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Synthesis and DNA-Damaging Properties of Cisplatin-*N*-Mustard Conjugates

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We report the synthesis of two novel cisplatin-*N*-mustard conjugates. In these compounds two potentially DNA-damaging molecules are combined and are separated by a spacer containing either one or four ethylene glycol units. We have shown that these conjugates are capable of forming novel clustered DNA adducts, thereby strongly increasing the lesion density in double-stranded DNA, which is thought to block DNA repair and translesion synthesis. Their ability to inhibit cell division was proven in an *E. coli* assay.

Introduction

Cisplatin and its analogues carboplatin and oxaliplatin (Figure 1) are highly important anticancer drugs, which are administered in at least 50% of all chemotherapies.[1] They are typically applied to treat testicular, prostate, ovarian, bladder, and cervical cancer.[2] The mechanism of action for all these compounds is generally believed to involve the covalent crosslinking of two adjacent nucleobases, mainly guanine. In the case of guanines, the main target is the nucleophilic N^7-position.[3] Enzymes such as DNA polymerases, which are the key proteins of the DNA replication machinery, and RNA polymerases are stalled at this DNA crosslink,[4] which induces programmed cell death.[5] The stalling of DNA polymerases is counteracted by special translesion polymerases, such as polymerase β, η, ζ, ε, or κ, which are able to copy DNA even in the presence of DNA damage. Polymerase η, for example, can read across cisplatin lesions.[6] This results in a decreased sensitivity towards cisplatin-based chemotherapies.[7] Mamenta et al. showed that in cisplatin-resistant ovarian cancer cells the cisplatin-damaged DNA is replicated up to 4.5 times more efficiently than damaged DNA in non-resistant cells. This proves that lesion tolerance is a major factor involved in cisplatin tolerance and resistance.[8]

A second pathway to acquiring resistance against cisplatin treatment is an enhanced DNA repair activity.[9] Schulte, Eastman, and Masuda et al. showed that increased resistance against cisplatin can be related to a more efficient DNA repair.[10] Both observations, increased DNA repair and increased lesion tolerance, demonstrate the need for improved cisplatin-based anticancer drugs.[11]

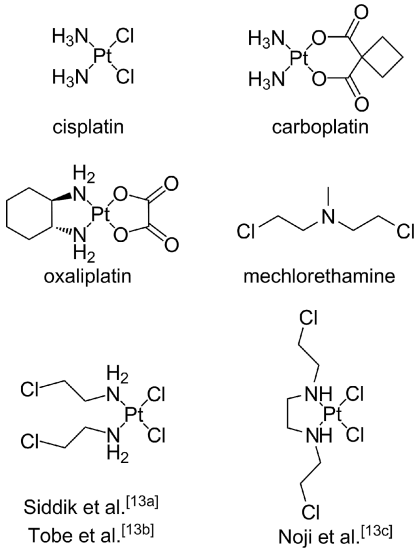

Figure 1. Structural formulae of cisplatin, carboplatin, oxaliplatin, mechlorethamine, and of known cisplatin-*N*-mustard conjugates.

We and others[12] have investigated the idea of linking cisplatin to additional molecules that can induce further DNA damage. Such conjugates are thought to induce clustered damage, which is believed to be less efficiently repaired and also less prone to translesion DNA synthesis. One approach is to combine cisplatin with *N*-mustards.[13] *N*-Mustards, such as mechlorethamine (Figure 1), crosslink two guanine residues also through their N^7-positions leading to cytotoxic 1,3-interstrand crosslinks.[14] Unlike cisplatin crosslinks, these lesions are intrinsically unstable. The covalent attachment of the *N*-mustard functionality to the 2′-deoxyguanosine (dG) residue destabilizes the glycosidic bond, which results in rapid depurination.[15] The abasic sites formed subsequently lead to strand breaks.[16] The cis-

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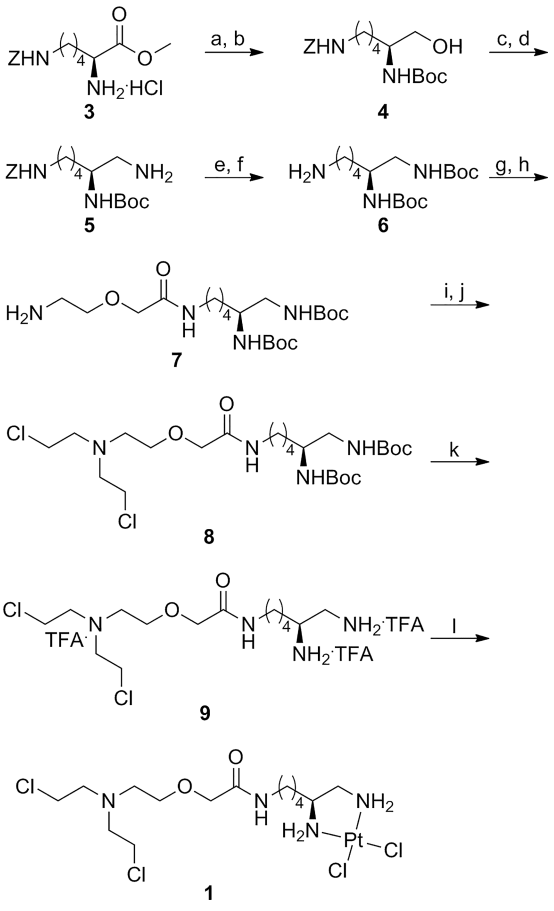
platin-*N*-mustard conjugates currently available coordinate the platinum center through the nitrogen of the *N*-mustard (Figure 1), which changes the reactivity of the *N*-mustard moiety quite dramatically.[13a–13c] The coordinating nitrogen lone pair is essential for activating the *N*-mustard for DNA alkylation via a reactive aziridinium intermediate.[17] Furthermore, information on how these compounds damage DNA is currently lacking.

We thought that it would be desirable to construct conjugates containing an intact *N*-mustard moiety and a cisplatin functionality as independent units linked by a flexible spacer containing one or four ethylene glycol units to counteract the known poor water solubility of cisplatin derivatives (see compounds **1** and **2**, Schemes 1 and 2). This idea requires the Pt^{II} coordination to be limited to the 1,2-diamino part of the molecule, which is challenging given the higher basicity of the tertiary amine of the *N*-mustard. We present herein the synthesis of the target compounds **1** and **2** and reveal how they interact with DNA through the use of LC-coupled high-resolution mass spectrometry (LC-ESI-HRMS).

Results and Discussion

The synthesis of complex **1** commenced with the Boc protection of the α-amino group of commercially available N^{ε}-(benzyloxycarbonyl)-L-lysine methyl ester hydrochloride (**3**) (Scheme 1). Reduction of the ester moiety with lithium borohydride gave alcohol **4** in excellent yield (96%). Compound **4** was converted into amine **5** in a two-step procedure. First, the alcohol functionality was substituted with phthalimide under Mitsunobu conditions, followed by the liberation of the amine by treatment with an ethanolic methylamine solution. Boc protection of the newly introduced amine functionality with subsequent cleavage of the benzyloxycarbonyl (Z) group yielded the bis-Boc-protected triamine **6**. The monoethylene glycol spacer was introduced by an amidation reaction with commercially available Z-protected 2-[2-(benzyloxycarbonylamino)ethoxy]acetic acid. After cleavage of the Z group by using molecular hydrogen, free amine **7** was obtained in good yield (86%). The two β-chloroethyl groups of the *N*-mustard functionality were introduced by applying a two-step procedure. First, the free amine in **7** was alkylated twice by using 2-bromoethanol. Subsequent chlorination of the two newly introduced alcohol groups using mesyl chloride yielded the reactive *N*-mustard derivative **8**. For the subsequent Boc deprotection of the two amines a methodology had to be developed that needed to be compatible with the sensitive mustard functionality. We finally achieved the deprotection of the two Boc protecting groups in the presence of the *N*-mustard moiety by using trifluoroacetic acid. This method allowed us to prepare the free ligand **9** as the tris(trifluoroacetic acid) salt. With ligand **9** in hand we started to elucidate suitable conditions for the desired complexation reaction with $K_2[PtCl_4]$, which was performed in deuteriated solvents to be able to directly monitor possible side-reactions by NMR spectroscopy. As expected, finding the optimal conditions for the coordination reaction turned out to be challenging. For the complexation, the primary and secondary amines needed to be deprotonated and as such these amines are nucleophiles, which could lead to the decomposition of the mustard moiety. In addition, the deprotonation of the tertiary amine of the *N*-mustard would result in an immediate activation of the *N*-mustard followed by decomposition of the free ligand. Indeed, this was observed. The reaction of the free ligand **9** under basic conditions led to the rapid decomposition of the compound, induced by activation of the *N*-mustard group.



Scheme 1. Reagents and conditions: a) Boc_2O, TEA, DMF, room temp., 25 h, 97%; b) $LiBH_4$, THF/DCM, 0 °C→room temp., 2 d, 96%; c) PPh_3, DBAD, phthalimide, PhMe, 80 °C, 8 h, 81%; d) $MeNH_2$, EtOH, room temp., 1 d, 74%; e) Boc_2O, KOAc, H_2O/MeCN, room temp., 3.5 h, 100%; f) 10% Pd/C/H_2 (1 bar), HOAc, MeOH, room temp., 6.5 h, 88%; g) 2-[2-(benzyloxycarbonylamino)ethoxy]acetic acid, EDC·HCl, 0 °C→45 °C, 4 d, 39%; h) 10% Pd/C/H_2 (1 bar), HOAc, MeOH, room temp., 5 h, 86%; i) $BrCH_2CH_2OH$, DIPEA, DMF, room temp., 8 d, 43%; j) Ms-Cl, TEA, 0 °C→room temp., 16 h, 46%; k) TFA, DCM, room temp., 3 h, 89%; l) $K_2[PtCl_4]$, D_2O, pH 1.0→8.0, room temp., 30 s, 67%; DBAD = di-*tert*-butyl azodicarboxylate.

Correct complexation was finally achieved by carefully controlling the pH of the reaction solution. In a typical experiment, ligand **9** and potassium tetrachloroplatinate(II) were solubilized in D_2O and the pH was carefully adjusted to pH 8.0 by the addition of a 1 M solution of NaOD. Un-

der these conditions we were able to obtain the desired cisplatin-*N*-mustard conjugate **1**. A high concentration of the free ligand **9** (0.1 M) was essential for the reaction, most likely to trigger the rapid coordination of the vicinal bisamine functionality followed by instantaneous precipitation of the product, which prevents the hydrolysis of the *N*-mustard functionality. Thus, the use of ligand **9** in excess was a second prerequisite for the successful synthesis of conjugate **1**.

Complex **1** was characterized by elemental analysis and far-IR spectroscopy (see the Exp. Sect.). Furthermore, the correct molecular mass of conjugate **1** was proven by HRMS (Figure 2). The recorded and calculated high-resolution mass spectra of compound **1** are in perfect agreement. The measured isotope pattern and mass values prove together with the elemental analysis and IR data that compound **1** was successfully prepared.

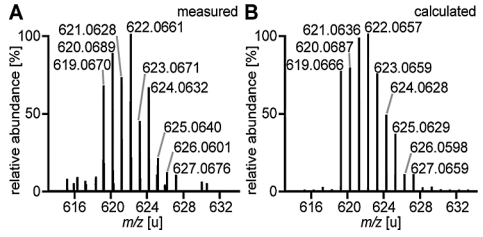


Figure 2. Recorded (A) and calculated (B) isotope patterns of the negative charged $[M - H]^-$ of **1**.

With the first cisplatin-*N*-mustard conjugate in hand we started to evaluate its DNA-damaging properties. To this end we incubated complex **1** with different double-stranded DNA oligonucleotides (dsODN, Table 1) having different sequences. Each duplex contains a d(GpG) motif (green) as the preferred binding site of cisplatin[18] and an additional d(GpNpC) [14b,14c,19] stretch (with N = A or T, blue), which is the preferred reaction site of the *N*-mustard. These two DNA sequences were separated by different numbers of AT base pairs (red) in order to investigate the distance required for the formation of clustered DNA damage.

Figure 3 (A) exemplarily shows an HPL chromatogram of dsODN 4 after incubation with a solution of conjugate **1** in 2% DMSO for 36 h at 37 °C. In dsODN 4 the d(GpG) and d(GpNpG) motifs are separated by five AT base pairs, the maximal distance for which the reaction of both the cisplatin and the *N*-mustard unit of conjugate **1** could be detected (see below). The very broad peak with several shoulders observed in the HPL chromatogram proves that multiple DNA adducts were formed in comparison with the sharp peak obtained when dsODN 4 was incubated in 2% DMSO alone as a negative control (Figure 3, B).

To gain deeper insights into the reaction products we then enzymatically digested the duplex dsODN 4 following its incubation with conjugate **1** by using nuclease P1 and Antarctic Phosphatase,[20] and subsequently analyzed the resulting mixture by LC-ESI-HRMS. We were able to identify two DNA lesions from the mass peaks observed at around *m*/*z* = 556 and 622 (Figures 4 and 5). The isotope pattern depicted in Figure 4 corresponds to the cisplatin–

Table 1. Sequences of double-stranded oligonucleotides used to investigate the DNA-damaging properties of the the newly synthesized cisplatin-*N*-mustard conjugates **1** and **2**.

#dsODN	Sequence
1	5′-d(AAA TTT TTA GGA GAC TTA TAT ATA TAT)-3′ 3′-d(TTT AAA AAT CCT CTG AAT ATA TAT ATA)-5′′
2	5′-d(AAA TTT TTA GGA AGA CTT ATA TAT ATA)-3′ 3′-d(TTT AAA AAT CCT TCT GAA TAT ATA TAT)-5
3	5′-d(AAA TTT TTA GGA AAG ACT TAT ATA TAT)-3′ 3′-d(TTT AAA AAT CCT TTC TGA ATA TAT ATA)-5′
4	5′-d(AAA TTT TTA GGA ATA AGA CTT ATA TAT)-3′ 3′-d(TTT AAA AAT CCT TAT TCT GAA TAT ATA)-5′
5	5′-d(AAA TTT TTA GGA ATA TAA GAC TTA TAT)-3′ 3′-d(TTT AAA AAT CCT TAT ATT CTG AAT ATA)-5′
6	5′-d(AAA TTT TTA GGA ATA TAT AAG ACT TAT)-3′ 3′-d(TTT AAA AAT CCT TAT ATA TTC TGA ATA)-5′

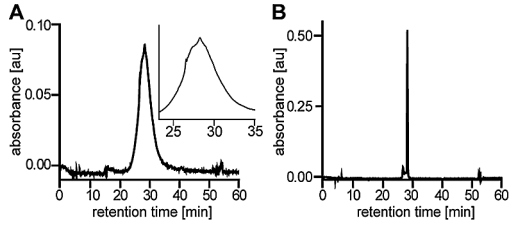


Figure 3. HPL chromatograms of dsODN 4 (A) incubated with conjugate **1** in 2% DMSO (inset: magnification of HPLC peak) and dsODN 4 (B) incubated in DMSO alone as a negative control; conditions: 36 h, 37 °C, pH 7.4.

1,2-d(GpG) DNA adduct **10**, formed from the reaction of the cisplatin unit with the two adjacent dG residues in the DNA duplex. This result shows that the cisplatin unit is indeed present in our conjugate and that it is reactive. The *N*-mustard is present in the hydrolyzed form, in agreement with the idea that this unit is more prone to hydrolysis than

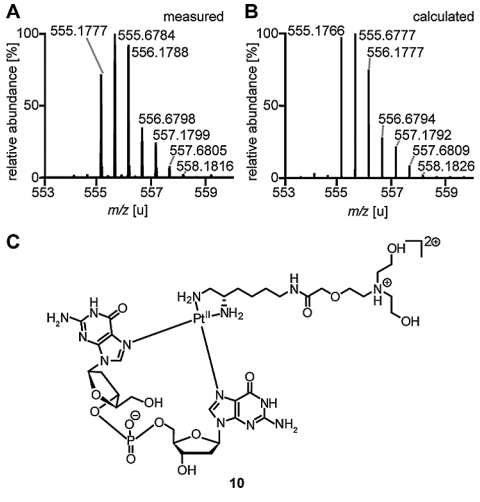


Figure 4. Recorded (A) and calculated (B) isotope patterns of DNA lesion **10** (C) obtained by LC-ESI-HRMS (retention time: 11.5–12.2 min). All the indicated signals are *z* = +2.

cisplatin.[21] It is evidenced that the *N*-mustard moiety did not react by the observation that both glycosidic bonds of lesion **10** are intact; we would have expected rapid cleavage of the glycosidic bond of the nucleotide if the *N*-mustard had reacted with the N^7-position.[15b] The formation of adduct **10** proves that the cisplatin unit can react in the normal way with DNA even in the presence of the *N*-mustard to give classical 1,2-intrastrand d(GpG) adducts.[3a,3c,18a,18c,22]

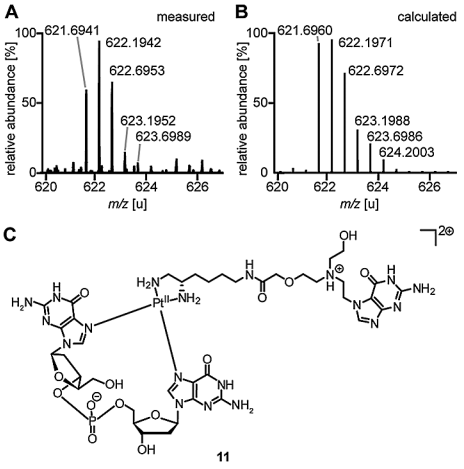
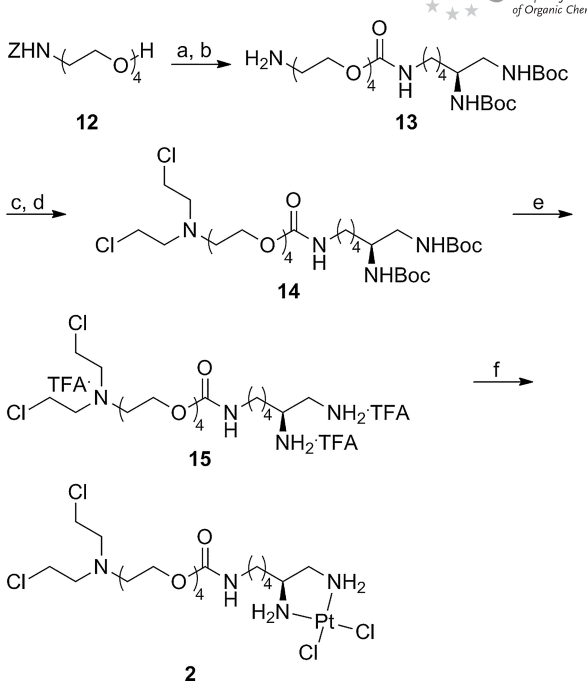


Figure 5. Recorded (A) and calculated (B) isotope patterns of DNA lesion **11** (C) obtained by LC-ESI-HRMS (retention time: 26.3–26.8 min). All the indicated signals are $z = +2$.

The molecular mass and isotope pattern of the signal at $m/z = 622$ (Figure 5) is best explained by DNA lesion **11**, which is formed by the reaction of the cisplatin moiety and one arm of the *N*-mustard with DNA. In this adduct **11**, one arm of the *N*-mustard reacted with a third dG residue, which subsequently underwent the expected cleavage of the glycosidic bond[15b] leading unavoidably to a strand break.[15a,16] This adduct shows us that the *N*-mustard unit in complex **1** is also active, as was hoped for. Formation of the new clustered lesion **11** was detected in all experiments performed with dsODN 1–4, proving that conjugate **1** can indeed form clustered DNA damage.

Encouraged by the ability to form clustered DNA damage we synthesized a cisplatin-*N*-mustard conjugate with a longer spacer between the two reactive groups to create a compound with greater flexibility. We hoped that with a more flexible connection between the cisplatin and the *N*-mustard the two cytotoxic moieties would react even more independently of each other, possibly resulting in new types of DNA lesions. We therefore prepared complex **2** featuring a tetraethylene glycol spacer (Scheme 2) to achieve greater flexibility but still to be able to generate a high density of DNA damage. The synthesis commenced with the coupling of Z-protected tetraethylene glycol derivative **12**, which was readily available from tetraethylene glycol in four synthetic steps according to literature procedures,[23] to amine **6** through a carbamate group.

To this end, the alcohol moiety of compound **12** was first treated with triphosgene and then coupled to amine **6**. Hydrogenolytic cleavage of the Z group yielded amine **13**.



Scheme 2. Reagents and conditions: a) 1. Triphosgene, activated charcoal, Et_2O, 0 °C → room temp., 16.5 h; 2. **6**, NaOH, THF, 0 °C → room temp., 21 h, 88%; b) 10% Pd/C/H_2 (1 bar), HOAc, MeOH, room temp., 5 h, 93%; c) $BrCH_2CH_2OH$, DIPEA, DMF, room temp., 4.5 d; d) Ms-Cl, TEA, 0 °C → room temp., 9 h, 40% over two steps; e) TFA, DCM, room temp., 2.5 h, 100%; f) $K_2[PtCl_4]$, D_2O, pH 1.0 → 8.0, room temp., 30 s, 97%.

The synthesis of conjugate **2** was completed by following the synthetic methodology reported in Scheme 1. The two β-chloroethyl moieties were introduced by the alkylation/chlorination sequence to yield the highly reactive *N*-mustard derivative **14**. The two Boc protecting groups were cleaved under mild conditions, and the free ligand **15** was coordinated to the platinum(II) center to obtain the cisplatin-*N*-mustard conjugate **2** in excellent yield (97%), which shows that the complexation reaction is broadly applicable. As reported above, tight control of the pH and a high concentration of the free ligand **15** were essential to prevent hydrolysis of the *N*-mustard functionality. Conjugate **2** was again characterized by elemental analysis, far-IR spectroscopy, and HRMS (see the Exp. Sect. and the Supporting Information).

To evaluate whether the more flexible tetraethylene glycol linker facilitates a broader range of DNA damage, conjugate **2** was treated with DNA duplexes dsODN 1–6 (Table 1). The oligonucleotides produced were analyzed as described above. The LC-ESI-HRMS analyses of the reaction of conjugate **2** with dsODN 1–5 in each case showed two signals, which, based on their isotope patterns, were assigned to adducts **16** and **17** (Figure 6 and Figure 7; for dsODN 6 only adduct **16** was observed). These adducts are similar to those already reported above. The fact that adduct **17** was observed in all the reactions with dsODN 1–5 shows that conjugate **2** can react with dG residues that are even separated by seven AT base pairs.

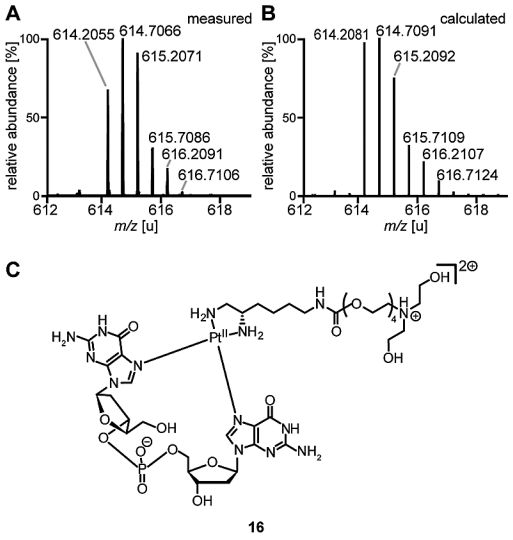


Figure 6. Recorded (A) and calculated (B) isotope patterns of DNA lesion **16** (C) obtained by LC-ESI-HRMS (retention time: 30.7–30.9 min). All the indicated signals are $z = +2$.

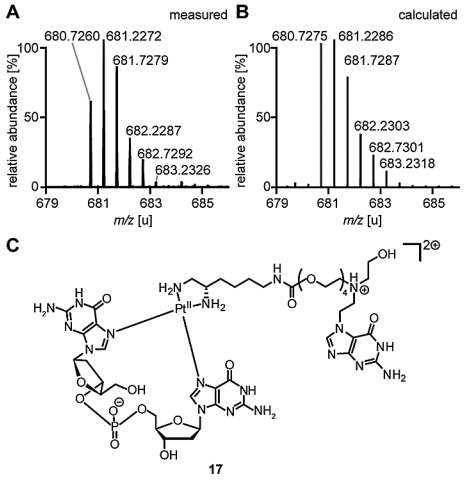


Figure 7. Recorded (A) and calculated (B) isotope patterns of DNA lesion **17** (C) obtained by LC-ESI-HRMS (retention time: 31.6–32.0 min). All the indicated signals are $z = +2$.

We also identified a third signal. As depicted in Figure 8, the mass spectrum allowed us to deduce the formation of a DNA adduct consisting of the conjugate **2** with three electrophilic positions bound to 2′-deoxyguanosine and guanine and the fourth one being hydrolyzed. Because the glycosidic bond of one of the 2′-deoxyguanosines is still intact we assumed that this one is bound to the cisplatin moiety through its N^7-position. The presence of a guanine without the ribose unit forces us to believe that a dG residue first reacted with the *N*-mustard moiety, but after depurination the cisplatin moiety has a chance to coordinate to the nucleophilic N^9-position of the guanine. This reaction sequence is supported by the work of Ali et al., who showed that cisplatin derivatives react preferentially with the N^9-position of N^7-methylguanine, if this site is accessible.[24] We therefore assume again that one arm of the *N*-mustard is hydrolyzed.[21]

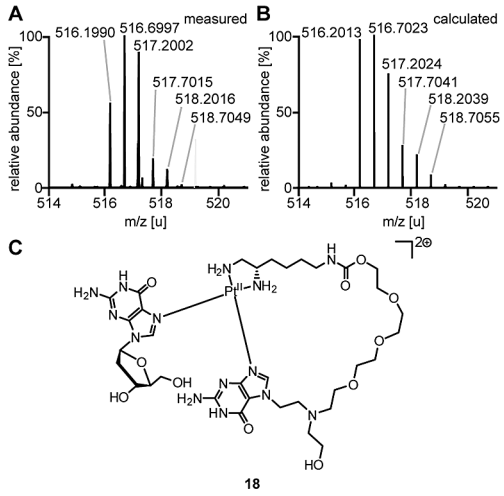


Figure 8. (A) Recorded and (B) calculated isotope patterns of (C) DNA lesion **18** obtained by LC-ESI-HRMS (retention time = 28.9–29.2 min). All the indicated signals are $z = +2$. The grey signal at $m/z = 519$ possesses the charge $z = +1$ and therefore does not belong to the isotope pattern of adduct **18**.

Although it is hard to elucidate lesion structures based only on MS data, the knowledge of the chemical reactivity of cisplatin compounds and *N*-mustards together with the high-resolution data gave some confidence that the indicated lesions **10**, **11**, and **16**–**18** are indeed formed. It is certainly safe to conclude that the cisplatin-*N*-mustard conjugates are able to form complex, clustered DNA lesions that are likely to be difficult to repair and therefore might block translesion synthesis. It should be noted that the cytotoxic action of conjugates **1** and **2** may not only be due to the cisplatin crosslink, but also to the cleavage of the glycosidic bond generating an abasic site that is prone to strand cleavage.

Encouraged by these findings, we next investigated the ability of the newly synthesized conjugates **1** and **2** to inhibit cell division, one of the key goals in antitumor therapy. We chose *E. coli* BL21(DE3) cells as a model system to mimic the experimental approach of Rosenberg and coworkers that originally led to the discovery of the antitumor effect of cisplatin.[25] For this purpose, we added a solution of **1**, **2**, or cisplatin (as a positive control) in DMSO to *E. coli* cells that had just entered the exponential growth phase. As a negative control DMSO alone was added. The cells were incubated at 37 °C and their morphology was investigated microscopically (for further experimental details see the Supporting Information). Figure 9 (A–D) show exemplary sections of cells after 9 h of incubation in DMSO alone, cisplatin, conjugate **1**, or conjugate **2**. It is clear that DMSO alone has no effect on the cell morphology in this experiment. The incubation of cells with cisplatin or conjugates **1** or **2** led to elongated bacteria (see arrows in Figure 9, B–D), most probably due to an inhibition of cell division, analogous to the results obtained by Rosenberg and co-workers.[25] This suggests that the newly synthesized cisplatin-*N*-mustard conjugates **1** and **2** can indeed inhibit cell division (although the effect seemed to be

slightly smaller than with cisplatin) and therefore they could be promising compounds for further biological investigations.

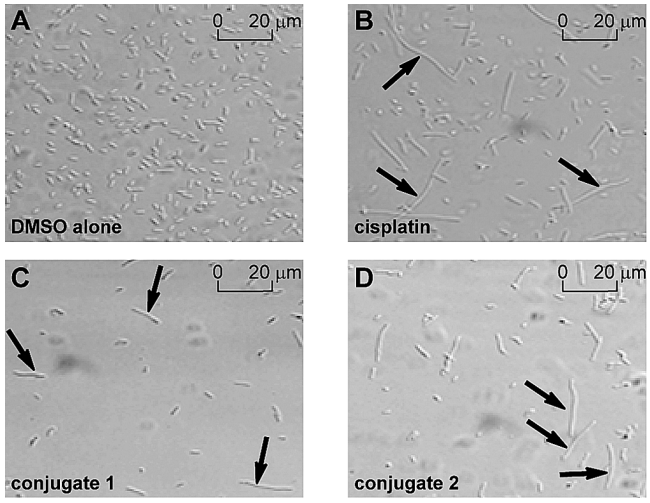


Figure 9. Microscopic pictures of *E. coli* cells incubated with DMSO alone (A), cisplatin (B), conjugate **1** (C), or conjugate **2** (D) at 37 °C for 9 h (for further experimental details see the Supporting Information).

Conclusions

We have synthesized two novel cisplatin-*N*-mustard conjugates **1** and **2** with the two different cytotoxic functionalities separated by one or four ethylene glycol units. Key to the synthesis was the tight control of the pH during the complexation reaction to prevent hydrolysis of the highly sensitive *N*-mustard functionality. Reactions of the complexes with DNA duplexes showed that the cisplatin as well as the *N*-mustard moiety are capable of reacting with guanine residues. The reactions lead to clustered DNA lesions that contain 1,2-d(GpG)–cisplatin adducts and abasic sites, which go hand-in-hand with the formation of strand breaks. Furthermore, the ability of these novel conjugates to inhibit cell division was shown by the elongation of *E. coli* cells when incubated with these compounds. We believe that our work will stimulate further research into new cisplatin-based anti-cancer drugs with novel modes of action.

Experimental Section

Conjugate 1: A 1 M solution of NaOD (0.3 mL) was added dropwise to a solution of the free ligand **9** (0.12 g, 0.17 mmol, 1.0 equiv.) and $K_2[PtCl_4]$ (16 mg, 39 µmol, 0.2 equiv.) in D_2O (1.23 mL) to increase the pH of the solution from 1.0 to 8.0. The precipitate formed was filtered off and washed with D_2O (0.6 mL) to obtain conjugate **1** as a pinkish solid (16 mg, 26 µmol, 67%), m.p. >280 °C (decomp.). IR (neat): $\tilde{\nu}$ = 2934 (m br), 1650 (s), 1542 (m), 1457 (m), 1438 (m), 1262 (w), 1119 (s), 1038 (m), 779 (m) cm^{-1}. FIR (PE): $\tilde{\nu}$ = 322 cm^{-1} (s br, Pt–Cl). $C_{14}H_{30}Cl_4N_4O_2Pt$ (623.32): calcd. C 26.98, H 4.85, N 8.99; found C 27.11, H 5.11, N 8.76.

Conjugate 2: The pH of a solution of the free ligand **15** (96 mg, 0.12 mmol, 1.0 equiv.) and $K_2[PtCl_4]$ (13 mg, 31 µmol, 0.3 equiv.) in D_2O (0.86 mL) was raised from pH 1.0 to 8.0 by the dropwise addition of a 1 M solution of NaOD (0.20 mL). The precipitate formed was collected and washed with D_2O to yield conjugate **2** as a pinkish solid (22 mg, 30 µmol, 97%), m.p. >240 °C (decomp.). IR (neat): $\tilde{\nu}$ = 2918 (m), 2850 (m), 1687 (m), 1540 (m), 1456 (m), 1258 (m), 1099 (s), 1038 (s), 799 (m), 720 (m) cm^{-1}. FIR (PE): $\tilde{\nu}$ = 332 (s br, Pt–Cl), 322 (s br, Pt–Cl) cm^{-1}. $C_{19}H_{40}Cl_4N_4O_5Pt$ (741.45): calcd. C 30.78, H 5.44, N 7.56; found C 30.85, H 5.35, N 7.26.

Acknowledgments

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