Folding of dinuclear platinum anticancer complexes within the cavity of para-
sulphonatocalix[4]arene

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Abstract

The binding of three dinuclear platinum complexes, where the bridging ligand of the complexes is $N,N'$-(alkane-1,1-diyl)diisonicotinamide ($n = 4, 6$ or $8$ for butane, hexane and octane, respectively) to the macrocycle para-sulphonatocalix[4]arene (sCX[4]) has been studied by $^1$H nuclear magnetic resonance (NMR) spectroscopy and molecular modelling. The NMR spectra show two important features, large upfield shifts of the methylene proton resonances of up to 1.8 ppm, which clearly places them within the shielding environment of the macrocycle's cavity, and a loss of chemical symmetry of the metal complexes with extra resonances observed upon sCX[4] binding. Molecular models of the platinum-sCX[4] host-guest complexes show significant folding of the metal complexes' aliphatic chain and a non-symmetrical interaction with the macrocycle. One side of the metal complexes forms three hydrogen bonds to sCX[4], whereas the opposite side of the metal complexes forms just one hydrogen bond, giving rise to the loss of chemical symmetry in the $^1$H NMR spectra. As the dinuclear platinum complexes are model anticancer drugs, the effect of sCX[4] binding was investigated in vitro in the human ovarian carcinoma cell line A2780 and its cisplatin-resistant sub-line A2780cp70. Whilst the free metal complexes are a magnitude of order more active than cisplatin in the A2780 cell line, they are all highly cross-resistant with cisplatin in the A2780cp70 line. Binding by sCX[4] has little affect on the metal complexes' cytotoxicity in the sensitive cell line, but has a large effect in the resistant cell line. The two shortest metal complexes become less active when bound by sCX[4], whereas the longest metal complex becomes more cytotoxic.
Introduction

Platinum-based anticancer drugs remain an integral component of many chemotherapy regimens despite their toxic side-effects and the ability of cancers to develop drug resistance against them.[1] Multinuclear platinum drugs are a class of chemotherapy agent which have attracted interest in their ability to overcome resistance developed by tumours to many other anticancer agents. The lead drug in this class is BBR3464,[2] a trinuclear complex, which was developed as far as Phase II in clinical trials. Recently, two dinuclear derivatives have been investigated as potential drug candidates.[3] Whilst these drugs are significantly more cytotoxic than cisplatin, they are rapidly degraded in vivo resulting in very little of the drug reaching tumours. Our group is interested in the use of macrocycles as protective delivery vehicles for multinuclear platinum drugs with a focus on the cucurbituril,[4, 5] cyclodextrin[6] and calixarene[6, 7] families of cavitands. One of the key benefits of macrocycle binding to platinum drugs is that their encapsulation confers steric protection, which slows or prevents the drug’s degradation by biological thiols like glutathione,[8-11] or sequestration by proteins like human serum albumin.[12-14]

Calixarenes are a family of macrocycles made from a hydroxyalkylation reaction between an aldehyde and phenol.[15] The resultant molecules, that generally contain 4, 6, 7, 8 or 9 subunits, form bowl shaped structures with a hydrophobic pocket and a extensively hydrogen bonded network of hydroxyl groups at its base.[15] The application of calixarenes to drug delivery stems from their ability to form host-guest complexes with a range of small molecule drugs and biological molecules.[16, 17] Such complexes are stabilised by hydrophobic effects and/or ion-dipole interactions or hydrogen bonds.
Native calixarenes are poorly soluble in aqueous solutions but functionalisation with sulphonate groups yields derivatives that are highly soluble. Para-sulphonated calixarenes (Figure 1), particularly the macrocycle made of four subunits (sCX[4]), have demonstrated many applications in drug delivery including: improved drug solubility, chemical stability, bioavailability, biodistribution and transport, and/or elimination of drug polymorphism in the solid state.[16, 18, 19] As an excipient for drugs, sCX[4] also displays relatively little cytotoxicity or toxicity in vivo.[20]

Figure 1. The chemical structure of para-sulphonatocalix[4]arene (sCX[4]), a molecular model showing sCX[4]’s bowl-shaped structure, and the chemical structure of the dinuclear platinum complexes used in this study; n = 1, 2 or 3. Counter ions for both molecules have been omitted.

Previously we have shown that sCX[4] is capable of forming host-guest complexes with different types of platinum anticancer complexes, including dinuclear agents.[6, 7] In our recent study involving a dinuclear platinum complex with a rigid bridging linker, however, we found that the sCX[4] bound the platinum complex in a side-on manner and provided no barrier to drug degradation.[7] Despite this, the strong association constant, and the ease with which sCX[4] can be functionalised with cancer targeting groups, made us hypothesise that the macrocycle may still be useful as a platinum drug delivery vehicle. The sulphonate groups of sCX[4] can readily be functionalised with groups which can be used to actively target tumours. A recent example includes
folate, a vitamin essential to cell growth,[21] and which was conjugated to sCX[4].[22] As such, we are interested in developing actively targeted sCX[4] derivatives which can be used to deliver multinuclear platinum drugs to cancers.

As a first step in our development of targeted calixarene-based delivery vehicles, in this paper we have investigated the binding of sCX[4] to a new group of dinuclear platinum complexes which have been shown to be up to 10-fold more active than cisplatin.[23] Their binding was examined by $^1$H and DOSY NMR from which molecular models were developed. The effect of sCX[4] on the cytotoxicity of the dinuclear platinum complexes was evaluated using in vitro growth inhibition assays with the human ovarian carcinoma cell line A2780 and its cisplatin-resistance sub-line A2780cp70.

**Experimental**

**Materials.** Para-sulphonatocalix[4]arene, diethyl ether, triethylamine, transplatin, D$_2$O (99.9%), isonicotinic acid and 1,6-diaminohexane were purchased from Sigma Aldrich. T3P® was purchased from Alfa Aesar. The metal complexes (1), (2) and (3) were made following a published method [23]. The characterisation data for 2 has not previously been reported and is given below.

**Characterisation data for the ligand of complex (2).** $^1$H NMR (d$_6$-DMSO, ppm): 8.72 (t), 8.70 (d, $J = 4.4$ Hz), 7.72 (d, $J = 4.6$ Hz), 1.53 (m), 1.34 (m). $^{13}$C NMR (d$_6$-DMSO): 165.1, 151.8, 142.1, 121.7, 29.45, 26.74. ESI-MS [M+H]$^+$ expected: 327.18 m/z, found: 327.60 m/z. Elemental analysis C$_{18}$H$_{22}$N$_4$O$_2$.

Expected: C, 66.24; H, 6.79; N, 17.17%; Found: C, 65.85; H, 6.71; N, 16.84%.
**Characterisation data for complex (2).** $^1$H NMR (D$_2$O, ppm): 8.97 (d, $J = 7.0$ Hz), 8.96 (t), 7.78 (d $J = 6.6$ Hz), 3.38 (m), 1.61 (m), 1.38 (m). $^{195}$Pt NMR (D$_2$O, ppm): -2301. ESI-MS [M+H]$^+$ expected: 855.16 m/z, found: 855.20; [M]$^{2+}$ expected: 427.08 m/z, found: 428.47 m/z. Elemental analysis:

C$_{36}$H$_{34}$Cl$_4$N$_8$O$_2$Pt$_2$.3H$_2$O. Expected: C, 22.05; H, 4.11; N, 11.43%. Found: C, 21.93; H, 4.09; N, 11.21%.

$^1$H NMR. One dimensional NMR spectra were obtained in D$_2$O on a JEOL JNM-LA400 referenced internally to the solvent peak at 4.78 ppm. Diffusion ordered spectroscopy experiments in D$_2$O were obtained on a Bruker Avance 400 using 600 µL of sample in a Wilmad NMR tubes rated for 400-500 MHz use. The pulse sequence used was the standard DOSY sequence provided by Bruker and analysed with the Bruker relaxation software using a non-linear least-squares fit of the data to the equation: $I = c(t^2 g^2 D^2 (1-D^2/3))$. Each diffusion coefficient was determined using a $\Delta$ of 80 ms, a $\delta$ of 5 ms, and recycle time of 5 s and at a fixed temperature of 25°C.

Molecular modelling. Calculations were performed on a dual-Xeon processor, workstation using the DMol$^3$ (Delley, Delley) program in Accelrys’ Materials Studio (Accelrys). Individual geometry optimisations for the three platinum complexes were undertaken at the LDA PWC level (Perdew) using a DND basis set (Double Numerical plus d-functions on all non-hydrogen atoms) in order to locate structurally stable conformers. Bond distances were determined using the measurement tool of the modelling software program: Accelrys Discovery Studio 3.1 visualizer.

In vitro growth assays. The cytotoxicity of the metal complexes and the platinum-sCX[4] host-guest complexes were conducted using MTT-based in vitro growth inhibition assays using the A2780 and A2780/cp70 human ovarian carcinoma cell lines as previously described [24]. The A2780 and A2780cp70 ovarian cancer lines were grown in RPMI media containing 10% foetal calf serum,
penicillin streptomycin and L-glutamate in a 5% CO$_2$ atmosphere. The cells were trypsinised, counted and adjusted to 500-1000 cells per well in 96 well plates). Metal complex/sCX[4] stock solutions were diluted with RPMI to prepare a dilution series based on the total platinum concentration (0.1 – 100 μM). 10 μL aliquots were taken from these solutions and added in triplicate to each well along with RPMI only. The plate was then cultured for 24h, after which MTT (50 μL of a 5 mg mL$^{-1}$ solution) was added to the 200 μL of medium in each well and the plates were incubated at 37 °C for 4 h in the dark. Medium and MTT were then removed and the MTT-formazan crystals dissolved in 200 μL DMSO. Glycine buffer (25 μL per well, 0.1 M, pH 10.5) was added and the absorbance measured at 570 nm in a multiwell plate reader.

Results and discussion

NMR. Proton NMR is possibly one of the most powerful techniques for examining the host-guest complexes of sCX[4]. The large changes in the chemical shift of guest resonances for metal complex protons located inside the sCX[4] cavity due to shielding effects can give information of binding location, depth and orientation as well as an indication of the exchange kinetics.
Figure 2. $^1$H NMR spectra of (a) free complex (1), (b) sCX[4] and (c) the 1:1 host-guest complex showing the additional metal complex (1) resonances and the large upfield shift of the metal complex methylene resonances due to their shielding within the sCX[4] cavity.

In the $^1$H NMR spectra each free metal complex (1-3) displays two doublet resonances at 8.95 and 7.76 ppm and either two or three resonances, representing the methylene protons, between 1.2 and 3.4 ppm (Figure 2). Free sCX[4] has three resonances, the aromatic protons are all magnetically equivalent and are observed as a singlet resonance at 7.52 ppm. The sCX[4] methylene proton resonance is observed at 3.29 ppm, and despite solvent exchange, freshly prepared samples have a broad hydroxyl resonance at 3.95 ppm.
Upon the addition of one equivalent of metal complex (1-3) to sCX[4] at ~1 mM in D$_2$O a white precipitate occurs, indicating the immediate formation of a host-guest complex. Despite this, enough complex remains in solution to obtain $^1$H NMR spectra. The binding of sCX[4] has a marked effect on the chemical shifts of the metal complex resonances. For all three metal complexes the aromatic protons are now no longer chemically equivalent and four doublet resonances are observed between 7.5 and 8.95 ppm (Figure 2). For the aliphatic resonances there are very large changes in their chemical shift and the observation of additional peaks in the $^1$H NMR spectra when the methylene protons become no longer chemically equivalent upon binding. In some cases there is significant broadening of the metal complex resonances as the host-guest complex goes into intermediate exchange kinetics on the NMR timescale.

For complex (1), the free metal complex has two aliphatic resonances at 3.40 and 1.66 ppm. Upon addition of sCX[4], as well as the additional two aromatic resonances, four aliphatic resonances are observed at 2.13, 1.22, -0.13 and -0.18 ppm (see Figure 2). For complex (2) four aromatic resonances are again observed and whilst free complex (2) has three aliphatic resonances at 3.4, 1.6 and 1.4 ppm, upon binding by sCX[4] five resonances are observed for the methylene protons. Two very broad peaks at 2.54 and 0.26 ppm, and three sharp resonances at 2.15, 2.03 and 1.17 ppm. Finally for complex (3), again the four aromatic resonances are observed upon sCX[4] binding and in the aliphatic region, there are numerous very broad and overlapping resonances between 0.2 and 3.1 ppm.

Overall, the very large changes in chemical shift are consistent with the binding of other platinum complexes to sCX[4] and clearly places the methylene chain of the three metal complexes within the sCX[4] cavity.[6, 7] Importantly, the extra metal complex resonances observed in both the aromatic
and aliphatic regions of the NMR indicate that the metal complexes are changing shape/conformation upon binding to the macrocycle in such a way that the two sides of the complexes are no longer magnetically equivalent.

The binding of complex (1) to sCX[4] was also examined using $^1$H diffusion ordered NMR spectroscopy (DOSY). The large amount of precipitate upon binding of complexes (2 and 3) excluded their analysis by DOSY. The diffusion coefficient of the free metal complex is $6.73 \pm 0.37 \times 10^{-10}$ m$^2$s$^{-1}$. Upon binding by sCX[4] the metal complex’s diffusion coefficient drops to $5.55 \pm 0.13 \times 10^{-10}$ m$^2$s$^{-1}$, which is consistent with an increase its hydrodynamic radius as the host-guest complex is formed. The diffusion coefficient of free sCX[4] has previously been determined as $3.21 \times 10^{-10}$ m$^2$s$^{-1}$ [6].

Molecular modelling. To further understand the binding of the three metal complexes to sCX[4], molecular models of each host-guest complex were generated using the results of the $^1$H NMR experiments which indicated placement of the metal complexes’ aliphatic chain centre most within the macrocycle cavity (Figure 3).

The models all indicate significant folding of the methylene chains. Each linker is considerably longer than the diameter of the sCX[4] cavity, and so the linker folds to maximise the hydrophobic interactions between itself and the inner surface of the macrocycle. The result is a significant shortening of the end-to-end length of all three complexes (Table 1).
Table 1. Relevant bond lengths of the free metal complexes and the metal complexes upon folding to form the sCX[4] host-guest complexes.

<table>
<thead>
<tr>
<th>Metal complex state</th>
<th>Bond lengths (Å)</th>
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<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Complex (1)</td>
<td>Complex (2)</td>
<td>Complex (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NH—NH</td>
<td>O—O</td>
<td>Pt—Pt</td>
<td>NH—NH</td>
<td>O—O</td>
<td>Pt—Pt</td>
</tr>
<tr>
<td>Free complex</td>
<td>6.23</td>
<td>10.35</td>
<td>19.9</td>
<td>8.75</td>
<td>12.8</td>
<td>22.2</td>
</tr>
<tr>
<td>Host-guest complex</td>
<td>5.90</td>
<td>5.50</td>
<td>17.9</td>
<td>7.01</td>
<td>6.88</td>
<td>19.1</td>
</tr>
</tbody>
</table>
The Pt-to-Pt length of complex (1) shortens by 11%, complex (2) by 16% and complex (3) by 20%.

Interestingly, for the free metal complexes the end-to-end lengths of the amide-nitrogen atoms are always shorter than the end-to-end lengths of the amide-oxygen atoms, but when binding to sCX[4] the folding of the linker causes the groups to rotate in such a way that the end-to-end lengths of the amide-oxygens are always shorter than the amide-nitrogens.

Figure 3. Energy minimised models of the three dinuclear platinum host-guest complexes with the sCX[4] macrocycle, showing from bottom to top complexes (1-3), respectively. The models show the significant folding of the metal complexes’ aliphatic chains to maximise hydrophobic effects with the inner surface of the macrocycle. Counter ions are not shown.
The host-guest complex is stabilised not just by hydrophobic effects but also by intra- and intermolecular hydrogen bonds (Figure 4, Table 2). Four intramolecular OH—O hydrogen bonds stabilise the base of the sCX[4] as is typical of this macrocycle. Four intermolecular hydrogen bonds are observed between the metal complexes and sCX[4]; three of these hydrogen bonds (N—O, NH—O and O—O) are from one side of the metal complexes with the other hydrogen bond (NH—O) observed from the other side of the metal complexes.

![Molecular model of complex (3) and sCX[4]](image)

Figure 4. The molecular model of complex (3) and sCX[4] showing the four hydrogen bonds that hold the bottom of the calixarene macrocycle together (dashed green lines) and the four intermolecular hydrogen bonds that stabilise the host-guest complex (solid green lines).


<table>
<thead>
<tr>
<th>Hydrogen bond</th>
<th>Complex (1) Length (Å)</th>
<th>Complex (2) Length (Å)</th>
<th>Complex (3) Length (Å)</th>
</tr>
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<tbody>
<tr>
<td>NH—O (1)</td>
<td>1.76</td>
<td>1.74</td>
<td>1.76</td>
</tr>
<tr>
<td>NH—O (2)</td>
<td>1.86</td>
<td>1.74</td>
<td>2.39</td>
</tr>
<tr>
<td>N—O</td>
<td>2.96</td>
<td>2.88</td>
<td>3.08</td>
</tr>
<tr>
<td>O—O</td>
<td>2.86</td>
<td>2.84</td>
<td>3.07</td>
</tr>
</tbody>
</table>
Cytotoxicity. As the metal complexes were designed as potential anticancer drugs and the ultimate goal is the use of sCX[4] as a targeted drug delivery vehicle, the cytotoxicity of the free metal complexes and their host-guest complexes with sCX[4] were examined using in vitro growth inhibition assays. Their IC$_{50}$ (the concentration of metal complex required to inhibit cell growth by 50%) was compared to the current platinum standard: cisplatin. All three of the metal complexes are a magnitude of order more active than cisplatin in the sensitive human ovarian carcinoma cell line A2780 (Table 3). Within the error of the experiments, binding to sCX[4] does not affect the cytotoxicity of the shortest (1) and longest (3) of the metal complexes. For complex (2) binding by sCX[4] appears to slightly increase the metal complex’s cytotoxicity.

**Table 3.** The in vitro cytotoxicity of the three platinum complexes with, and without, one equivalent of sCX[4] in the human ovarian carcinoma cell line A2780 and its cisplatin resistant sub-line A2780cp70. Rf is the resistance factor and is determined by the IC$_{50}$ in the resistant line divided by the IC$_{50}$ in the sensitive line.

<table>
<thead>
<tr>
<th>Metal complex</th>
<th>IC$_{50}$ ($\mu$M)</th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A2780</td>
<td>A2780cp70</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.22 ± 0.03</td>
<td>3.82 ± 0.21</td>
</tr>
<tr>
<td>sCX[4]$^+$</td>
<td>&gt;1500</td>
<td>1500</td>
</tr>
<tr>
<td>1</td>
<td>0.029 ± 0.012</td>
<td>4.94 ± 0.09</td>
</tr>
<tr>
<td>1—sCX[4]</td>
<td>0.030 ± 0.004</td>
<td>12.41 ± 1.08</td>
</tr>
<tr>
<td>2</td>
<td>0.041 ± 0.005</td>
<td>2.72 ± 0.77</td>
</tr>
<tr>
<td>2—sCX[4]</td>
<td>0.026 ± 0.004</td>
<td>3.75 ± 0.50</td>
</tr>
<tr>
<td>3</td>
<td>0.028 ± 0.011</td>
<td>2.84 ± 0.15</td>
</tr>
<tr>
<td>3—sCX[4]</td>
<td>0.052 ± 0.018</td>
<td>0.576 ± 0.025</td>
</tr>
</tbody>
</table>

a. Data taken from reference [7].
The A2780cp70 is a sub-line of A2780 which has been developed to express resistance to cisplatin treatment. This is observed by the much higher cisplatin IC₅₀ in this cell line, 3.82 µM, compared with the sensitive cell line, 0.22 µM. The resistance factor (Rf) is a measure of a drug’s activity in the matched cell lines, with an Rf value of 1 or lower indicating an ability to circumvent resistance. All three metal complexes lose their activity in the A2780cp70 cell line and do not overcome resistance. There does however appear to be a general trend demonstrating that the longer the length of the metal complex, the more active it is in the resistant cell line to the extent that the host-guest complex of complex (3) and sCX[4] has a smaller resistance factor than cisplatin.

These in vitro assays are used only as an initial cytotoxicity screen as we have discovered over the last five years that the total effect of delivery vehicles on the activity of platinum anticancer complexes can only be properly evaluated in vivo. This is due to the nature of the in vitro assay which has relatively short drug exposure times, grows cancer cells as monolayers rather than solid tumours and does not allow for the dynamics of drug distribution and uptake in a living animal. In many cases drug delivery vehicles have no effect on metal complex cytotoxicity in vitro, but in several cases the addition of the delivery vehicles results in significantly improved cytotoxicity in vivo. As such, the results obtained in this study provide preliminary cytotoxicity data, but only in vivo experiments can definitively examine the effect on sCX[4] on the metal complexes.

**Conclusions**

In this work the formation of host-guest complexes of three dinuclear platinum complexes with the bowl-shaped macrocycle para-sulphonatocalix[4]arene has been studied. All three complexes bind the macrocycle so that the aliphatic chain of their bridging linker resides in the cavity. Such binding results in significant folding of the metal complexes such that their end-to-end length is reduced by...
as much as 20%. Binding is stabilised by hydrophobic forces between the metal complexes’ bridging ligand and the inner surface of the macrocycle’s cavity, and four intermolecular hydrogen bonds; three on one side of the metal complexes and one on the opposite side. The result is a loss of chemical symmetry of the metal complex which is observed in the $^1$H NMR spectra. Binding of the macrocycle to the metal complexes has little, to no, effect on their in vitro cytotoxicity in the human ovarian carcinoma cell line A2780, but a significant effect in the cisplatin-resistant cell line A2780cp70. The results of this work provide important knowledge on the nature of the host-guest complex formation of flexible dinuclear platinum complexes and can now be used to develop more advanced drug delivery vehicles where sCX[4] can be conjugated with tumour targeting groups to better deliver multinuclear platinum drugs in chemotherapy.

References