Novel platinum(II)-based anticancer complexes and molecular hosts as their drug delivery vehicles†‡

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Platinum(II)-based DNA intercalators where the intercalating ligand is 1,10-phenanthroline or a phenanthroline derivative and where the ancillary ligand is either achiral (*e.g.* ethylenediamine) or chiral (*e.g.* diaminocyclohexane) show a range of cytotoxicities with a defined structure–activity relationship. The most cytotoxic are those that contain methylated-phenanthroline ligands and 1*S*,2*S*-diaminocyclohexane (*S*,*S*-dach) as the ancillary ligand. We have developed a new purification method using Sep-Pak® C-18 reverse phase columns, which means these metal complexes can be made faster and cheaper compared to published methods. Platinum(II)-based complexes containing imidazole, pyrrole and β-alanine subunits, that are capable of recognising specific DNA base-pair sequences have also been synthesised. These include linear or hairpin polyamide ligands that can recognise DNA sequences up to seven base-pairs in length and contain single platinum centres capable of forming monofunctional adducts with DNA. We have now synthesised and characterised, by ^{1}H and ^{195}Pt NMR, ESI-MS and elemental analysis, the first *dinuclear* platinum(II) DNA sequence selective agent. Finally, using ^{1}H NMR we have examined the encapsulation of our platinum(II)-based DNA intercalators by cucurbit[6]uril (CB[6]). Encapsulation by CB[6] was found to not significantly change the cytotoxicity of five platinum(II)-based DNA intercalators, indicating it may have utility as a molecular carrier for improved drug delivery.

Introduction

Cisplatin, carboplatin and oxaliplatin (Fig. 1) are used to treat a narrow variety of human cancers, particularly: head, neck, ovarian, testicular, lung and colorectal cancers.[1] Carboplatin is generally used where treatment by cisplatin is tolerated poorly, whereas oxaliplatin is used to treat specific cisplatin-resistant cancers.[2]

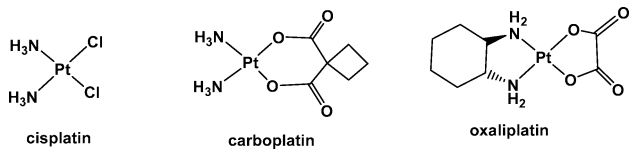

Fig. 1 The chemical structures of the clinical agents cisplatin, carboplatin and oxaliplatin.

Unfortunately, cisplatin is restricted in use by high dose limiting side effects and the intrinsic or acquired resistance cancers may have to treatment.[3] Resistance to cisplatin and similarly to carboplatin, can result from several mechanisms, and includes: decreased cellular uptake/increased drug efflux, increased drug deactivation arising from increased intracellular glutathione concentration, and enhanced DNA adduct repair/tolerance.[3] More effective drugs can be developed by (1) lowering the toxic side-effects by decreasing their reactivity with non-DNA biological nucleophiles, (2) the use of drug delivery vehicles to target specifically cancerous cells, leaving normal cells unharmed, (3) increasing the efficiency of DNA binding inside the cell, and (4) design of drugs that form novel adducts with DNA, different to those of cisplatin, which are still able to prevent DNA transcription and replication, but are not recognised and removed by DNA repair proteins.[4–11]

To this end, our group has been investigating new drugs as well as macrocyclic compounds capable of acting as molecular hosts for use as drug delivery vehicles. This includes the synthesis of platinum(II)-based DNA intercalators;[12–18] that act as agents capable of intercalating into the DNA double helix rather than forming coordinate covalent adducts with DNA, as cisplatin does. We are also synthesising platinum(II) complexes which are able to bind to specific DNA base-pair sequences, for increased DNA binding affinity.[19–21] Finally, we have examined cucurbit[*n*]urils (CB[*n*], where $n = 6$, 7 or 8) as molecular hosts for our platinum(II)-based DNA intercalator agents.[22] In the interest of driving an informative Dalton Discussion, in this paper we discuss our past research work as a foundation for the reporting of our most recent advances in our drug design and synthesis, and the ability of CB[6] to act as a molecular host for improved delivery of our platinum DNA intercalators. We also discuss where we believe platinum(II)-based drug design and delivery is heading and our plans for the future.

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Experimental

Materials and general methods

Fmoc–lysine–(Fmoc)–OH was purchased from Auspep, Australia. β-Alanine and γ-buytric acid were purchased from Fluka. 1*S*,2*S*-Diaminocyclohexane, 1,10-phenanthroline, 3,4,7,8-tetramethyl-1,10-phenanthroline, 5,6-dimethyl-1,10-phenanthroline and 4,7-dimethyl-1,10-phenanthroline were purchased from the Aldrich Chemical Company. Sep-Pak® (2 g) C-18 reverse phase columns were purchased from Waters. Dowex® anion exchange resin and 5% foetal bovine serum were obtained from Sigma-Aldrich. Potassium tetrachloroplatinate(II) was purchased from Precious Metals Online. Dimethyl sulfoxide (D, 99.9%), dimethylformamide (D, 99.9%) and deuterium oxide (D, 99.9%) were purchased from Cambridge Isotope Laboratories. Cucurbit[6]uril was made as previously described.[23] The heterocyclic 1-methylimidazole and 1-methylpyrrole rings for the polyamide were made as previously described.[19,21]

ESI-MS

Positive ion ESI mass spectra were acquired using a Micromass (Wyntheshawe, UK) Quattro Micro™ spectrometer equipped with a Z-spray probe. Solutions containing concentrations ranging between 10 and 50 μM were injected into the instruments at a flow rate of 10 μL min^{-1}. The source and desolvation temperatures were 150 and 120 °C, respectively. The capillary tip potential and cone voltage were 2500 and 50 V, respectively. Between 10 and 50 acquisitions were summed to obtain spectra, which were calibrated against a standard CsI solution (750 mM) over the same m/z range.

Cytotoxicity assays

Growth inhibition cytotoxicity experiments were performed as previously described.[24]

NMR

NMR spectra were obtained on a 300 MHz Varian Mercury or a 400 MHz Bruker Avance spectrometer, in d_6-DMSO, d_7-DMF or D_2O, referenced internally to the solvent. NMR were run at 35 °C for DMSO and 25 °C for D_2O/DMF unless otherwise stated. ^{195}Pt NMR were externally referenced to K_2PtCl_4 (−1631 ppm; D_2O, 25 °C, $\Xi = 21.4$ MHz) at an operating frequency of 85.88 MHz.[25,26] For one-dimensional spectra, a spectral width of 5000 Hz was used with 50 000 data points, a d1 of 0.5 s and an acquisition time of 3 s.

Improved synthesis of platinum(II)-based DNA intercalators

K_2PtCl_4 (~0.1–1.0 g) and the ancillary ligand (1 mol. equiv.) are stirred together at r.t. in water (100 mL). After stirring for 48 h, the resultant yellow precipitate is collected by filtration and washed with water, ethanol and diethyl ether. The metal complex [Pt(ancillary ligand)Cl_2] (~0.1–1.0 g) is then refluxed in water (~100–500 mL) with the intercalating ligand (1.1 mol. equiv.) for at least 24 h. The clear, yellow solution is filtered then concentrated by rotary evaporation to ~20–50 mL. A Waters (2 g) C18-reverse phase Sep-Pak® is activated with methanol (10 mL) and washed with water (20 mL) before the metal complex solution is loaded onto the column and eluted with water (~20–30 mL) as a yellow band, leaving an orange-black band on the head of the column. The column is then washed with 90% CH_3CN–9% H_2O–1% trifluoroacetic acid, eluting another yellow band, which is rotary evaporated to dryness, dissolved in a minimum amount of water and eluted with water on fresh Sep-Pak®, leaving an orange band on the head of the column. The two water fractions are combined and then either lyophilised or rotary evaporated to dryness (yield 80–90%).

Solid phase synthesis

Machine assisted synthesis was performed on a Symphony Quartet 3.21 protein synthesiser on a 0.28 mmol scale, as previously described.[21] Each cycle of monomer addition involved a dichloromethane (DCM) wash, a dimethylformamide (DMF) wash, deprotection with 20% piperidine–DMF for 3 min, draining the reaction vessel, a DMF wash, deprotection for 17 min, a DCM wash, 2 × DMF washes, draining the reaction vessel, coupling for 3.5 h (10 h when coupling to an imidazole ring), coupling for 1 h, and finally, draining the reaction vessel. Activated transplatin was coupled to the polyamide in the presence of triethylamine (TEA, 0.27 mL, 2.0 mmol) for 15 h in the dark, after which the resin was washed with 1 × DMF, 2 × brine, 2 × water, 2 × DMF, 3 × DCM and dried for 1 h. The activated acids were added manually to the reaction vessel at the end of every deprotection cycle. The cycle was interrupted, the reaction vessel vented, and the activated acid added before the cycle was resumed.

Activation of Fmoc–lysine–(Fmoc)–OH

Fmoc–lysine–(Fmoc)–OH (0.37 g, 0.62 mmol) was dissolved in DMF (7 mL). *o*-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (0.21 g, 0.56 mmol) was added followed by diisopropylethylamine (0.31 mL, 1.8 mmol) and the solution stirred for 10 min.

Synthesis of DNHLSP-6

The metal complex was prepared by machine-assisted synthesis protocols. Once synthesised, it was cleaved from the resin by addition of a solution of DCM (10 mL), trifluoroethanol (TFE, 2.4 mL) and acetic acid (1.2 mL) before it was shaken very gently for 1.5 h. The resin was removed by filtration and washed with TFE–DCM (1 : 4, 6 mL) before it was subjected to the cleaving conditions twice more. The yellow-brown filtrate was collected and the solvent was reduced in volume under pressure to ~2 mL. Cold diethyl ether (4 mL) was added and the mixture left in the fridge for 1.5 h to precipitate a yellow solid. The solvent was decanted and water (3 mL) was added to the solid before the solution was lyophilised to yield **DNHLSP-6** (0.27 g, 77%) as a brown-yellow solid. Anal. calc. for $C_{46}H_{71}Cl_4N_{23}O_{10}Pt_2 \cdot 5H_2O$: C, 31.97; H, 4.72, N, 18.64%. Found: C, 31.65; H, 4.65; N, 18.97%. ^{1}H NMR 300 MHz (d_6-DMSO): δ 10.80 (s, 1H), 9.83 (s, 1H), 9.81 (s, 1H), 9.76 (s, 1H), 9.66 (s, 1H), 9.54 (s, 1H), 8.21 (t, 1H, $J = 5.8$ Hz), 7.96 (t, 1H, $J = 5.4$ Hz), 7.64 (s, 1H), 7.62 (s, 1H), 7.51 (s, 2H), 7.21 (d, 1H, $J = 1.4$ Hz), 7.17 (d, 1H, $J = 1.4$ Hz), 7.15 (d, 1H, $J = 1.4$ Hz), 7.01 (d, 1H, $J = 1.4$ Hz), 6.85 (d, 1H, $J = 1.4$ Hz), 6.83 (d, 1H, $J = 1.5$ Hz), 5.79 (t, 2H), 5.62 (t, 2H), 4.03 (s, 3H), 4.00 (s, 3H), 3.96 (s, 3H), 3.84 (s, 3H), 3.83 (s, 3H), 3.80 (s, 3H),

3.33 (m, 6H), 2.47 (t, 2H, $J = 7.1$ Hz), 2.38 (t, 2H, $J = 7.5$ Hz), 2.29 (t, 2H, $J = 7.4$ Hz), 1.82 (m, 4H, $J = 7.1$ Hz), 1.58 (bm, 2H). ^{195}Pt NMR 85 MHz (d_7-DMF): δ −2424 (bs), −2426 (sh).

Encapsulation of platinum(II)-based DNA intercalators in CB[6]

The metal complexes **56MESS**, **47MESS**, **3478MESS**, **5MESS** and **PHENSS** (0.01–0.03 g) were added to CB[6] (1 mol. equiv.) and NaCl (15 to 30 mol. equiv.) in water (~200 mL) and heated (60–70 °C) until the solution went clear, colourless. The solution was stirred with heating for a further 3–4 h, then stirred overnight at room temperature. The solvent was removed by rotary evaporation or lyophilisation to yield the products as yellow solids. **3478MESS-CB[6]**. Anal. calc. for $C_{58}H_{66}Cl_2N_{28}O_{12}Pt{\cdot}12H_2O{\cdot}15NaCl$: C, 25.74; H, 3.35; N, 14.49%. Found: C, 26.25; H, 3.39; N 13.93%. **PHENSS-CB[6]**. Anal. calc. for $C_{54}H_{58}Cl_2N_{28}O_{12}Pt{\cdot}12H_2O{\cdot}27NaCl$: C, 19.35; H, 2.47; N, 11.70%. Found: C, 19.39; H, 2.50; N, 11.69%. **47MESS-CB[6]**. Anal cal. for $C_{56}H_{62}Cl_2N_{28}O_{12}Pt{\cdot}3H_2O{\cdot}16NaCl$: C, 26.13; H, 2.66; N, 15.23%. Found: C, 26.49; H, 3.09; N, 15.30%. **5MESS-CB[6]**. Anal. calc. for $C_{55}H_{60}Cl_2N_{28}O_{12}Pt{\cdot}9H_2O{\cdot}20NaCl$: C, 22.76; H, 2.71; N, 13.51%. Found: C, 22.90; H, 2.95; N, 13.55%. **56MESS-CB[6]**. Anal. calc. for $C_{56}H_{62}Cl_2N_{28}O_{12}Pt.10H_2O.29NaCl$: C, 19.44; H, 2.39; N, 11.33%. Found: C, 19.27; H, 2.51; N, 11.32%.

Results and discussion

Platinum(II)-based DNA intercalators

Over the last seven years we have developed over 60 platinum(II)-based DNA intercalators and examined their utility as anti-cancer agents, principally through the use of growth inhibition cytotoxicity assays using the L1210 murine leukaemia cell line and DNA binding experiments. These agents typically contain 1,10-phenanthroline or phenanthroline derivatives as the intercalating ligand and either chiral or achiral ancillary ligands (Fig. 2).

Previously we have developed a metallointercalator naming method which uses a five to eight digit identifying label and allows easy reference to the metal complexes in text and tables.[17] The first one or two numbers indicate the position of substitution on the phenanthroline ligand, if applicable. The next two or three letters indicate the substitution group and the final two letters indicate either the chirality of our active 1,2-diaminocyclohexane ligand or the use of ethylenediamine. For example, [Pt(4,7-dimethyl-1,10-phenanthroline)(ethylenediamine)]$^{2+}$ is abbreviated as **47MEEN**, [Pt(5-nitro-1,10-phenanthroline)(1*R*,2*R*-diaminocyclohexane)]$^{2+}$ is **5NORR**, [Pt(1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane)]$^{2+}$ is simply **PHENSS** and [Pt(5-chloro-1,10-phenanthroline)(ethylenediamine)]$^{2+}$ is **5CLEN**.

New platinum(II)-based DNA intercalator purification method

Previously our preferred method for the synthesis of these DNA intercalators was by reacting K_2PtCl_4 with one equivalent of the intercalating ligand in DMSO, then refluxing the subsequent [Pt(intercalating ligand)Cl_2] complex with two equivalents of ancillary ligand in water. This method can require numerous purification steps, including precipitation from water as a perchlorate salt, then conversion back to the chloride salt with Dowex® anion exchange resin, and usually further purification by liquid–liquid

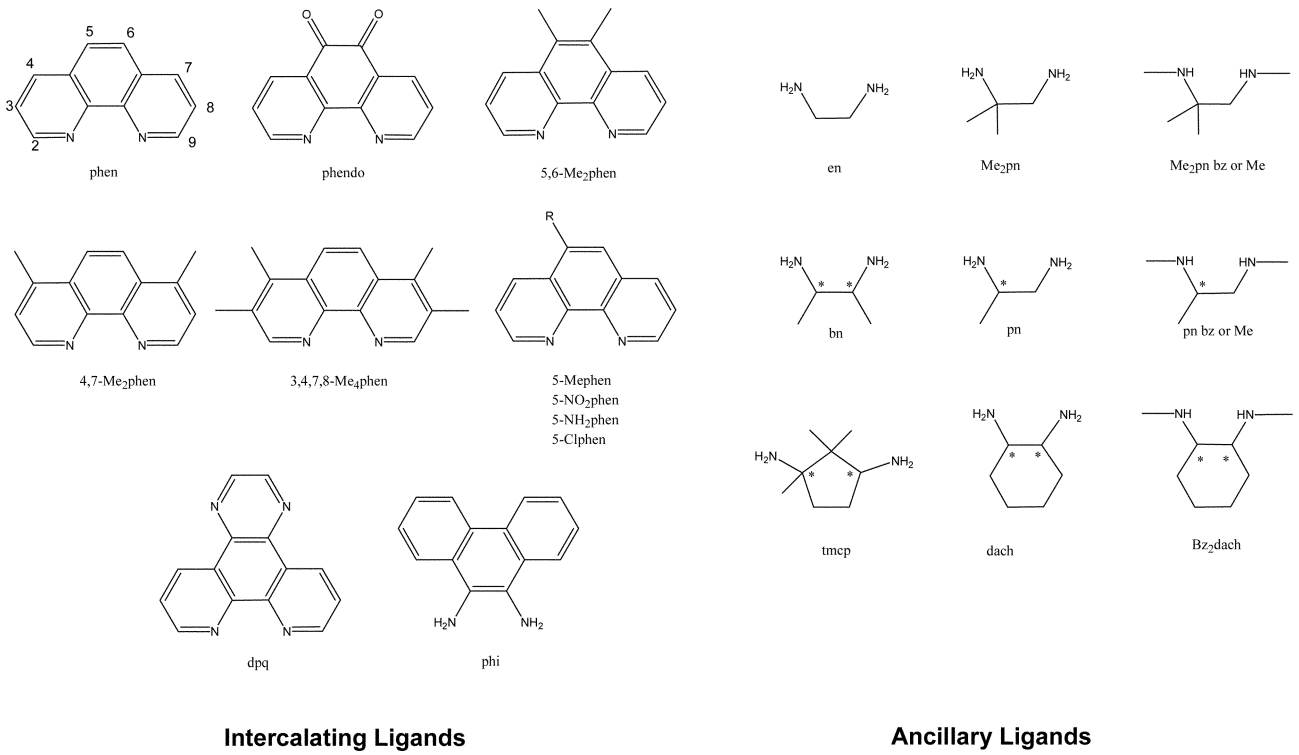


Fig. 2 The chemical structures of the 1,10-phenanthroline and phenanthroline-based intercalating ligands, and the chiral and achiral ancillary ligands used during our investigation of platinum(II)-based DNA intercalators as anticancer agents. R = methyl, nitro, amino or chloro. * Indicates a chiral centre (either *S* or *R*). The numbering scheme used for the intercalating ligand protons is shown on 1,10-phenanthroline.

extraction with toluene. Often, the precipitation and extraction steps need to be repeated several times to ensure purity. This is labourious and reduces the yield with each additional purification step.

We can now report a new method for synthesis and purification. K_2PtCl_4 is reacted with one equivalent of ancillary ligand in water. The subsequent [Pt(ancillary ligand)Cl_2] complex is refluxed with 1.1 equivalents of intercalating ligand overnight, to produce a clear, yellow coloured solution. This solution was then reduced to a minimum volume without precipitation of the metal complex. A Waters C18-reverse phase Sep-Pak® (2 g) was activated with methanol, washed with water and the DNA intercalator solution loaded onto the column in 10 mL batches. Elution with water (*ca.* 20–30 mL) yielded the product which was then lyophilised. Occassionally, the solution needs to be eluted a second time to ensure purity. Sep-Pak® columns can be reused for subsequent batches, once impurities have been removed from the column using 90% acetonitrile–9% water–1% trifluoroacetic acid. These impure fractions can also be rotary evaporated to dryness, redissolved in water and eluted on a fresh Sep-Pak® to yield more product, increasing the overall yield by as much as 10%.

This new method has two advantages. First, it uses less ancillary ligand, which is generally more expensive than the intercalating ligand due to the cost of resolving the chiral ligand. Second, the new method uses just one purification step, eliminating the need to precipitate from solution as perchlorate salts. Additionally, purification of the products by the new method takes between 2 and 4 h depending on the batch size (up to 500 mg), as opposed to the previous method which took around 1 to 2 weeks to attain the same level of purity. Larger batch sizes (1 to 2 g) can be completed if a larger reverse phase Sep-Pak® (*i.e.* 10 g) is used.

In vitro cytotoxicity

From cytotoxicity growth inhibition assays our three best platinum(II)-based DNA intercalators are [(5,6-dimethyl-1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane)platinum(II)]$^{2+}$ (**56MESS**), [(5-methyl-1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane)platinum(II)]$^{2+}$ (**5MESS**) and [(1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane)platinum(II)]$^{2+}$ (**PHENSS**), although all DNA intercalators tested to date which contain *S*,*S*-dach ancillary ligands display higher cytotoxicity than cisplatin in the L1210 murine leukaemia cell line (Table 1). Our lead drug **56MESS** is *ca.* 100-fold more cytotoxic than cisplatin. This family of DNA intercalators also displays excellent cytotoxicity in a number of human cancer cell lines, including some that are sensitive to cisplatin treatment and others that have one or more modes of resistance to cisplatin (see ESI‡). Before the discovery of **56MESS** and **5MESS** the complex **PHENSS**[15] was tested in several human cancer cell lines and displayed potent cytotoxicity in the A-427 (human lung cancer), RT-112 and RT-4 (human bladder cancer), KYSR-70 (human oesophagus cancer), SISO (human cervical cancer), MCF-7 (human breast cancer), 2008 (human ovarian carcinoma), C13*5 (human ovarian carcinoma with acquired resistance to cisplatin), SKOV-3 (human ovarian carcinoma with intrinsic resistance to cisplatin), 5637 (bladder cancer) and 5637/DDP (bladder cancer with acquired resistance to cisplatin) cell lines.[15]

Table 1 Cytotoxicity, expressed as IC_{50} (μM), of our leading platinum(II)-based DNA intercalators in the L1210 murine leukaemia cell line. IC_{50} is defined as the required concentration of drug to inhibit cellular growth by 50%

Metal complex	IC_{50}/μM	Metal complex	IC_{50}/μM
Cisplatin	1.0	**47MESS**	0.13[a]
56MESS[a]	0.0092	**5NHSS**	0.32[b]
5MESS[b]	0.033	**DPQSS**	0.33[b]
5CLSS[b]	0.13	**5NOSS**	0.57[b]
PHENSS[c]	0.13	**3478MESS**	0.87[a]

[a] New data based on previously published[28] metal complexes using the new purification method. [b] Data taken from reference 17. [c] Data taken from reference 15.

From these cytotoxicity tests we have determined a set of structure–activity rules for this family of metal complexes.[15,17] For high cyotoxicity and the ability to overcome cisplatin resistance the 1,10-phenanthroline ligand should contain methyl groups in either the five or both the five and six positions. The most active ancillary ligand is 1,2-diaminocyclohexane (dach). In all cases platinum(II)-based DNA intercalators containing the dach ligand in an *S*,*S* conformation are more cytotoxic than the *R*,*R* conformation, in the cell lines tested.[15,17] Achiral ligands (like ethylenediamine) impart the least cytotoxic potential. Other ancillary ligands we have examined include 2*R*,3*R*-, 2*S*,3*S*- and *meso*-butane-2,3-diamine, 1*R*-, 1*S*- and *meso*-propane-1,2-diamine, *N*,*N*′-dimethyl-1*R*-propane-1,2-diamine, *N*,*N*′-dimethyl-1*S*-propane-1,2-diamine, and 1*S*,3*R*- and 1*R*,3*S*-1,3-diamino-1,2,2-trimethylpentane.[13,27]

The exact mechanism(s) by which these complexes exert their cytotoxicity and overcome cisplatin resistance, however, is still not clear. Their binding to DNA has been studied by molecular modelling, ^{1}H NMR with the oligonucleotoide d(GTCGAC)$_2$, and circular dichroism, UV-Vis and fluoresence studies with calf-thymus DNA. From these experiments we know that these metal complexes are able to intercalate double stranded DNA with a preference for GT/CA sequences with the ancillary ligand in the DNA minor groove.[12–15] Interestingly, there are no significant differences between the DNA binding of the highly cytotoxic (**5MESS**), moderately cytotoxic (**5MERR**) and poorly cytotoxic [(5-methyl-1,10-phenanthroline)(ethylenediamine)platinum(II)]$^{2+}$ (**5MEEN**) complexes.[17] This indicates that either DNA binding is not the mechanism by which these complexes exert their cytotoxicity, or, that their DNA binding does induce apoptosis, but their ability to reach DNA as the cellular target is determined by intracellular transport factors which differ based on the subtle structural changes between the active and inactive metal complexes.[17] We are currently completing *in vitro* cytotoxicity and cellular uptake studies with **56MESS**, **56MERR** and **56MEEN** in the LoVo human adenocarcinoma colorectal cancer cell line and hope to report the results in the near future.

Platinum(II) DNA sequence selective complexes

When 1-methylimidazole (Im), 1-methylpyrrole (Py) and 1-methylhydroxypyrrole (Hp) heterocyclic rings are coupled into polyamide linear or hairpin chains they produce compounds that are capable of recognising and binding specific DNA base-pair sequences. Each pair of heterocyclic rings recognises a specific base-pair in DNA: Im/Py and Py/Im pairings distinguish between G:C and C:G base pairs respectively, while Hp/Py and Py/Hp

recognise T:A and A:T base pairs. A combination of Py/Py is selective for both A:T and T:A base pairs.[29]

To this end we are examining the utility of platinum(II)-polyamide complexes as agents that will show enhanced DNA adduct formation compared to cisplatin. The enhanced DNA affinity will be facilitated through preferential binding to extended telomere (TTAGGG) sequences commonly found on the ends of chromosomal DNA in cancer cells.[30] Previously, we have synthesised four DNA sequence selective complexes with linear and hairpin polyamide ligands (Fig. 3). This includes two mononuclear platinum complexes with linear, three-ring polyamide ligands, selective for 5′-TGTCA-3′ DNA sequences. These two metal complexes, **DJ1953-2** and **DJ1953-6**, unwind supercoiled DNA by 13°, inhibit RNA transcription, form coordinate covalent adducts with DNA and show a magnitude of order increase in binding affinity over a double mismatch DNA sequence.[20] Unfortunately, neither metal complex displayed cytotoxicity better than cisplatin in the L1210 murine leukaemia cell line.

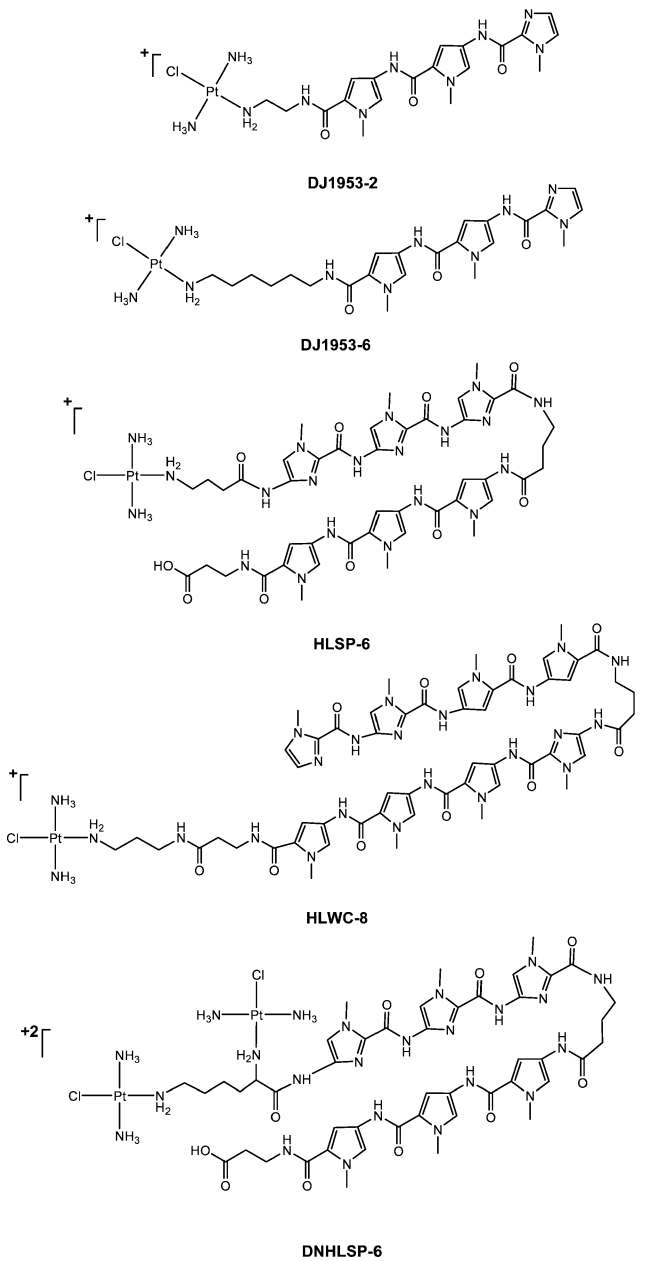


Fig. 3 The chemical structures of the five platinum(II) DNA sequence selective complexes synthesised using either wet chemistry or solid phase synthesis. Anions have been omitted for clarity, but are generally chlorides.

We have also examined the best method of synthesis for more complex mononuclear platinum complexes with six and eight ring polyamide hairpin ligands by wet chemistry or solid phase synthesis.[21] Solid phase synthesis is more economical, efficient and faster than wet chemistry and is the preferred method in our group for the synthesis of platinum(II) DNA sequence selective agents.[21] We can now report the synthesis and characterisation of a new *dinuclear* platinum(II) DNA sequence selective complex, **DNHLSP-6**.

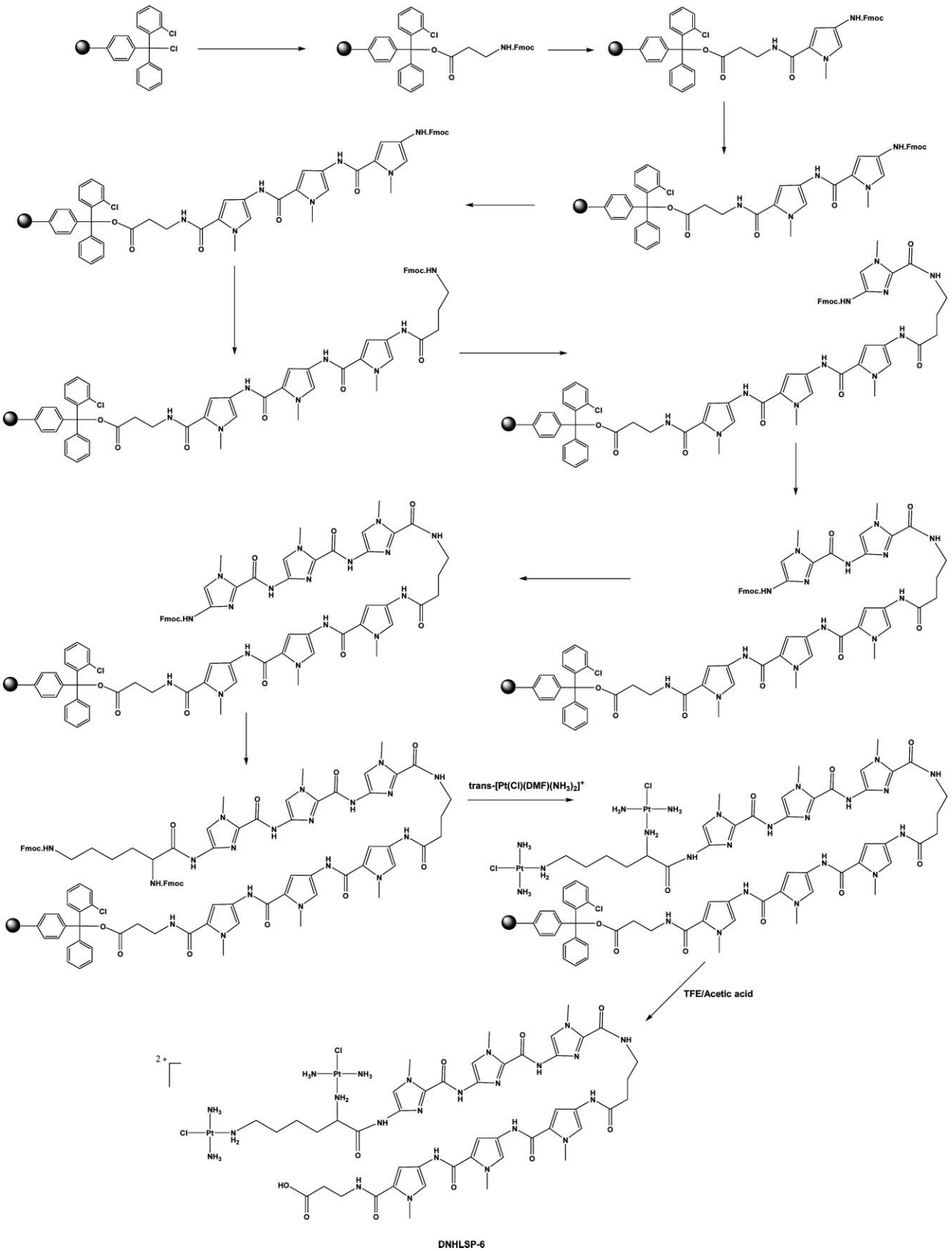
DNHLSP-6 was synthesised to determine whether a dinuclear platinum(II) DNA sequence selective agent would demonstrate higher cytotoxicity compared to similar mononuclear platinum(II) complexes. In this case, **DNHLSP-6** contains a BBR3005-like component (a drug already shown to have cytotoxicity better than cisplatin in sensitive and cisplatin-resistant cell lines),[5,6] with two *trans*-chloroplatinum groups separated by a 1,5-diaminopentane chain. It is expected that like BBR3005, **DNHLSP-6** is also able to form flexible, long range, DNA interstrand adducts, which would be able to bypass removal by DNA repair proteins and thus display higher cytotoxicity than cisplatin.

The synthesis of **DNHLSP-6** was accomplished using an adaptation of our published solid phase synthesis method by sequential coupling of Im, Py, γ-butyric acid and β-alanine on a Symphony Quartet Protein Synthesiser (Scheme 1).[21] The major difference was the use of the di-Fmoc–lysine–OH linker instead of 4-(Fmoc–amino)butyric acid. This allows the hairpin polyamide to coordinate to two individual platinum centres through two individual amine groups after the removal of both Fmoc protecting groups.

In the ^{1}H NMR spectrum (see ESI‡) of **DNHLSP-6**, there are six amide singlets between 9.54 and 10.8 ppm. The two triplets at 7.96 and 8.21 ppm correspond to the two amide bonds on the aliphatic chains. The nine aromatic hydrogens appear as three singlets and six doublets between 6.83 and 7.64 ppm. The four NH_3 groups attached to the platinum appear as two triplets at 5.62 and 5.79 ppm. The six methyl groups appear as six singlets between 3.80 and 4.03 ppm. The OH of the carboxylic acid at 3.33 ppm is at the same chemical shift as that of water in d_6-DMSO and thus could not be individually observed. The remaining groups which consists of 9 × CH_2, a CH and 2 × NH_2 appear as triplets and multiplets between 1.82 and 3.5 ppm.

In the ^{195}Pt NMR spectrum, **DNHLSP-6** shows a single platinum resonance at −2424 ppm with a shoulder at a higher frequency (~2426 ppm), indicating two chemically similar, but separate, platinum groups (see ESI‡). The chemical shift is consistent with similar metal complexes with PtN_3Cl coordination spheres, where N = two *trans* ammine ligands and one primary amine ligand.[21,31]

In the electrospray ionisation mass spectrum of **DNHLSP-6**, there are two prominent ion peaks which occur at *m*/*z* 784.4 and 1566.4 (see ESI‡). The peak at *m*/*z* 784.4 corresponds to the parent metal complex with a 2+ charge. The peak at *m*/*z* 1566.4 corresponds to the deprotonated metal complex with a charge of +1. Fragmentation ions are also observed. The peak at *m*/*z* 1303.1



Scheme 1 Solid phase synthesis of **DNHLSP-6**. Each step (except the addition of the platinum) represents the removal of the Fmoc-protecting group using 20% piperidine followed by coupling of the rings or linker.

can be assigned to the metal complex with a 1+ charge from the loss of one *trans*-chlorodiammine platinum group.

Elemental analysis shows that despite **DNHLSP-6** not displaying any solubility in water (it is soluble only in DMF and DMSO) the solid produced by lypholisation contains five waters of crystallisation.

The cytotoxicity of **DNHLSP-6** is currently being examined using growth inhibition assays in a variety of human cancer cell lines.

Cucurbit[*n*]urils as drug delivery vehicles

Like the clinical agents cisplatin, carboplatin and oxaliplatin both our platinum(II)-based DNA intercalator complexes and our platinum(II) DNA sequence selective complexes are expected to exhibit high dose limiting side-effects. Primarily, these side-effects occur because platinum chemotherapeutic drugs do not discriminate well between normal and cancerous cells. As such, we are examining ways of specifically delivering our platinum(II)-based drugs only to cancerous cells, using novel drug delivery vehicles.

Cucurbit[*n*]urils (CB[*n*], where $n = 5$, 6, 7, 8 or 10), are small barrel shaped molecules that have an internal hydrophobic cavity and symmetrical, hydrophilic portals and have been shown as suitable molecular hosts for neutral and charged mono- and multinuclear platinum(II) anticancer agents (Fig. 4).[32–34]

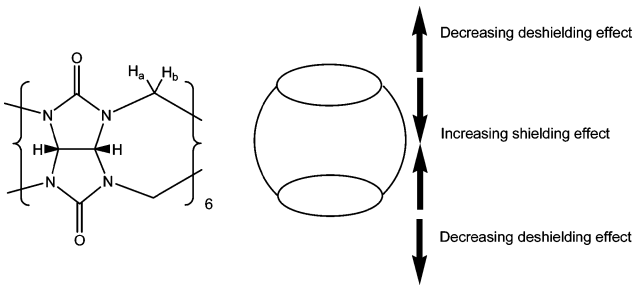


Fig. 4 The chemical structure of cucurbit[6]uril (CB[6]).

Previously, we have studied the encapsulation of the metallointercalators [(5-chloro-1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane)platinum(II)]$^{2+}$ (**5CLSS**), [(5-chloro-1,10-phenanthroline)(1*R*,2*R*-diaminocyclohexane)platinum(II)]$^{2+}$ (**5CLRR**) and [(5-chloro-1,10-phenanthroline)(ethylenediamine)platinum(II)]$^{2+}$ (**5CLEN**) with CB[6], CB[7] and CB[8].[22] All three platinum(II)-based DNA intercalators are bound over the ancillary ligand with slow to intermediate exchange kinetics on the NMR timescale. The different sized cucurbiturils are found to affect the cytotoxicity in a size specific manner, CB[7] and CB[8] encapsulation resulted in a dramatic unwanted decrease in cytotoxicity but interestingly CB[6] was found to increase the cytotoxicity of **5CLSS** by 2.5-fold.[22] Therefore it was of interest to determine whether CB[6] would also increase the cytotoxicity of other *S*,*S*-dach containing platinum(II)-based DNA intercalators.

We can now report on the encapsulation by CB[6] of five of our platinum(II)-based DNA intercalators, **56MESS**, **5MESS**, [(3,4,7,8-tetramethyl-1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane)platinum(II)]$^{2+}$ (**3478MESS**), [(4,7-dimethyl-1,10-phenanthroline)(1*S*,2*S*-diaminocyclohexane)platinum(II)]$^{2+}$ (**47MESS**) and **PHENSS**, and their subsequent cytotoxicity in the L1210 murine leukaemia cell line. The metallointercalators were chosen because of the different position and number of methyl groups on the phenanthroline ligand, which provide alternate and competing binding sites for CB[6] encapsulation of the ancillary ligand, and because of the range of the metal complexes' cytotoxicities.

Unfortunately, CB[6] is relatively insoluble in aqueous solutions (<1 mM) and requires the use of alkali earth metals such as caesium, sodium or lithium (~20 mM) for concentrations high enough to study host–guest interactions by NMR. As such, in this study it was necessary to dissolve both the platinum(II)-based DNA intercalators and CB[6] with between 15 and 20 equivalents of NaCl. NaCl was chosen in preference to CsCl or LiCl as saline solutions are routinely used to deliver platinum drugs intraveneously in the clinic. When **56MESS**, **5MESS**, **47MESS** or **PHENSS** are added to 0.1 to 1.1 equivalents of CB[6] there are significant and specific shifts of the metal complex resonances in the ^{1}H NMR spectra (see Fig. 5). The *S*,*S*-dach resonances shift to a lower frequency between 0.5 to 1.0 ppm, whilst the phenanthroline resonances move slightly to a higher frequency or are not affected. Previous research has established that shifts to lower frequencies (upfield) are observed for protons of guest compounds shielded inside the cucurbituril cavity[32] and shifts to higher frequencies (downfield) are observed for those guest protons deshielded by being outside, but close to, the cucurbituril portals.[32] These results therefore clearly indicate that for **56MESS**, **5MESS**, **47MESS** and **PHENSS**, CB[6] encapsulates the metal complexes over the *S*,*S*-dach ligand (Fig. 6). The ^{1}H NMR spectra also demonstrate that this encapsulation process is slow on the NMR timescale. When 0.5 equivalent of CB[6] is added to the metal complexes, resonances representing free and bound metal complex are observed simultaneously (see Fig. 5).

An interesting feature of the ^{1}H NMR spectra are the double set of resonances for the H_a and H_b CB[6] protons, but only a single methine resonance. The portal diameter of CB[6] is smaller than the width of *S*,*S*-dach, and as such, one of the portals has to stretch to accommodate the ligand within the cavity. As such, the two portals would no longer be equivalent and thus lead to the extra set of CB[6] resonances observed. Interestingly, the amine resonances of these metal complexes display significant shifts to lower frequencies of *ca.* 0.6 to 0.75 ppm. These lower frequency shifts are indicative that they are located within the CB[6] cavity, most likely hydrogen bonding with the oxygens of *one* of the portals. This hydrogen bonding could also explain the extra set of H_a and H_b CB[6] resonances. Importantly, when dissolved in D_2O without CB[6] the amine resonances of the platinum(II)-based DNA intercalators exchange with the solvent within 1–2 min, but with an equimolar concentration of CB[6], the amine resonances are still observable one to two weeks after dissolution. This indicates that the CB[6]–metal complex species is particularly long-lived and that encapsulation by CB[6] greatly increases the steric hinderance of nucleophiles toward the platinum atom as the entire *S*,*S*-dach ligand is located in the CB[6] cavity.

When the platinum(II)-based DNA intercalators and CB[6] are combined in a ratio of 1 : 1 (as measured by resonance integration) the ^{1}H NMR spectra indicate that two species of metal complex are present. The major species represents full encapsulation of the *S*,*S*-dach ligand within CB[6], with a second set of metal complex resonances representing free metal complex. As stated previously,

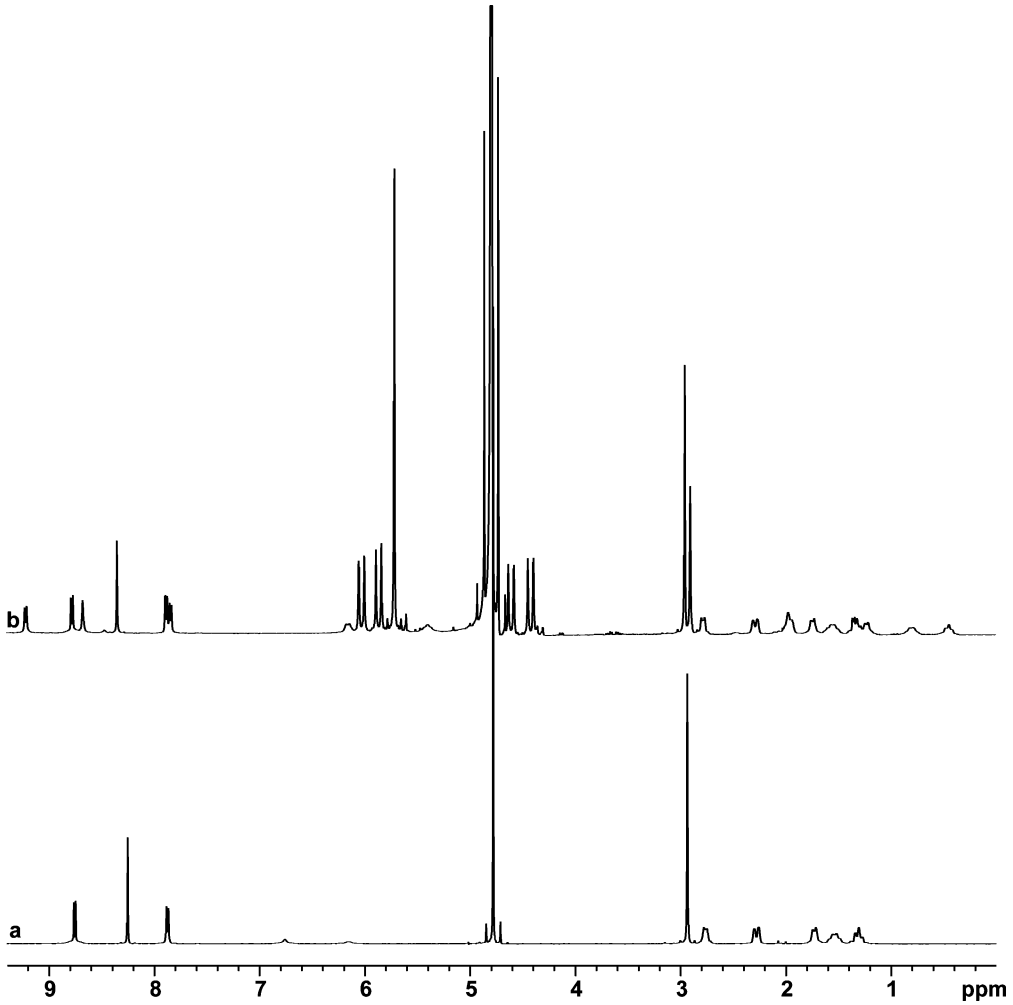


Fig. 5 ^{1}H NMR spectra of (a) **47MESS** in D_2O and (b) **47MESS** with 0.5 equivalent of CB[6] in ~20 mM NaCl, showing both free and bound metal complex resonances and the large shifts to a lower frequency of the *S*,*S*-dach amine and aliphatic protons, which is evidence of the ancillary ligand being located within the CB[6] cavity. These spectra are indicative of similar spectra for **56MESS**, **5MESS** and **PHENSS** with CB[6].

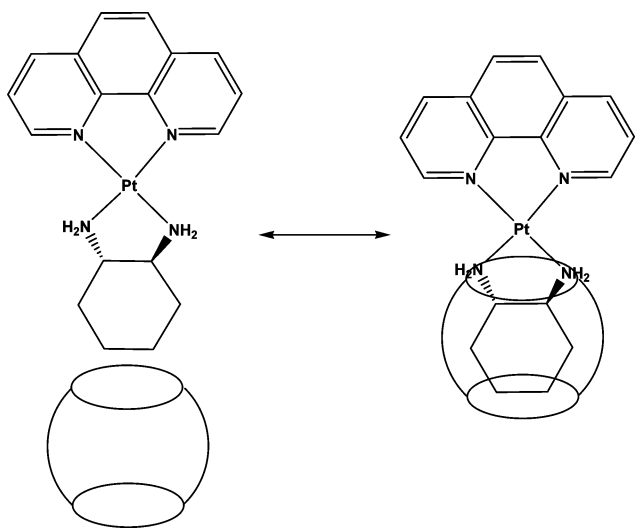


Fig. 6 A schematic diagram showing the exchange process between a free platinum(II)-based DNA intercalator (**PHENSS**) and where it is encapsulated over the ancillary ligand by a CB[6] molecule. Anions and charges have been omitted for clarity.

it is likely that one of the CB[6] portals must stretch to permit entry of the *S*,*S*-dach ligand, and therefore there is a thermodynamic cost to do so. As such, at any given moment there appears to be an equilibrium between free and bound metal complex, where the ratios of each are approximately 10 and 90%, respectively. Unfortunately, efforts to determine the binding constants and/or exchange rate of the DNA intercalator–CB[6] complex by UV-Vis, circular dichroism and exchange NMR have been unsuccessful.

When **3478MESS** is added to an equimolar concentration of CB[6], three sets of CB[6] resonances are observed in the ^{1}H NMR. We assign these to three different species, in slow exchange, between free CB[6], CB[6] bound around *S*,*S*-dach and CB[6] bound around the methyl groups of the 3,4,7,8-Me_4phen ligand (Fig. 7).

In the aliphatic region of **3478MESS**, two sets of phenanthroline methyl resonances were observed (four peaks in total). One set of resonances are from the methyl groups being deshielded inside the CB[6] cavity (**A**). The other resonances result from the two remaining species; the free metal complex (**C**) and the metal complex with CB[6] bound over *S*,*S*-dach (**B**) (see Fig. 7).

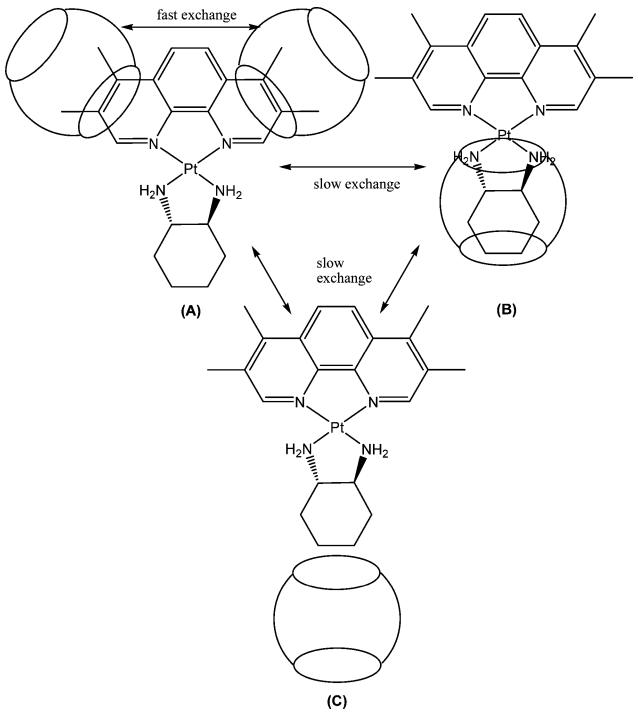


Fig. 7 A schematic diagram displaying the three proposed modes of CB[6] encapsulation of **3478MESS**. Anions and charges have been omitted for clarity.

Two sets of *S*,*S*-dach resonances were also observed in the aliphatic region. One set results from CB[6] encapsulation over the ancillary ligand (**B**), and the other set of resonances resulted from the other two species; free metal complex (**C**) and the metal complex with CB[6] encapsulated over two phenathroline methyl groups (**A**). These latter two species have equivalent *S*,*S*-dach proton resonance chemical shifts, due to the CB[6] having little effect on their chemical environments. Five resonances integrating to a total of six protons were observed in the aromatic region of the **3478MESS** ^{1}H NMR spectrum. These protons could not be assigned as the degree of shifting of each signal in each binding mode was unknown, however the spectra did confirm that there were indeed three different species of platinum(II)-based DNA intercalator in the solution.

Elemental analysis indicates that at a ratio of 1 : 1 platinum(II)-based DNA intercalator to CB[6], all complexes contain waters of crystallisation. **3478MESS** and **PHENSS** both precipitate with 12 water molecules, **47MESS** with three water molecules, **5MESS** with nine and **56MESS** with 10 waters of hydration. These results are not unexpected however, as all cucurbit[*n*]urils are known, from X-ray crystal structures, to readily form hydrated solids.[23] CB[5–10] typically form solids with between eight and 30 waters of hydration, and sometimes acids (typically HCl) as well.[23]

The platinum(II)-based DNA intercalators **56MESS**, **5MESS**, **47MESS**, **3478MESS**, **PHENSS** and **5CLSS** were tested for cytotoxicity in the absence and presence of one mole equivalent of CB[6] (Table 2). For **5CLSS** and **3478MESS** CB[6] encapsulation resulted in a small increase in cytotoxicity, **56MESS**, **47MESS** and **PHENSS** display a small decrease in cytotoxicity, whereas **5MESS** displays no significant change (within experimental error). These results indicate that because CB[6] does not significantly change the cytotoxicity of these platinum(II)-based DNA intercalators, it may have utility as a molecular host for improved drug delivery.

Table 2 Cytotoxicity, expressed as IC_{50} (μM), of our methyl-group containing platinum(II) DNA intercalators with CB[6] in the L1210 murine leukaemia cell line. IC_{50} is defined as the required concentration of drug to inhibit cellular growth by 50%. Values were determined from between 3 and 5 experiments, and reported ± standard errors

Metal complex	IC_{50} free drug/μM	IC_{50} with CB[6]/μM
Cisplatin	1.0	n/a
56MESS	0.0092 ± 0.0008	0.011 ± 0.004
5MESS	0.033[a] ± 0.014	0.038 ± 0.02
47MESS	0.13 ± 0.01	0.17 ± 0.01
5CLSS[b]	0.13 ± 0.003	0.05 ± 0.008
PHENSS	0.25 ± 0.02	0.41 ± 0.01
3478MESS	0.87 ± 0.03	0.60 ± 0.05

[a] Data taken from reference 17 [b] Data taken from reference 22.

Future work

Our future work continues to develop further our family of platinum(II)-based DNA intercalators. This includes new intercalating ligands like pyridocarbazole,[35] other chiral ancillary ligands like 1*R*,2*R*-, 1*S*,2*S*- and *meso*-1,2-bis(4-fluorophenyl)-1,2-ethanediamine, and the replacement of the central platinum atom with other square-planar transition metals, such as palladium(II). We are also working towards expanding our platinum(II) DNA sequence selective family of metal complexes by creating water soluble multinuclear complexes. One such compound includes a trinuclear platinum complex where the terminal platinum groups are linked to the central platinum group through two separate hairpin polyamides. This complex will have a 4+ charge and be capable of recognising DNA sequences up to 12 base pairs in length; specifically this will mean the compound would be capable of recognising and binding tandem duplex telomere sequences, d(TTAGGGTTAGGG).

Despite our good results with cucurbiturils, their use is limited by their poor water solublity and their ability to transport only one drug molecule at a time. We are therefore examining other molecular hosts such as cyclodextrins, calix[*n*]arenes and dendrimers. These additional molecular hosts will allow us to examine in detail several different factors that may affect drug transport and release; *i.e.* flexible *vs* rigid structure, chiral *vs* achiral, sub-nanometre *vs* nanometre size, and water solubility/lipophilicity. We also plan to examine the cellular uptake and distribution of cucurbit[*n*]urils (where $n = 6$, 7 or 8) in order to better understand how they affect drug cytotoxicity. With this information we hope to be able to design a molecular host that will not only transport drugs to, and within, the cell, preventing their degradation and deactivation, but also develop molecular hosts that can specifically recognise and bind cancerous cells.

Conclusions

In this paper we have discussed the three main thrusts of our research into improved platinum(II)-based anticancer drug design and delivery. From a family of over 60 platinum(II) DNA intercalator drugs, one metal complex **56MESS** displays up to

100-fold better cytotoxicity compared to cisplatin in the L1210 murine leukaemia cell line.

We have synthesised five platinum(II)-based DNA sequence selective agents, including a new *dinuclear* platinum(II) complex. These compounds form the basis of further drug design of more complex, water soluble platinum(II)-based DNA sequence selective agents capable of enhanced DNA sequence recognition and higher cytotoxicity. Finally, our platinum(II)-based DNA intercalators are encapsulated over the ancillary ligand by CB[6], and despite the presumed strong binding, have little, to no, affect on the metal complexes' cytotoxicity. These results form the basis of our fledgling drug delivery research and we plan to expand this work to other molecular hosts such as cyclodextrins, calix[*n*]arenes and dendrimers.

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