Oxa-aza Crown Ethers as Ligands for Mixed-Ligand Cisplatin Derivatives and Dinuclear Platinum Anticancer Drugs

Bart A. J. Jansen,[a] Peter Wielaard,[a] Hans den Dulk,[b] Jaap Brouwer,[b] and Jan Reedijk*[a]

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In the search for novel platinum anticancer drugs, cisplatin derivatives and dinuclear platinum complexes containing oxa-aza crown ether and oxa-diaza crown ether ligands have been prepared. The cisplatin derivatives *cis*-[$PtCl_2(NH_3)$(1,4,7,10,13-pentaoxa-16-azacyclooctadecane-*N*)] (**AO18**) and *cis*-[$PtCl_2(NH_3)$(1,4,7,10-tetraoxa-13-azacyclopentadecane-*N*)] (**AO15**), and the dinuclear cationic platinum complexes [{*trans*-$PtCl(NH_3)_2$}$_2$(μ-1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-*N*,*N*′)]$(NO_3)_2$ (**DAO18**) and [{*trans*-$PtCl(NH_3)_2$}$_2$(μ-1,4,10-trioxa-7,13-diazacyclopentadecane-*N*,*N*′)]$(NO_3)_2$ (**DAO15**) were investigated for their cytotoxic properties, cellular uptake and intracellular DNA binding in A2780 human ovarian cancer. The cisplatin derivative **AO15** shows the highest cytotoxic activity, whereas the cationic dinuclear complexes **DAO18** and **DAO15** display a disappointing lack of biological activity at concentrations up to 100 μM. In the intracallular DNA platination experiments, the fifteen-membered rings were found to have a threefold higher intracellular DNA binding compared to their 1eight-membered analogues for the neutral cisplatin derivatives as well as for the cationic dinuclear complexes. The crown ether complex with the most effective binding to DNA, **AO15**, also shows the highest cytotoxicity against A2780 cancer cells. It is remarkable that, although **AO15** binds more effectively to DNA than cisplatin, its cytotoxic effect is much lower.

Introduction

The success of the anticancer drug cisplatin [*cis*-$PtCl_2(NH_3)_2$] has inspired the search for new platinum complexes with anticancer properties.[1−3] In the current paper, new platinum complexes based on aza crown ethers are described for their cytotoxic properties and binding to intracellular DNA. Binding to DNA is assumed to be a major mechanism of action,[4] leading to the death of cancer cells in platinum anticancer therapy.[5]

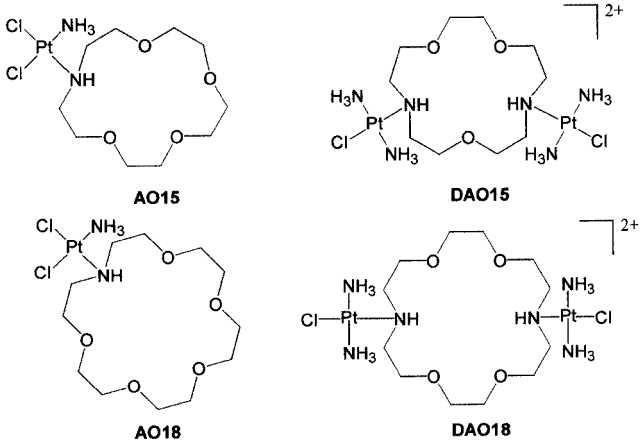
Although crown ethers are not known to possess medicinal properties themselves, they may improve drug uptake and transport properties.[6,7] Crown ethers are known to form cationic complexes with alkali metal ions and tertiary amines.[8] The special complexing properties of crown ethers have led to applications in drug delivery systems[9,10] and as targeting functionalities incorporated in drug derivatives and DNA-binding agents.[11−18] It was shown in DNA-binding studies that the positive charge of cation−crown ether complexes increases the affinity of crown ether linked compounds with the polyanionic phosphate backbone of DNA.[11,19] Thus, crown ether derivatized drugs may gain an increased interaction with DNA by the formation of cationic complexes with ions that are abundant in cells, such as sodium (5−15 mM) or potassium (140 mM).[20]

The groups of Keppler[21] and Sohn[22] have reported complexes where a crown ether is linked to a platinum atom through a functionalized bipyridine ligand. The bipyridine−crown ether compounds are biologically active and overcome cisplatin resistance. Sohn[22] also prepared carboplatin analogues of theses complexes by replacing the chloride leaving groups with 1,1-cyclobutanedicarboxylate. Similar to carboplatin, these complexes have a significantly lower cytotoxicity than the corresponding cisplatin (chloride) derivatives, probably due to their lower reactivity. A dinuclear platinum complex with a polycarboxylato crown ether as a bridging ligand has been reported by Fruhauf and Zeller.[23,24] The crown ether acts as a leaving group, releasing aquated species of cisplatin. The complex shows remarkable activity and DNA-binding properties in sensitive ovarian tumor cells as well as cisplatin-resistant cell lines.

In the platinum−crown ether complexes described above, the platinum atom is linked through a spacer or to pendant coordinating groups. In the current chapter, two types of platinum complexes are presented (Scheme 1), where the

[a] Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University,
P. O. Box 9502, 2300 RA Leiden, Netherlands
Fax: (internat.) + 31-71/527-4671
E-mail: reedijk@chem.leidenuniv.nl
[b] Molecular Genetics, Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University,
P. O. Box 9502, 2300 RA Leiden, Netherlands

platinum atom is directly coordinated to the nitrogen atom in an aza crown ether. The first type comprise mixed-amine cisplatin derivatives of the general formula *cis*-$[PtCl_2(NH_3)(L)]$, where L are azacrown ethers. The second type are cationic dinuclear *trans* complexes using diaza crown ethers as linkers, similar to the anticancer active cationic dinuclear *trans*-(diaminoalkyl)platinum complexes as developed by Farrell.[25–28] The cytotoxic properties of the complexes are investigated, and the results are related to the efficiency of DNA binding in A2780 human ovarian cancer cells.



Scheme 1. Oxa-aza crown ether cisplatin derivatives **AO18** and **AO15** and the cationic oxa-diaza crown ether dinuclear platinum complexes **DAO18** and **DAO15**

Results and Discussion

Synthesis

Both cisplatin derivatives[29,30] and dinuclear oxa-aza crown ether platinum complexes[25] could be prepared employing standard synthesis methods.

The reactions of $Ph_4P[Pt(NH_3)Cl_3]$ with the appropriate oxa-aza crown ether produced the asymmetric cisplatin derivatives **AO18** and **AO15**. Yields after recrystallization from methanol were found to be 15% and 39%, respectively. The ^{195}Pt NMR shifts of **AO18** and **AO15** are at $\delta = -2222$ and -2231 ppm, respectively, both in the typical range for $PtCl_2N_2$ complexes.[31]

The dinuclear complexes **DAO18** and **DAO15** were obtained in 24% and 51% yield from the reaction of silver nitrate activated *trans*-$PtCl_2(NH_3)_2$ with the appropriate diamine linker. The oxa-diaza crown ether complexes have the platinum atom coordinated to a secondary amine group. The ^{195}Pt NMR chemical shifts of **DAO18** ($\delta = -2408$ ppm) and **DAO15** ($\delta = -2424$ ppm) are somewhat higher than those of related alkyldiamine complexes where the platinum atom coordinates to primary amines ($\delta \approx -2385$ ppm).[25,32]

The formation of all four platinum complexes is quantitative, as can be monitored by ^{195}Pt NMR, the final yields are relatively low. The solubility properties of the crown ether complexes resulted in losses in the purification steps. Elemental analysis results are not completely satisfactory for all complexes, apparently the samples still include some impurities that were not removed even with repeated recrystallization. Possibly, the impurities are related to degraded crown ether moieties, although these were not apparent from the ^1H NMR spectroscopic data. The ^{195}Pt NMR spectra showed only a single signal even after overnight measurements. The inclusion of considerable amounts of highly active platinum species like cisplatin, that could influence the outcome of the cytotoxicity assays, can be ruled out on basis of these data.

Cytotoxicity Assays

All complexes were tested for cytotoxicity by determination of the IC_{50} values in the human ovarian cancer cell line A2780 and the cisplatin-resistant A2780cisR line. Cytotoxicity results are summarized in Table 1. In the A2780 cell line, the neutral cisplatin derivative **AO15** is the most active oxa-aza crown ether platinum complex, followed by **AO18**. Interestingly, in a recently published series of crown ether analogues of actinomycin D, the 15-crown-5 derivatives were also found to be biologically more active than the 18-crown-6 compounds.[18] The dinuclear diazacrown ether complexes show a disappointing lack of cytotoxic effect. In the cisplatin-resistant cell line, all oxa-aza crown ether complexes failed to induce a cytotoxic effect at concentrations as high as 100 μM. When compared with the established platinum anticancer drug cisplatin, none of the oxa-aza crown ether complexes appears to be a potential anticancer drug.

Table 1. Cytotoxicity results in human ovarian cancer A2780 and the cisplatin-resistant derivative A2780cisR

Compound	IC_{50} (μM)	
	A2780	A2780cisR
cisplatin	0.37	2.4
AO18	66	> 100
AO15	21	> 100
DAO18	> 100	> 100
DAO15	> 100	> 100

Intracellular DNA Platination

A2780 cells were incubated for 1 h at drug concentrations of 200 μM. After the DNA was extracted from the cells, the drug-to-base pair ratio (r_b) was determined by UV and FAAS. The results are presented in Figure 1.

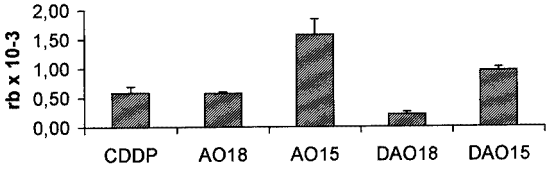


Figure 1. DNA binding (r_b = drug-to-basepair ratio) of cisplatin and aza crown ether platinum complexes in A2780 cells after a 1 h incubation at 200 μM

For both the mononuclear and dinuclear complexes, the 15-crown-5 derivatives show a higher binding to DNA than the corresponding 18-crown-6 compounds. The complex with the highest biological activity of the oxa-aza crown ether complexes studied, **AO15**, also shows the highest binding to DNA in the A2780 cells. The difference in lipophilicity may play an important role here, as it influences the permeation of the cell membrane. Interestingly, a comparable result was found for a series of crown ether derivatized phenoxazone drugs,[18] were the fifteen-membered compounds were also more effective in biological activity and intracellular DNA binding than the eighteen-membered derivatives.

It is remarkable that although **AO15** binds more effectively to DNA than cisplatin, its IC_{50} is much higher. Apparently, not only the amount of DNA damage is important for the cytotoxic effect, but also the type of molecule that binds DNA.

For the dinuclear cationic complexes, the fifteen-membered ring compound **DAO15** performs better in intracellular DNA binding than the eighteen-membered ring compound **DAO18**. Both show a reduced DNA binding compared to the corresponding neutral cisplatin derivatives. **DAO18** binds even less than cisplatin. This is remarkable, because in several cell studies related cationic polynuclear platinum complexes with alkyldiamine linkers were shown to have a much higher affinity for DNA than cisplatin.[28,33−35] Active uptake mechanisms,[32] that are thought to transport cationic polynuclear platinum complexes into cells, may not facilitate the transport of the crown ether compounds.

Conclusion

For both neutral cisplatin derivatives and the cationic dinuclear complexes, the fifteen-membered rings were found to have an increased binding to intracellular DNA compared to their eighteen-membered counterparts. The complex with the highest affinity for DNA, **AO15**, also showed the highest cytotoxicity of the series against A2780 cancer cells. Although **AO15** was found to bind more effectively to intracellular DNA than cisplatin, its cytotoxicity was found to be significantly lower. The effectivity of DNA binding appears not to be the determining factor for cytotoxicity. Apparently, the crown ether moiety plays a role here, but its interactions with for instance DNA repair systems would require further study.

Due to charge interactions, most cationic polynuclear platinum complexes show a significantly higher affinity for DNA than neutral platinum complexes, like cisplatin.[28,33−35] Surprisingly, the cationic dinuclear complexes with oxa-diaza crown ethers exhibit a lower DNA binding in the A2780 cells than cisplatin and its mono-aza crown ether derivatives. Although the efficiency of transport by diffusion probably depends on the lipophilicity of the linker, it has also been suggested that, due to their resemblance of natural polyamines like spermine and spermidine, cationic dinuclear complexes are taken up through active transport mechanisms.[34,35] The present results suggest that the linear shape of the linker is important for these molecules to enter the cell.

Experimental Section

General: Oxa-aza crownethers and oxa-diaza crown ethers were either a gift from Dr. L. G. A. van de Water (Leiden Institute of Chemistry), or obtained from Acros Chemicals, the Netherlands. K_2PtCl_4 was obtained as a loan from Johnson-Matthey. The complex $Ph_4P[PtCl_3(NH_3)]$ was prepared from *cis*-$[PtCl_2(NH_3)_2]$ using literature procedures.[29,30] Solvents and other chemicals were obtained from Acros and Aldrich. NMR spectra were recorded with a Bruker DPX 300 spectrometer with a 5-mm multi-nucleus probe. ^1H NMR spectra were determined with respect to external TMS. ^{195}Pt spectra were calibrated using K_2PtCl_4 as an external reference at $\delta = -1614$ ppm. Reaction mixtures could be monitored using ^{195}Pt NMR by adding 10% of a deuterated solvent (D_2O or $CDCl_3$) for the locking signal. C, H and N analyses were performed with a Perkin−Elmer 240B microanalyzer. Platinum concentrations were determined by using graphite-oven flameless atomic absorption spectroscopy (FAAS), based on a previously published method.[28] Measurements were carried out with a Perkin−Elmer 3100 AAS apparatus, equipped with a platinum hollow cathode lamp and an AS-60 graphite-oven autosampler. For each determination, 20 μL of sample was injected. The used furnace program was: drying 120 °C/90 s, ashing 1300 °C/60s, 20 °C/15s, atomization and measurement 2650 °C/5s, purging 2600 °C/5 s. The furnace was purged with argon gas.

Synthesis

***cis*-[$PtCl_2(NH_3)$(1,4,7,10,13-pentaoxa-16-azacyclooctadecane-*N*)] (AO18):** To a solution of 102 mg (0.155 mmol) of $Ph_4P[Pt(NH_3)Cl_3]$ in 5 mL of methanol a solution of 40 mg (0.152 mmol) of aza-18-crown-6 (1,4,7,10,13-pentaoxa-16-azacyclooctadecane) in 5 mL of methanol was added. The mixture was stirred for 48 h at room temperature. The intensity of the yellow/orange color of the solution gradually decreased. After 48 h, the volume was reduced to 5 mL by rotary evaporation and the mixture was allowed to stand overnight at −20 °C. A yellow precipitate was formed. The product was filtered off, washed with diethyl ether and dried in air. A second crop was obtained by reducing the volume further by rotary evaporation. The solution was allowed to stand overnight at −20 °C. The product was recrystallized from a minimum amount of methanol. Yield: 13 mg (15%). ^1H NMR (MeOD): $\delta = 2.5$ (m, 2 H), 2.83 (m, 2 H), 3.5 (m, 18 H), 3.78 (m, 2 H), 5.09 (m, 2 H), 5.23 (s, br, 1 H) ppm. ^{195}Pt NMR (MeOD): $\delta = -2222$ ppm. $C_{12}H_{28}Cl_2N_2O_5Pt$ (546.3): calcd. C 26.38, H 5.17, N 5.13; found C 25.37, H 5.28, N 5.04.

***cis*-[$PtCl_2(NH_3)$(1,4,7,10-tetraoxa-13-azacyclopentadecane-*N*)] (AO15):** Complex **AO15** was prepared according to the procedure as described for **AO18**, with 33.3 mg (0.15 mmol) of aza-15-crown-5 (1,4,7,10-tetraoxa-13-azacyclopentadecane). Yield: 29 mg (39%). ^1H NMR (D_2O): $\delta = 2.70$ (m, 2 H), 3.25 (m, 2 H), 3.70 (m, 16 H), 4.00 (br, 1 H), 5.10 (br, NH) ppm. ^{195}Pt NMR (D_2O): $\delta = -2231$ ppm. $C_{10}H_{24}Cl_2N_2O_4Pt$ (502.3): calcd. C 23.91, H 4.82, N 5.58; found C 23.70, H 4.56, N 5.77.

[{*trans*-$PtCl(NH_3)_2$}$_2$(μ-1,4,10,13-tetraoxa-7,16-diazacyclooctadecane-*N*,*N*′)]$(NO_3)_2$ (DAO18): To a solution of 302 mg (1.0 mmol) of *trans*-$[PtCl_2(NH_3)_2]$ in 5 mL of DMF a solution of 162 mg (0.95

mmol) of $AgNO_3$ in 5 mL of DMF was added. The solution was stirred in the dark overnight at room temperature. The precipitated AgCl was filtered off and a solution of 98 mg (0.37 mmol) of 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane (diaza-18-crown-6) in 5 mL of DMF was added to the yellow filtrate. The solution was stirred for 72 h at 40 °C. Subsequently, diethyl ether (400 mL) was added to the solution and an off-white precipitate appeared immediately. The solution was allowed to stand overnight at 4 °C and the precipitate was filtered off. The product was suspended in methanol and stirred for 1 h at 40 °C. Insoluble material was filtered off. Diethyl ether (400 mL) was added to the filtrate, a white precipitate appeared immediately. The mixture was allowed to stand overnight at 4 °C. The white product was filtered off, washed with diethyl ether and dried under vacuum. Yield: 81 mg (24%). ^{1}H NMR (D_2O): δ = 3.06 (m, 6 H), 3.70 (m, 8 H), 3.94 (m, 8 H), 4.20 (m, 2 H), 5.1 (s, br, 2 H), 4.75 (m, 2 H) ppm. ^{195}Pt NMR (D_2O): δ = −2408 ppm. $C_{12}H_{38}Cl_2N_8O_{10}Pt_2$ (915.5): calcd. C 15.74, H 4.18, N 12.24; found C 15.57, H 4.44, N 11.95.

[{*trans*-PtCl(NH$_3$)$_2$}$_2$(μ-1,4,10-trioxa-7,13-diazacyclopentadecane-*N*,*N*′)](NO$_3$)$_2$ (DAO15): The complex was prepared according to the procedure for **DAO18**, using 66.8 mg (0.31 mmol) of 1,4,10-trioxa-7,13-diazacyclopentadecane (diaza-15-crown-5) as the linker. Yield: 136 mg (51%). ^{1}H NMR (D_2O): δ = 3.15 (m, 12 H), 3.8 (m, 4 H), 4.2 (br, 2 H), 4.4 (m, 4 H) ppm. ^{195}Pt NMR (D_2O): δ = −2424 ppm. $C_{10}H_{34}Cl_2N_8O_9Pt_2$ (895.5): calcd. C 13.78, H 3.93, N 12.86; found C 14.40, H 3.86, N 12.90.

Cytotoxic Studies: A2780 and A2780cisR human ovarium cell lines were a gift from Dr. J. M. Perez (Universidad Autónoma de Madrid, Spain). The cell lines were grown as monolayers in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (Gibco, Paisley, Scotland), penicillin (100 units/ml: Duchefa, Netherlands) and streptomycin (100 μg/ml: Duchefa, Netherlands) in a humidified 6% CO_2, 94% air atmosphere. Cells were passed after trypsinization when the plates were 80−90% full. Growth inhibition by the platinum compounds was determined using an MTT-based assay.

Intracellular DNA Binding Studies: For the uptake/DNA binding experiment, A2780 cells were grown in 6-cm plates with 4 mL of DMEM medium, until a concentration of approximately 1×10^7 cells was reached. At this point, the medium was replaced by 4 mL of serum-free DMEM medium, as the binding of platinum complexes to proteins in the serum might influence the uptake efficiencies. Fresh stock solutions of the platinum complexes were added to a concentration of 200 μM. Each experiment was performed in duplicate. After 1 h of incubation (37 °C, 6% CO_2, dark), the medium was removed and the cells were washed twice with 1 mL of PBS (0.1 M phosphate buffer in 0.15 M NaCl). The cells were then trypsinized and centrifuged at 1000 G for 10 min. At this point, the cell pellets may be stored at −20 °C for future use. DNA was isolated from the cells by using the following procedure. Cell pellets were suspended in 300 μL of buffer (10 mM tris, pH = 8, 150 mM NaCl, 10 mM edta). 5 μL of proteinase K solution was added and mixed well with the cells. Finally, 50 μL of 10% SDS was added and the resulting mixture was heated at 50−55 °C for 1.5 h. The solutions were transferred to 2-mL Eppendorf Phaselock tubes, and extracted with 300 μL of chloroform/phenol/amyl alcohol (24:25:1), and subsequently with 300 μL of chloroform/amyl alcohol (24:1). DNA was precipitated by adding 600 μL of isopropyl alcohol to the water layer. The DNA pellet was washed once with cold 70% ethyl alcohol, and then dissolved in 1 mL of milli-Q water. The DNA concentration was determined by measuring the UV absorption at 260 nm. From this absorption, the concentration of base pairs was calculated, using an average molar extinction coefficient per base pair of ε_{260} = 16800 $M^{-1}cm^{-1}$. The platinum concentration was measured by FAAS. From the platinum concentration, the actual drug concentration was derived. Combining FAAS and UV results, the drug molecules per base pair ratio (r_b) was calculated.

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