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Adamantane–platinum conjugate hosted in β-cyclodextrin: Enhancing transport and cytotoxicity by noncovalent modification

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ABSTRACT

This work reports the synthesis of a complex of a carboplatin analog having tethered adamantane that is encapsulated in the hydrophobic cavity of β-cyclodextrin (βCD) and its cytotoxic activity towards human neuroblastoma cells (SK-N-SH). We found that this inclusion complex of βCD adamantane carboplatin analog exhibited higher cytotoxicity towards SK-N-SH cells than carboplatin itself, and the inclusion complex exhibited a higher binding to plasmid pBR322 deoxyribonucleic acid (DNA) than carboplatin. Confocal fluorescence images of SK-N-SH cells treated with βCD having an attached fluorescein isothiocyanate (FITC)-tag exhibited fluorescence in the vicinity of the nuclei of the neuroblastoma cells. Direct measurements of the platinum content in SK-N-SH cells using inductively coupled plasma mass spectrometry (ICP-MS) indicated that the uptake rate of carboplatin was about 4 times higher than βCD adamantane carboplatin analog inclusion complex. When compared to carboplatin, we believe that the higher cytotoxicity of inclusion complex towards SK-N-SH cells is due to its higher DNA binding ability as compared to carboplatin, and more efficient delivery to the nucleus of the cell. This work suggests that the advantage of deliberate noncovalent modification with βCD through host-guest chemistry may also be broadly applicable to other anticancer agents as well.

Sugar derivatives including cyclodextrins and other benign molecules have been used in drug formulations often by simple mixing, for improving the drug properties such as solubility, stability and bioavailability.[1–5] This work explores the enhancement of the cytotoxic effect of a carboplatin analog compound by using a host–guest chemistry to noncovalently tether a dicarboxylate platinum(II) chelate with β-cyclodextrin. Platinum-based compounds are an important class of active chemotherapeutic agents for treating various cancers,[6–9] yet the mechanisms of drug action are still unraveling.[10–12] Recent work has shown that in the case of cisplatin and carboplatin, a platinum carbonato complex rather than the reactive mono-aqua complex may be responsible for biological effects in cells.[13,14] In spite of this growing understanding, the development and optimization of the pharmaceutical properties of platinum-based therapeutic agents has been largely empirical.[15–18] In this work, we explore the strategy of noncovalent modification via host–guest chemistry to enhance the cytotoxicity of a carboplatin analog. We used the cyclic sugar β-cyclodextrin (βCD) to encapsulate an adamantane-derivatized dicarboxylate that was complexed with a platinum(II) ion.

Noncovalent modification of pharmacologically active agents to enable host–guest chemistry is a relatively unexplored approach for controlling drug activities, and it offers potential enhancement of desired properties such as increased water-solubility, cellular uptake and targeted delivery without severely altering the efficacy of the agents.[19,20] Cyclodextrins have been widely used for improving drug properties such as solubility, stability and bioavailability.[2,3,5] Recently, Maxfield and co-workers discovered that βCD has a high propensity to be taken up by mammalian cells, presumably through pinocytosis.[21] This discovery suggests the possibility of enhancing the potency of platinum(II)-based compounds by noncovalent modification of the agent with βCD. We synthesized an adamantane–dicarboxylate which, after complexation with believed to be βCD forms a host–guest complex, which is further reacted with *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2]^{+2}$ to afford **6** (Scheme 1). The binding of adamantane with βCD is stoichiometric in water, with one adamantane group hosted in the hydrophobic annular cavity of βCD. We examined the cytotoxicity of this host–guest complex against neuroblastoma (SK-N-SH) cells, and compared the cytotoxicity of the carboplatin analog to that of carboplatin.

Scheme 1 shows the synthesis of the βCD adamantane carboplatin analog host–guest complex (**6**). Briefly, tosylation of adamantane alcohol (**1**),[22] afforded the tosylate (**2**). Displacement of tosyl group in (**2**) with diethylmalonate gave the diethyl ester (**3**), which on hydrolysis provided the diacid (**4**). We noted that direct coupling of (**4**) and *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2]^{+2}$ (**5**),[23,24] gave a grey solid that was insoluble in all solvents studied (see Supplementary data, Scheme S1). The presence of ^{15}N label in the platinum diaqua complex facilitated the characterization of the

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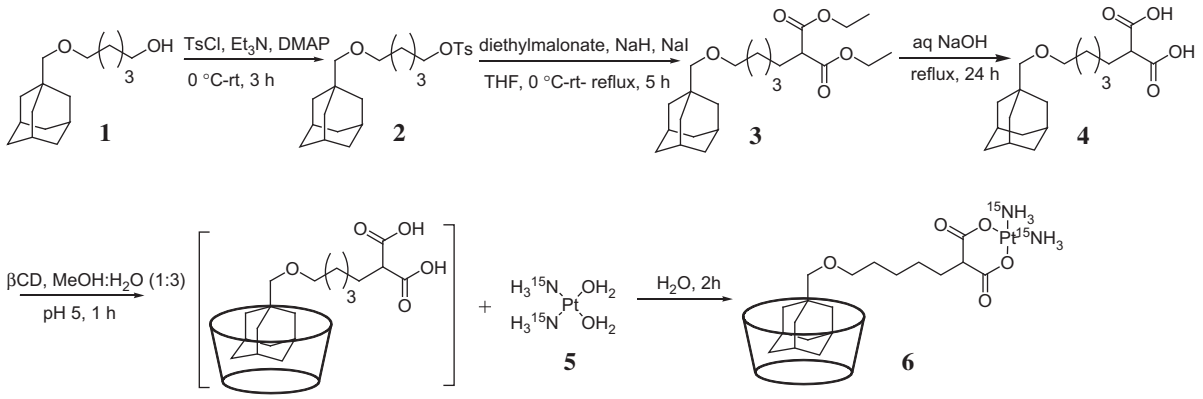

Scheme 1.

analog using heteronuclear single quantum coherence [^{1}H, ^{15}N] HSQC NMR. Because cyclodextrins are known to form water soluble host-guest complexes with hydrophobic groups,[2] we tried using βCD to possibly extract the product from the grey solid into water. Addition of βCD to an aqueous suspension of the grey solid did not result in detectable product. However, treating the diacid (**4**) first with 1 equiv of βCD, followed by addition of *cis*-$[Pt(^{15}NH_3)_2(H_2O)_2]^{+2}$ (**5**), resulted in the desired water-soluble conjugate (**6**). These details of the synthesis suggest that the exchange rate of the βCD adamantane host–guest complex is likely slow, and that using βCD likely provides a general approach to render hydrophobic small molecules soluble in water. The chemical shift (δ, ppm) in HSQC for ^{1}H = 4.13 and ^{15}N = −82.0, was similar to those observed for carboplatin (Fig. 1).[24]

Because **6** and carboplatin share a common functional group, we studied the cytotoxicity of both compounds towards human neuroblastoma cells (SK-N-SH).[14] The neuroblastoma (SK-N-SH) cells were grown for 24 h in 96-well plates, and the cells were then treated with different concentrations of **6** or carboplatin for an exposure time (length of exposure of the cells to the agent) of 1 h. Viabilities were assayed immediately following exposure to the agent and culturing cells in fresh medium for 24, 48 and 72 h, referred to as the recovery time.[25] At each recovery time, the number of live cells was determined by a colorimetric cell

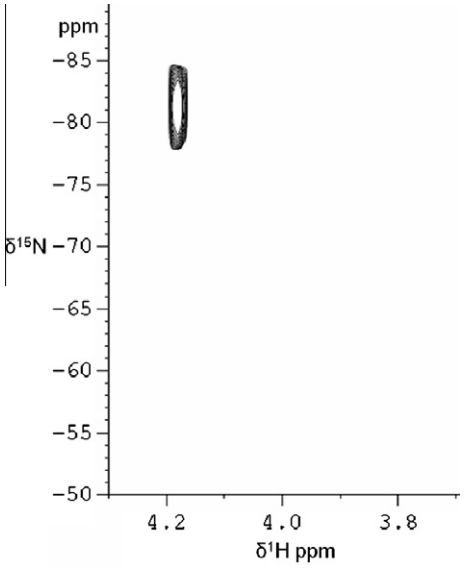

Figure 1. [^{1}H, ^{15}N] HSQC NMR of βCD adamantane carboplatin analog host–guest complex (**6**) in 5% D_2O/H_2O: δ (ppm) ^{1}H = 4.13, δ ^{15}N = −82.0.

counting assay (CCK-8 assay).[26] The assay utilizes a water soluble tetrazolium salt, which is reduced by dehydrogenases in the live cells to give a yellow colored product (formazan). The amount of the formazan dye produced in the culture medium is directly proportional to the number of living cells. The survival (%) was calculated by using the equation (OD_{450} sample)/(OD_{450} control) × 100, where the sample OD was obtained from the cells treated with **6** or carboplatin, and the control OD was obtained from cells.[27]

Figure 2 shows the plots of survival (%) of neuroblastoma (SK-N-SH) cells as a function of concentration for **6** and carboplatin at different recovery times. Immediately after exposure to carboplatin (0 h recovery time), there was no observable toxic effect and the survival (%) does not change with drug concentration. However for longer recovery times, the cells exhibit an unusual dose dependent behavior to carboplatin. The cytotoxicity of carboplatin increases in the drug concentration range, 0 to ~500 μM, reaches a maximum at ~500 μM and decreases in the range, 500–1000 μM (Fig. 2A). Recently it was shown that carboplatin exists in a monomer–dimer equilibrium in aqueous solution, and there is evidence that the same equilibrium may also exist in culture media.[28] Since the concentration of a species in a monomer–dimer equilibrium is dependent on the total concentration of the agent in the medium, it is possible that the reduced cytotoxicity of carboplatin observed at high concentration is due to the presence of the dimer which may be less toxic to the cells than the monomeric form of the drug. Figure 2B shows the survival (%) of **6** as a function of concentration and recovery time which sharply contrasts with that of carboplatin. While being non-toxic at all concentrations for a recovery time of 0 h, **6** exhibited the typical dose–response expected at longer recovery times with the longest recovery time (72 h) producing the greatest amount of cell death. Comparison of the survival (%) for **6** with carboplatin indicates that overall **6** is more toxic to the neuroblastoma cells than is carboplatin.

We also examined the effects of different exposure times of **6** and carboplatin on neuroblastoma cells at a recovery time of 0 h (viabilities were measured immediately following exposure). Confluent layers of SK-N-SH cells were incubated with 500 μM of each agent for 2, 4, 6, 8 and 12 h in 96-well plates. After each exposure time, medium containing **6** or carboplatin was replaced with fresh culture medium, and the number of live cells was immediately determined by CCK-8 assay. Figure 3 shows that the survival (%) for both carboplatin and **6** for exposure times up to 12 h, gives the expected dose–response effect, as is shown in the low concentration range of Figure 2, with **6** being more toxic to the cells than carboplatin.

Maxfield and coworkers recently discovered that endocytosis of βCD is responsible for cholesterol reduction in Niemann–Pick type

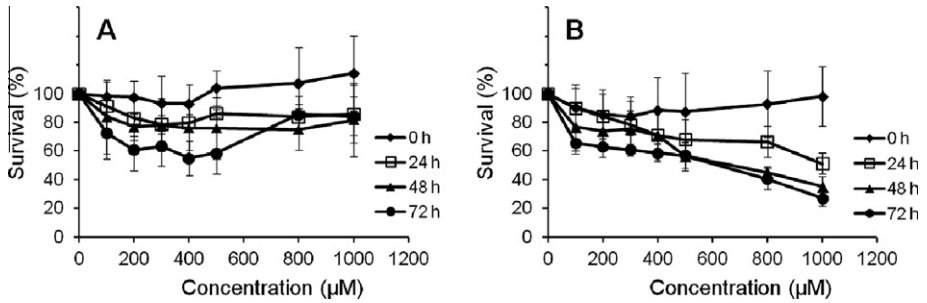

Figure 2. Plots of survival (%) of SK-N-SH cells treated with (A) carboplatin, and (B) **6**, as a function of concentration with different recovery times at 0, 24, 48 and 72 h.

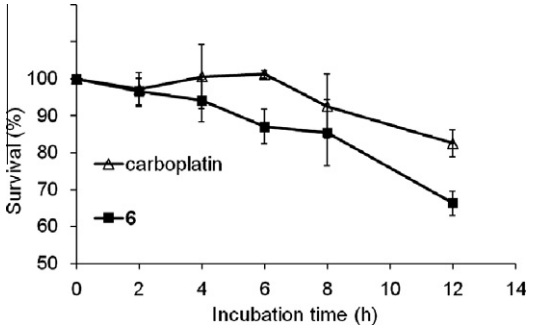

Figure 3. Survival (%) of SK-N-SH cells when treated with 500 μM carboplatin and **6** for different incubation times of 2, 4, 6, 8, and 12 h with a recovery time of 0 h.

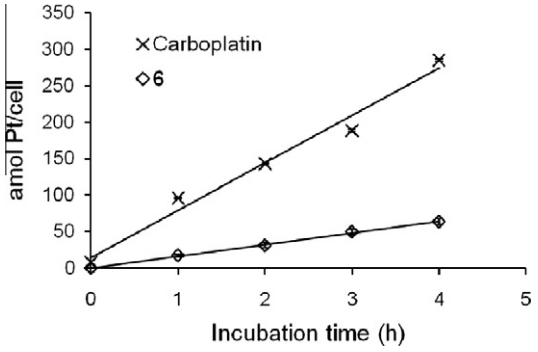

Figure 5. Platinum (amol) taken up per SK-N-SH cell, when exposed to carboplatin (500 μM) and **6** (500 μM) as a function of time. The rate of uptake (amol of Pt $cell^{-1}$ h^{-1}) was found to be 16.0 ± 0.56 and 64.9 ± 1.4 for cells treated with **6** and carboplatin, respectively.

C mutant cells.[21] This effect is believed to be due to the internalization of cyclodextrin in the cells through fluid phase pinocytosis.[21,29,30] To validate the internalization of βCD in SK-N-SH cells, we synthesized fluorescein isothiocynate (FITC) tagged cyclodextrin.[31] Confocal fluorescence images showed that all SK-N-SH cells treated with FITC-tagged βCD exhibited green fluorescence (Fig. 4), suggesting that FITC-tagged βCD were efficiently internalized into the cancer cells. Interestingly, the FITC-tagged βCD appeared to be localized around the nucleus of the cells.

Because **6** is more cytotoxic than carboplatin towards SK-N-SH cells, we examined the uptake rate at which both compounds enter the cells using inductively coupled plasma mass spectrometry (ICP-MS).[14,32] Six-well plates with each well containing 3.5×10^5 of SK-N-SH cells suspended in culture medium were incubated at 37 °C for 24 h to allow the cells to adhere to the surface of the well. The number of viable cells in the wells was measured by light microscopy using a hemocytometer and trypan blue. The cells in the wells were treated with 500 μM of **6** or carboplatin for different incubation times of 0, 1, 2, 3, and 4 h. The cells were trypsinized, pelleted by centrifugation, and the pellets were washed twice with phosphate buffered saline (PBS, 10 mM). The cells were lysed in 70% nitric acid at 50 °C for 48 h and the cell lysate was analyzed for platinum by ICP-MS. The uptake rate was determined by calculating the amount of platinum taken up by the cell in units of attomoles (10^{-18}) of platinum which was plotted versus incubation time (Fig. 5). The uptake rate of platinum per cell, in attomoles (10^{-18}) per hour, was calculated from the total number of viable cells (number of viable cells in each well in the 6-well plates, before treatment with **6** and carboplatin were 2.04×10^5 and 3.85×10^5, respectively) and the number of moles of platinum that

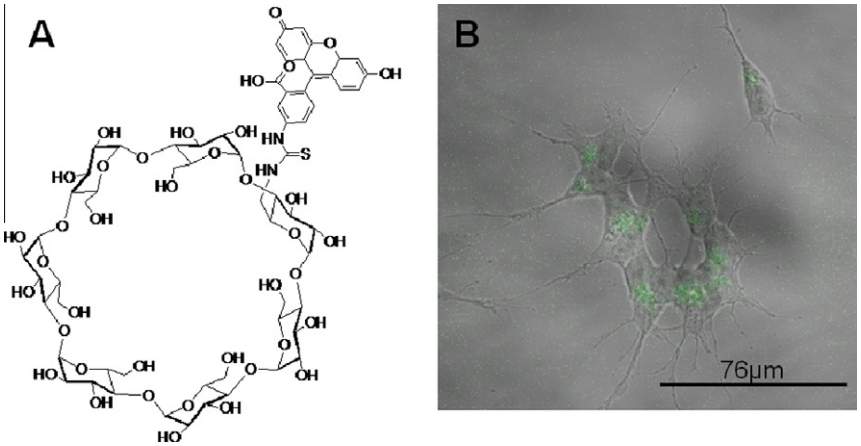

Figure 4. (A) Structure of FITC-tagged βCD, (B) confocal fluorescence image of SK-N-SH cells incubated with 150 nM of FITC-tagged βCD.

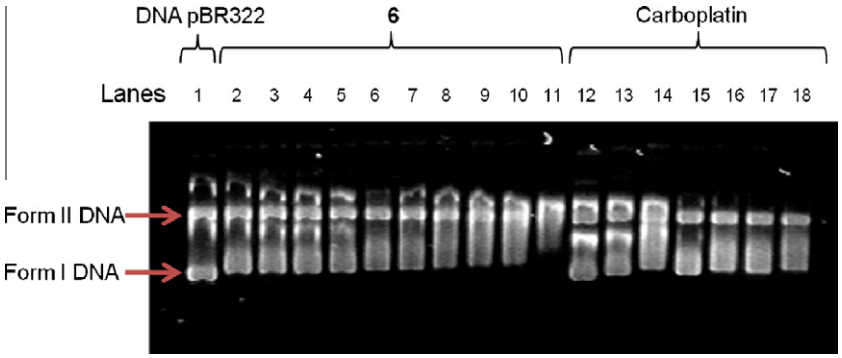

Figure 6. Agarose gel electrophoresis image of pBR322 DNA (33.3 μM base pairs) after incubation with **6** or carboplatin in 10 mM HEPES, pH 7.4 for 24 h. Lanes 2 through 11 contained 30, 35, 40, 45, 50, 60, 70, 80, 90, and 100 μM of **6**, and lanes 12 through 18 contained 30, 40, 50, 70, 80, 90 and 100 μM of carboplatin. Lane 1 (control) contained only pBR322 DNA.

each cell took up from the culture medium per hour.[14] The uptake rates of **6** and carboplatin at 500 μM by SK-N-SH cells were found to be 16.0 ± 0.56 and 64.9 ± 1.4 amol of Pt cell^{-1} h^{-1}, respectively. Comparing the uptake of platinum (Fig. 5) and the survival (%) of SK-N-SH cells (Fig. 3) after 4 h incubation shows that while the uptake of carboplatin was ~4.4 times higher than that of **6**, there was a decrease of ~6% in the survival of SK-N-SH cells treated with **6**, and no observable decrease of survival for cells treated with carboplatin.

These data indicate that the uptake rate of carboplatin by neuroblastoma cells is ~4 times higher than **6**, while the cytotoxicity experiments show that **6** is more cytotoxic than carboplatin. Hence the amount of platinum entering the cell alone cannot be the basis for the different cytotoxic effects of the compounds. However, considering that the main target for the platinum agents is believed to be DNA, carboplatin could be more susceptible to reaction with, and inactivation by, components in the cytosol than is the case for **6**.

Because binding to DNA is believed to be a key element for platinum-based therapeutic agents,[33] we examined and compared the binding of carboplatin and **6** to plasmid pBR322 DNA using agarose gel electrophoresis.[34–36]

Plasmid DNA (pBR322) exists in different chemical forms—covalently closed, nicked and linear. Form I DNA is a supercoiled closed circular DNA, and Form II DNA is a nicked circular DNA without supercoiling, and thus have different mobilities in an agarose gel. Form I DNA is wound into a compact structure, and hence is the fastest moving conformation of the plasmid. Once the plasmid DNA is treated with the platinum agents, binding and unwinding of Form I DNA will cause a decrease in the mobility of Form I DNA.[33] After incubating pBR322 for 24 h with different concentrations (30–100 μM) of **6** or carboplatin, the image of the agarose gel (Fig. 6) shows that the mobility of closed circular Form I DNA decreased with an increase in concentrations of **6** and carboplatin, but decrease in mobility of Form I with **6** is greater than the case for carboplatin.

Since the leaving ligands for both compounds are expected to be the dicarboxylate, the DNA binding unit in both cases is most likely *cis*-$\{Pt(NH_3)_2\}^{+2}$ which, in comparison to cisplatin, would bind to and unwind supercoiled Form I closed circular DNA (negative writhe) through the formation of a 1,2 intrastrand crosslink.[6] For cisplatin, platinum binding unwinds Form I DNA, which reduces the supercoiling (writhe approaches zero), causing the closed circular DNA to become more open and 'doughnut-like' in nature, and hence causing a decrease in the electrophoretic mobility of the platinated DNA in the gel. Since mobility is approximately inversely proportional to platinum loading on the DNA, Figure 6 suggests that, during the 24 h incubation period, more platinum from **6** binds DNA than in the case for carboplatin. For example, the DNA in lane 11 (**6** at 100 μM) migrated less than in lane 18 (carboplatin at 100 μM). As shown in Figure 6, carboplatin and **6** have little effect on the mobility of the nicked circular, Form II DNA (no supercoiling) in the gel. We note that βCD alone does not show any binding to pBR322 DNA (see the Supplementary data).

Collectively, the results of this study suggest that, when compared to carboplatin, the higher cytotoxicity of **6** towards neuroblastoma cells is not related to the amount of platinum that enters the cell, but perhaps to the more effective transport of **6** to the nucleus via the appended βCD moiety followed by efficient binding to nuclear DNA. Other than binding nuclear DNA, platinum based complexes have demonstrated the potency for targeting and inhibiting protein activities.[37–41] For example, a number of platinum(II) complexes have been reported to inhibit human thioredoxin reductase,[39] mammalian topoisomerases[40] and matrix metalloproteinase (MMP-3),[41] by undergoing ligand-substitution reactions with reactive amino acid residues. Studies of these agents in vivo including binding to DNA as well as to potential protein targets will further decipher the mechanism of the cytotoxicity of these βCD hosted agents.

This work reports the synthesis of a βCD encapsulated adamantane–platinum host–guest complex, **6**. Confocal fluorescence indicated the internalization of βCD at locations close to nuclei of cells. The cytotoxicity assays showed that **6** was more toxic than carboplatin toward neuroblastoma (SK-N-SH) cells. Agarose gel electrophoresis confirmed the binding of **6** to pBR322 DNA, while βCD alone does not bind DNA, and showed that **6** exhibited a higher level of binding than carboplatin. Interestingly, the ICP-MS measurement indicated that carboplatin had a higher uptake rate than **6** into the cells. These results suggest that factors such as intracellular transport, receptor binding, and heterogeneous distributions of agents inside the cells likely play more important roles than just the cellular uptake for cytotoxic effects. This work suggests that using deliberate host-guest chemistry can be a powerful strategy to influence the binding activities and other pharmaco-properties of a therapeutic agent. We believe that noncovalent modification can be integrated into drug design, formulation, as well as other functions such as targeted delivery and combined drug therapy, rather than treating them as separate topics.

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Supplementary data

Supplementary data (details of synthesis and characterization of synthetic products and other experimental details) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.10.006.

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