found to be slightly more activating than 2-hydroxypyridine and 3-hydroxypyridine at least in the parent cell line. This means that the tripalladium complex in which each of the two terminal palladium ions is bound to two 2-hydroxypyridine ligands and the central metal ion is bound to two 4-hydroxypyridine ligands may be significantly more active. As noted earlier, another tripalladium complex in which each of the three palladium ions is bound to two 2-hydroxypyridine ligands may also be significantly more active. However, this remains to be confirmed as the suggested compounds have not yet been prepared.

Generally, comparing the levels of Pd–DNA binding of tripalladium complexes with the corresponding values for cisplatin at 2 and 24 h, the values at 24 h were lower than those at 2 h for the tripalladium complexes, however, the converse was observed for cisplatin. The results indicate that the tripalladium complexes bind with DNA much faster than cisplatin. Triplatinum complexes, such as BBR3464, TH1 and CH9, have also been reported to bind faster with cellular DNA than cisplatin.[6,10,17] The decrease in the level of Pd–DNA binding with an increase in time may indicate the occurrence of increased DNA repair. It is reasonable to assume that the Pd–DNA adducts may be mended by excision repair, which is known to be one of the dominant mechanisms of resistance applying to the platinum-based anticancer drugs.[18]

The levels of Pd–DNA binding are found to vary significantly among the trinuclear compounds. At 2 h, the highest levels of metal–DNA binding apply to MH6 in A2780 and A2780$^{cisR}$ cell lines, whereas in A2780$^{ZD0473R}$ the highest level applies to MH4. After 4 h, the highest levels of metal–DNA binding apply to MH8 in A2780 and A2780$^{cisR}$ cell lines, whereas in A2780$^{ZD0473R}$ the highest level applies to MH4, as was the case after 2 h. At 24 h, the highest level of metal–DNA binding applies to MH6 in A2780 cell lines, to MH3 in A2780$^{cisR}$ cell line and to MH8 in A2780$^{ZD0473R}$ cell line.

## Conclusions

Although palladium compounds are much more reactive than the corresponding platinum compounds making them generally therapeutically inactive but toxic, the present study shows that significant antitumor activity can be introduced in trinuclear palladium complexes when their reactivity is lowered by the presence of sterically hindered ligands such as 2-hydroxypyridine, 3-hydroxypyridine and 4-hydroxypyridine bound to the central palladium ion. When bound to the central metal ion, 4-hydroxypyridine is more activating than 2-hydroxypyridine and 3-hydroxypyridine, suggesting that noncovalent interactions, such as hydrogen bonding and ionization of planaramine ligands, may also be a key determinant of antitumour activity in addition to steric effects.

## Experimental Section

Potassium tetrachloropalladate ($K_2[PdCl_4]$), *N,N′*-dimethylformamide (DMF), $(CH_3)_2NCHO$, 2-hydroxypyridine, 3-hydroxypyridine, 4-hydroxypyridine, cisplatin and 1,6-diaminohexane were obtained from Sigma Chemical Company (St. Louis, USA). Acetone and silver nitrate ($AgNO_3$) were obtained from Ajax Chemicals (Auburn, Australia). Methanol, ethanol and dichloromethane were obtained from Merck Pty Ltd (Kilsyth, 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 pellets was obtained from Astral Scientific (Australia). In electrophoresis, KODAK Gel Logic 100 imaging system (GL 100) was used for taking gel pictures, and images were analyzed using Kodak molecular imaging software (KODAK MI software)

### Chemistry

#### *Synthesis*

Tripalladium compounds MH3, MH4 and MH5 were prepared using the step-up method of synthesis, branching out from the central palladium unit. TH5, TH6 and TH7 served as the central units for MH3, MH4 and MH5, and are respectively: bis(3-hydroxypyridine)-dichloropalladium(II), bis(2-hydroxypyridine)dichloropalladium(II) and bis(4-hydroxypyridine)dichloropalladium(II). TH5, TH6 and TH7 were prepared according to previously described procedures.[1] Briefly, $K_2[PdCl_4]$ (1 mmol, 0.326 g) was dissolved in milliQ water (7.5 mL) and treated with concentrated HCl (0.25 mL). The solution was warmed to 50 °C for 15 min and further treated slowly with a solution of the appropriate planaramine ligand (10 mmol, 0.951 g)

(3-hydroxypyridine in the case of TH5, 2-hydroxypyridine in the case of TH6 and 4-hydroxypyridine in the case of TH7) in DMF (2 mL). After stirring for 30 min at 50 °C, HCl (6 M, 40 mL) was added to the reaction, which was then refluxed for 5 h. The reaction was cooled to room temperature and then cooled in an ice bath for 1 h resulting in the formation of yellow precipitate that was collected by vacuum filtration, washed with mQ water and ethanol, and air dried to give TH5 (0.252 g, 69%), TH6 (0.271 g, 74%) or TH7 (289 g, 79%). TH5, TH6 and TH7 were characterized by microanalysis and spectral studies.

Transpalladin–DMF filtrate (obtained by replacement of chloro ligands of transpalladin by DMF), required for the synthesis of MH3, MH4 and MH5, was prepared as follows. Transpalladin (1.0 mmol, 0.212 g) was dissolved in DMF (16 mL) and treated with a solution of $AgNO_3$ (0.1664 g, 0.98 mmol) in mQ water (4 mL). The mixture was stirred in the dark for 20 h at room temperature. It was then centrifuged at 5500 rpm for 10 min to remove AgCl. The supernatant (transpalladin–DMF filtrate) was collected by vacuum filtration and refrigerated until used. TH5, TH6 or TH7 (0.25 mmol, 0.091 g) was dissolved in DMF (3 mL) by stirring at 30–40 °C for ~30 min. Meanwhile, a solution of 1,6-diaminohexane (0.5 mmol, 0.058 g) in mQ water (1.5 mL) was acidified with HCl (1 M, 500 μL) and stirred at 40 °C for 15 min. This solution was then added to the freshly prepared (within 30 min) solution of TH5, TH6 or TH7 at 40 °C resulting in the formation of a yellow precipitate. The mixture was stirred at 40 °C for 15 min, then treated with aq NaOH (1 M, 250 μL) and stirred for a further 5 min at 40 °C. Transpalladin–DMF filtrate (0.25 mmol) was prepared as described previously and added dropwise to the mixture followed by stirring for 5 min at 40 °C. Further aq NaOH (1 M, 250 μL) was added to the mixture and stirred for another 5 min at 35 °C. After dropwise addition of further transpalladin–DMF filtrate (0.25 mmol) at 40 °C, the mixture was stirred at 40 °C for 3 h. The solution was filtered, and the volume of filtrate reduced to 3 mL using a vacuum concentrator (Javac DD150 Double stage High Vacuum Pump, Savant RVT 4104 Refrigerated Vapor Trap and Savant Speed Vac 110 Concentrator). $CH_2Cl_2$ (20 mL) was added and the mixture was placed in ice for 2 h resulting in the formation of yellow precipitate of MH3, MH4 or MH5. The precipitate was collected by vacuum filtration, washed with ice-cold mQ water and methanol, and air dried at room temperature over a period of 24 h. Repeated dissolution in DMF and precipitation by adding $CH_2Cl_2$ was carried out to improve the purity. The steps in synthesis are shown in Scheme 1.

### *Characterization*

Microanalysis: C, H, N and Cl were determined using microanalysis facility available at the Australian National University. A Carlo Erba 1106 automatic analyzer was used for the determination of C, H and N, and Cl was determined by titration with standardized mercuric nitrate. Palladium was determined by graphite furnace atomic absorption spectroscopy (AAS). Infrared, mass and $^1$H NMR spectra were used to aid structural characterization of MH3, MH4 and MH5. Infrared spectra were recorded using a Bruker IFS66 spectrometer. To obtain mass spectra of MH3, MH4 and MH5, solutions of compounds were made in 10% DMF and 90% methanol and then sprayed into a Finnigan LCQ ion trap mass spectrometer available in the School of Chemistry, The University of Sydney. The flow rate was 0.2 mL min$^{-1}$ consisting of 50% methanol and 50% water. The $^1$H NMR spectra of MH3, MH4 and MH5 dissolved in deuterated DMSO were recorded in a Bruker DPX400 spectrometer using a 5 mm high precision Wilmad NMR tube at 300 K (±1 K). To determine molar conductivity, 1 mM solutions of MH3, MH4 and MH5 in 1:1 mixture of DMSO and mQ water were progressively diluted with mQ water to give concentrations ranging from 0.5 μM to 1.0 mM. The conductivity values were measured using a PW9506 digital conductivity meter. Molar conductivity values were plotted against concentration to obtain the value at zero concentration.[19]

**MH3**: $^1$H NMR (400 MHz, [D$_6$]DMSO): $\delta = 11.4$ (br s, due to OH); 9.76 (t, due to CH ortho); 7.26 (s, due to CH meta); 4.82 (t, due to $NH_2$); 2.45 (t, due to DMSO); 2.35 (t, due to NH); 1.67 (d, due to $CH_2$); 1.62 (q, due to $CH_2$); 1.37 (t, due to $CH_2$); 1.30 (s, due to $CH_2$); 1.25 (s, due to $CH_2$); 0.88 (t, due to $CH_2$); IR (KBr): $\tilde{\nu} = 3268$, 3216, 2854, 1616, 1585, 1509, 1449, 1384, 536 cm$^{-1}$; MS (ESI) *m/z*: 899 = [{NH2-(CH2)$_6$NH$_2$}Pd(C$_5$H$_5$NO){NH$_2$(CH$_2$)$_6$NH$_2$}PdNH$_3$Cl$_3$−H]$^+$; 844 = [Pd-{(NH$_3$)$_2$}{NH$_2$(CH$_2$)$_6$NH$_2$}Pd(C$_5$H$_5$NO)$_2$}{NH$_2$(CH$_2$)$_6$NH$_2$}Pd{(NH$_3$)$_2$}Cl−H]; 727 = Pd{(NH$_3$)$_2$}{NH$_2$(CH$_2$)$_6$NH$_2$Pd{NH$_2$(CH$_2$)$_6$NH$_2$Pd{(NH$_3$)$_2$Cl$_3$; Anal. calcd for $C_{22}H_{54}Cl_6N_{10}O_2Pd_3$ (%): C 25.8, H 5.3, N 13.7, Cl 20.8, Pd 31.2, found: C 25.5 ± 0.4, H 5.3 ± 0.4, N 13.5 ± 0.4, Cl 20.4 ± 0.4, Pd 31.1 ± 1.0.

**MH4**: $^1$H NMR (400 MHz, [D$_6$]DMSO): $\delta = 8.48$ (s, due to OH); 8.33 (q, CH ortho); 8.04 (s, due to CH ortho); 7.48 (q, CH meta); 7.42 (d, CH para); 3.53 (s, due to $NH_3$); 2.93 (p, due to $CH_2$); 2.76 (p, due to $CH_2$); 2.65 (br, due to $CH_2$); 1.4 (br, due to $CH_2$); IR (KBr): $\tilde{\nu} = 3268$, 2923, 1585, 1385, 1220, 574 cm$^{-1}$; MS (ESI) *m/z*: 1024 = [Pd-(C$_5$H$_5$NO)$_2$}{NH$_2$(CH$_2$)$_6$NH$_2$}+H], 847 = [Pd{(NH$_3$)$_2$}{NH$_2$(CH$_2$)$_6$NH$_2$}Pd-$_3$)$_2$}{NH$_2$(CH$_2$)$_6$NH$_2$}Pd(C$_5$H$_5$NO)$_2$}{NH$_2$(CH$_2$)$_6$NH$_2$}Pd{(NH$_3$)$_2$}Cl+2H], 727 = [Pd{(NH$_3$)$_2$}{NH$_2$(CH$_2$)$_6$NH$_2$Pd{NH$_2$(CH$_2$)$_6$ NH$_2$Pd{(NH$_3$)$_2$Cl$_3$], 550 = [Pd{NH$_2$(CH$_2$)$_6$ NH$_2$}Pd{NH$_2$(CH$_2$)$_6$NH$_2$}Pd−2H]; Anal. calcd for $C_{22}H_{54}Cl_6N_{10}O_2Pd_3$ (%): C 25.8, H 5.3, N 13.7, Cl 20.8, Pd 31.2, found: C 25.7 ± 0.4, H 5.1 ± 0.4, N 13.4 ± 0.4, Cl 20.5 ± 0.4, Pd 30.6 ± 1.0.

**MH5**: $^1$H NMR (400 MHz, [D$_6$]DMSO): $\delta = 11.6$ (s, due to OH), 7.94 (s, due to CH ortho); 7.57 (d, due to CH meta); 7.36 (t, due to CH meta), 6.32 (d, due to CH para); 5.96 (d, due to NH); 3.51 (t, due to $NH_3$); 2.97 (s, due to $NH_2$); 2.88 (s, due to $NH_2$); 2.72 (s, due to $CH_2$); 2.58 (s, due to DMSO); 2.39 (s, due to $CH_2$); 2.22 (t, due to $CH_2$); 1.56 (t, due to $CH_2$); 1.26 ppm (t, due to $CH_2$); IR (KBr): $\tilde{\nu} =$ 3236, 3136, 2929, 2855, 1655, 1610, 1491, 1438, 1384, 1280, 1151, 1114, 582, 531, 491 cm$^{-1}$; MS (ESI) *m/z*: 844 = [Pd{(NH$_3$)$_2$}{NH$_2$-$_3$)$_2$}{NH$_2$(CH$_2$)$_6$NH$_2$}Pd(C$_5$H$_5$NO)$_2$}{NH$_2$(CH$_2$)$_6$NH$_2$}Pd{(NH$_3$)$_2$}Cl−H], 790 = [Cl$_3$Pd{NH$_2$(CH$_2$)$_6$NH2}Pd(C$_5$H$_5$NO){NH$_2$(CH$_2$)$_6$NH$_2$}Pd{(NH$_3$)$_2$}+2H], 729 = [Pd{(NH$_3$)$_2$}{NH$_2$(CH$_2$)$_6$NH$_2$}Pd{NH$_2$(CH$_2$)$_6$NH$_2$}(NH$_3$)$_2$Cl$_3$+2H], 551 = [Pd{NH$_2$(CH$_2$)$_6$NH$_2$}Pd{NH$_2$(CH$_2$)$_6$NH$_2$}Pd−H]; Anal. calcd for $C_{22}H_{54}Cl_6N_{10}O_2Pd_3$ (%): C 25.8, H 5.3, N 13.7, Cl 20.8, Pd 31.2, found: C 25.4 ± 0.4, H 4.2 ± 0.4, N 13.3 ± 0.4, Cl 20.6 ± 0.4, Pd 31.1 ± 1.0.

## Biology

### Interaction with DNA

The interaction of MH3, MH4 and MH5 with salmon sperm DNA (ssDNA) and pBR322 plasmid DNA was studied using gel electrophoresis.[20] Further experiments in which pBR322 plasmid DNA was first interacted with varying concentrations of compounds followed by BamH1 digestion were carried out to gain information on change in DNA conformation. The amount of DNA was kept constant while the concentrations of compounds were varied.

### *ssDNA*

Stock solutions of ssDNA prepared by dissolving 10–15 mg of ssDNA in 10 mL of 0.05 M Trizma buffer at pH 8 to give DNA con-

centrations ranging from 1 mg mL$^{-1}$ to 1.5 mg mL$^{-1}$ and stored at −17 °C until needed. Varying amounts of MH3, MH4, MH5, and cisplatin in solution were added to 4 μL aliquots of ssDNA solution (at 1.5 mg mL$^{-1}$) and the total volume was made up to 20 μL by adding mQ water so that the concentrations of compounds ranged from 1.25 μM to 60 μM. A DNA blank was prepared by adding 16 μL mQ water to 4 μL of ssDNA. The mixtures were incubated in a shaking water bath at 37 °C for 4 h following which 16 μL aliquots of drug–DNA mixtures were loaded onto the 1% agarose gel and electrophoresis was run in TAE buffer for 2 h at 80 V cm$^{-1}$ at room temperature.

#### *pBR322 plasmid DNA*

Varying amounts of compound in solution were added to exactly 1.5 μL of supplied pBR322 plasmid DNA in solution to give concentrations ranging from 1.25 μM to 60 μM. The total volume was made up to 20 μL with 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 which the reaction was quenched by rapid cooling to 0 °C for 20 min. The samples were thawed and mixed with 4 μL of marker dye (0.25% bromophenol blue and 40% of sucrose). Each sample (17 μL) 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.[21] Electrophoresis was carried out in TAE buffer containing ethidium bromide at 80 V for 3 h at room temperature.

#### *BamH1 digestion*

A set of drug–DNA mixtures, prepared as 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 mixture were added 3 μL of 10× digestion buffer SB followed by 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, visualized under UV light and photographed as described previously.

#### Cytotoxicity assay

Cytotoxicity of MH3, MH4 and MH5 against human ovarian cancer cell lines: A2780, A2780$^{cisR}$ and A2780$^{ZD0473R}$ along with that for cisplatin (that served as the reference) was determined using MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] reduction assay.[23,24] The method used for cell subculture was a modification of that described by Freshney et al.,[23] 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.[9] 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% sodium bicarbonate, and 2 mM glutamine without antibiotics), depending on the growth characteristics of the cell line, were seeded into flat-bottomed 96-well culture plates in 10% FCS/RPMI 1640 culture medium. The plate was incubated for 24 h at 37 °C in a humidified atmosphere to allow cells to attach. MH3, MH4, MH5 and cisplatin were first dissolved in a minimum amount of DMF and then diluted to the required concentrations with mQ water and finally filtered to sterilize. A serial five-fold dilutions of the drugs (ranging from 0.004 μM to 40 μM) in 10% FCS/RPMI 1640 medium were added to equal volumes of the cell culture in quadruplicate wells that were then left to incubate under normal growth conditions for 72 h. The inhibition of the cell growth was determined using the MTT reduction assay. Four hours after the addition of the MTT solution (50 μL per well of 1 mg mL$^{-1}$ MTT solution), the yellow formazan crystals produced from the reduction of MTT were dissolved in 150 μL of DMSO and read with a Bio-Rad Model 3550 Microplate Reader. The $IC_{50}$ values were obtained from the results of quadruplicate determinations of at least three independent experiments.

#### Drug uptake and binding with DNA

MH3, MH4, MH5 and cisplatin at 50 μM final concentration were added to culture plates containing exponentially growing A2780, A2780$^{cisR}$ and A2780$^{ZD0473R}$ cells in 10 mL 10% FCS/RPMI 1640 culture medium (cell density = $1\times10^6$ cells mL$^{-1}$). The cells containing the drugs were incubated for 2, 4, 24 and 72 h, at the end of which cell monolayers were trypsinized and cell suspensions (10 mL) were transferred to centrifuge tube and spun at 3500 rpm for 2 min at 4 °C. The experiment was carried out for both TH8 and cisplatin. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and the pellets were stored at −20 °C until assayed. At least three independent experiments were performed.

#### Drug accumulation in cells

Following drug incubation the cell pellets were suspended in 0.5 mL of 1% triton-X, held on ice then sonicated. Total intracellular platinum contents were determined by graphite furnace AAS. To determine platinum–DNA binding levels, high-molecular weight DNA from a cell pellet was isolated using JETQUICK Blood DNA Spin Kit/50 according to the modified protocol of Bowtell et al.[25] Platinum content 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.[26]

## Abbreviations

Cisplatin: *cis*-dichlorodiamminplatinum(II). ZD0473: *cis*-amminedichloro(2-methylpyridine)platinum(II). MH3: {*trans*-PdCl$(NH_3)_2$}$_2${*trans*-Pd(3-hydroxypyridine)$_2${$H_2N(CH_2)_6NH_2$}$_2Cl_4$. MH4: {*trans*-PdCl$(NH_3)_2$}$_2$-{*trans*-Pd(2-hydroxypyridine)$_2${$H_2N(CH_2)_6NH_2$}$_2Cl_4$. MH5: {*trans*-PdCl$(NH_3)_2$}$_2${*trans*-Pd(4-hydroxypyridine)$_2${$H_2N(CH_2)_6NH_2$}$_2Cl_4$. MH5: [bis(4-hydroxypyridine)dichloropalladium(II)]. TH5: bis[3-hydroxypyridine]dichloropalladium(II). TH6: bis[2-hydroxypyridine]dichloropalladium(II). TH7: bis[4-hydroxypyridine]dichloropalladium(II). EDTA: Ethylene diamine tetraacetic acid. Tris: Tris-hydroxymethylaminomethane. Tris-HCl: Tris-hydroxymethylaminomethane hydrochloride. Hepes: (*N*-[2-Hydroxymethyl]piperazine-*N*′-[2-ethanesulfonic acid]. 1xTAE buffer: 0.05 M Tris base + 0.05 M glacial acetic acid + 1 mM EDTA, pH 8.0. MTT: 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide.

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