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Graphic abstract:

Top-down MS analysis provided sequential and complementary fragments, being more efficient than ladder-sequencing MS to discriminate binding sites of a ruthenium anticancer complex bearing a bulky ligand to oligonucleotides.
Identification and Discrimination of Binding Sites of an Organoruthenium Anticancer Complex to Single-Stranded Oligonucleotides by Mass Spectrometry

Suyan Liu, Kui Wu,* Wei Zheng, Yao Zhao, Qun Luo, Shaoxiang Xiong, Fuyi Wang*

We herein report the identification of binding sites of an organometallic ruthenium anticancer complex [(η⁶-biphenyl)Ru(en)Cl]+ (I) to single-stranded oligodeoxynucleotides (ODNs), 5′-CCCA₂G₃C₄CC-3′ (I) and 5′-CCC₃G₄A₅CCC-3′ (II), by mass spectrometry. The MS analysis of exonuclease ladders demonstrated that the 5′-exonuclease bovine spleen phosphodiesterase digestion of mono-ruthenated I and II by complex I was arrested solely at A₄ and partially at C₃ and G₄, respectively, and that the 3′-exonuclease snake venom phosphodiesterase digestion of the ruthenated ODNs was retarded solely at G₃ and G₄, respectively, due to the ruthenation. These results did not allow unambiguous identification of ruthenation sites on the metallated ODNs. In contrast, tandem mass spectrometry analysis with CID fragmentation of the mono-ruthenated ODNs provided sequential and complementary [a – B]/w fragments, leading to unambiguous identification of G₃ in I and G₄ in II as the ruthenation sites on the ODN adducts, which is in line with the high selectivity of this complex towards guanine base as reported previously. These findings suggest that caution should be raised with regards to the identifications of binding sites of metal complexes, in particular ones with bulky ligands like biphenyl in complex I, to DNA by MS analysis of exonuclease ladders of the metallated adducts because the bulky ligands may take such an orientation that they block the exonuclease cleavage of 5′- or 3′-side the phosphodiester bonds adjacent to the binding sites, leading to digestion stalling at the nucleotides before the binding sites.

Introduction

Organometallic ruthenium complexes in the formula of [(η⁶-arene)Ru(en)Cl]+, where arene = benzene, p-cymene, biphenyl (I), dihydroanthracene or tetrahydroanthracene, etc. and en = ethylenediamine, are a family of promising anticancer drug candidates which are cytotoxic both in vitro and in vivo, even active against cisplatin-resistant cancer cells.1, 2 As for cisplatin,3 which is one of the most used anticancer drugs, DNA is thought to be a potential target for the Ru(II) arene complexes4–7 which preferentially bind to N7 of guanosine but have low affinity to N3 of thymidine and little affinity to N3 of cytidine and adenosine.5 A great number of reports have demonstrated that the binding sites of cisplatin to DNA and the structural alternation of DNA duplex upon such bindings play a crucial role in the mechanism of action of the metallodrug.3 Therefore the exact localization of DNA metallation by metal-based anticancer drugs/candidates and the structural consequence of the DNA metallation have attracted increasing attention.

Cisplatin has been demonstrated to bind to guanine selectively, forming 1,2-G,G-intrastranded crosslinked DNA adducts which account for about 65% of the total platinated DNA products.3, 8 However, it has been shown that the bulky intercalation unit 1-[2-(acridin-9-ylamino)ethyl]-1,3-dimethylthiourea (ACRAMTU-S) in an analog of cisplatin, [PtCl(en)(ACRAMTU-S)(NO₃)]₂, could alter the binding-site specificity of Pt(II), leading to formation of more A-bound adducts which renders this complex a non-guanine specific platinum-based DNA modifier and accounting for the different biological activity of this mono-functional platinum complex.9 The binding sites of ruthenium-based anticancer drug candidates KP1019, NAMI-A and RAPTA-T to different duplex oligonucleotides was also studied and compared with those of platinum-based chemotherapeutics such as cisplatin, carboplatin and oxaliplatin.10 It has been showed that a strong preference for guanine bases was established irrespective of the sequence of oligonucleotide for these Ru(II)/Ru(III) complexes, perhaps responsive for the distinct mechanism of action of ruthenium-based anticancer complexes.11, 12

With the virtues of high sensitivity, low sample consumption and chemical specificity, electrospray ionisation mass spectrometry (ESI-MS) has become one of the most powerful tools for characterizing the diverse interactions, in particular elucidating the interaction sites of metal complexes with DNA.13–15 Generally, there are two types of complementary mass spectrometric approaches for such work: MS analysis of the exonuclease ladders of DNA adducts and...
MS/MS analysis of DNA adducts. The former one, also termed as ladder sequencing MS, involves in digests of metallated DNA adducts by exonuclease, followed by MS or LC-MS analysis. While in the latter approach, also termed as top-down MS, the DNA adducts were directly introduced into mass spectrometer and fragmented under various excitation techniques. These two methods have been successfully applied to study the interactions of diverse metal complexes with DNA. We have recently demonstrated that the combination of the two MS approaches are even more powerful in identifying the binding sites of ruthenium arene anticancer complexes to single-stranded oligodeoxynucleotides (ODNs) and found the novel thymine binding sites.

It has been previously shown that the exonuclease digestions could be arrested at the sites where metal complexes such as platinum22 and ruthenium17, 18 anticancer complexes coordinated to DNA bases, or chemical carcinogens such as polycyclic aromatic hydrocarbons (PAH)23, 24 and 4 - (methylnitrosamino) - 1 - (3 - pyridyl) - 1 - butanone (NNK)25 covalently bound to DNA bases. This provides structural information for localization of the binding/modification sites on DNA. However, when we recently studied the interaction between a ruthenium arene anticancer complex [(η⁶-biphenyl)Ru(en)Cl][PF₆] (I) and a 22-mer human telomeric ODN 5'-A₃G₅G₅G₅(TTAGGG)₃] by LC-MS, we observed arresting of the 5'-exonuclease digestion of the ruthenated adduct at A₁ and A₁₆. These seem to suggest that adenine base in DNA is also a binding site for complex I, inconsistent with previous reports, where complex I was showed to bind selectively to guanine base, being highly discriminatory between G and A bases. To address this controversial issue, in the present work, two short single-stranded ODNs, 5'-CCCA₄G₅CCC-3' (I) and 5'-CCCG₄A₅CCC-3' (II), were synthesised and reacted with complex I, and the ruthenated adducts were then characterised by ladder-sequencing and top-down MS analysis. The results demonstrated that the guanine bases in both ODN strands are the selective binding sites for complex I, but the biphenyl ligand of the G-bound I orient over the 5'-side, leading to arresting of 5'-exonuclease cleavage at the 5'-side nucleotides before the binding sites.

Materials and methods

Chemicals

[(η⁶-biphenyl)Ru(en)Cl][PF₆] (I)[PF₆]; en = ethylenediamine) was synthesized as described in the literature. HPLC-purified oligodeoxynucleotides (ODNs) 5'-CCCA₄G₅CCC-3' (I) and 5'-CCCG₄A₅CCC-3' (II) were obtained as sodium salts from AppliChem (Germany). Bovine spleen phosphodiesterase (BSP) was bought from Sigma and snake venom phosphodiesterase (SVP) from Orientoxin (Shandong, China). The dialysis bag (1 kDa) was purchased from Viskase (USA). Aqueous solutions were prepared using MilliQ water (MilliQ Reagent Water System).

Sample preparation

The stock solutions of complex I (0.5 mM) and ODNs (1 mM) were prepared by dissolving the complex and ODNs, respectively, in deionised water, and then diluted as required prior to use.

To identify the binding sites of complex I on ODNs, the mixture of complex I with each ODN (molar ratio [Ru]/[ODN] = 0.2) was incubated at 310 K for 24 h was dialysed against deionised water for 12 h to remove the unbound ruthenium complex. After freeze-dried, the samples were re-dissolved in 20 µL water, and partially digested by BSP or SVP. The digestions were carried out at 310 K with 0.8 µL (16 mU) of SVP in 10 mM Tris buffer containing 20 mM MgSO₄ (pH 8.8), or with 2.8 µL (28 mU) of BSP in 20 mM NH₄Ac buffer (pH 6.7), and then analysed by HPLC-ESI/MS.

High performance liquid chromatography (HPLC)

An Agilent 1200 series quaternary pump and a Rhodyne sample injector with a 20 µL loop, an Agilent 1200 series UV-Vis DAD detector and Chemstation data processing system were used. The mobile phases were water containing 20 mM TEAA (solvent A) and acetonitrile containing 20 mM TEAA (solvent B). The separation of the digests was carried out by using a C18 reversed-phase column (2.0 × 100 mm, Varian, Inc.) with a flow rate of 0.2 mL min⁻¹. The gradient was as follows (B): 1% from 0 to 5 min, 1% to 20% from 5 to 30 min, 20% to 80% from 30 to 32 min, 80% from 32 to 37 min, and resetting to 1% at 37 min. For the online HPLC-ESI-MS assays, a splitting ratio of 2/5 was used to introduce eluents into the mass spectrometer (Micromass Q-TOF, Waters).

Electrospray ionisation mass spectrometry (ESI-MS)

Negative ESI-MS spectra were obtained with a Micromass Q-TOF mass spectrometer (Waters) equipped with a Masslynx (ver 4.0) data processing system for analysis and post processing. The spray and cone voltages were 3.3 kV and 35 V, respectively. The collision energy was set up to 5 eV. The desolvation temperature was 353 K and the source temperature 413 K. Nitrogen was used as both cone gas and desolvation gas with a flow rate of 50 L h⁻¹ and 500 L h⁻¹, respectively. The spectra were acquired in the range of m/z 200 ~ 2000. The mass accuracy of all measurements was within 0.01 m/z unit, and all m/z data are the mass-to-charge ratios of the most abundant isotopomer for the observed ions. For ESI-MS/MS analysis, [M – 3H]⁺ was selected as the parent ions for collision induced dissociation and the collision energies were set at the range of 16 to 22 eV, and the spectra were acquired in the range of m/z 200 ~ 2000.

Docking Analysis

The binding models were constructed using Sybyl X 1.1 program (Tripos Inc.), running on Dual-core Intel(R) E5300 3.00 GHz, RAM Memory 2 GB under the Windows XP Professional operating system. The Gasteiger-maniulated, and the entire Ru ligand could be rotated a rotatable bond around which the biphenyl moiety could be manipulated, and the entire Ru ligand could be rotated independently of the DNA structure. Then the Gasteiger-
Huckel charges was added to the ODN complexes and they were energy-minimized using the Tripos force field with a distance-dependent dielectric and Powell gradient algorithm with an energy convergence value of 0.05 kcal·mol⁻¹.

Results and discussion

Firstly, the reaction mixture of complex I and ODN I at a molar ratio of [I]/[I] = 0.2, where the low molar ratio was applied to maintain the binding specificity, was analysed by LC-ESI-MS, which showed that the reaction produced only a mono-ruthenated adduct (Figure S1 in the Electronic supplementary information). Then, the dialysed reaction mixture was partially digested by 5′-exonuclease BSP, followed by LC-MS analysis of the exonuclease ladders. As shown in Figure 1a, two ruthenated ladders were observed at m/z 879.66 and 890.65, respectively, which correspond to mono-ruthenated adduct F 4 = 5′-A4G5CCC-3′ and I′ = [η6-bip]Ru(phen)3⁺. The sodium ion added to the ODN fragment raised from the synthetic ODN which was provided as sodium salt. These results indicated that the BSP digestion was retarded at the adenine base (A4) in the mono-ruthenated I, similar to that occurred in the BSP digestion of the ruthenated 22-mer human telomeric ODN 5′-A3G6G4G3(TTAGGG)3.26 No resistance at A4 to BSP digestion was observed for free ODN I (data not shown), thus the arrest at A4 is attributed to the binding of complex I to either A4 or G5, because the binding at A4 or G5 may prevent the phosphodiester bond between A4 and G5 from cleaving by BSP as did the covalent modification of ODNs by PHAs.22, 24, 27, 28

To complement the identification of the binding sites of complex I on I, 3′-exonuclease SVP was also applied to digest the aforementioned mono-ruthenated I. Two negative ions containing characteristic ruthenium isotopes (Figure 1b) were observed which are assignable to doubly-charged ladders F 4′+1’ (observed (obs.) m/z 879.66, calc. m/z 879.66) and {[F 4′+1’]+Na}2⁻ (obs. m/z 890.65, calc. m/z 890.65), respectively, where F 4′ = 5′-CCCA4G5-3′ (Figure S3). This suggests that complex I probably bound to either G5 or A4, leading to missing of SVP digestion of the phosphodiester bond between G5 and A4. However, neither MS analysis of 5′-exonuclease BSP ladders nor MS analysis of 3′-exonuclease SVP ladders can allow unambiguous localization of the binding site of complex I on the mono-ruthenated I.

To further verify the effectiveness of exonuclease digestion for characterisation of binding sites of ruthenium arene complexes on ODNs, single-stranded ODN II (5′-CCCA4G5CCC-3′), an analogue of ODN I with only variation at the order of G and A bases was also synthesised and reacted with complex I at [I]/[II] = 0.2. The formation of mono-ruthenated II was confirmed by LC-MS (Figure S4). Then, the mono-ruthenated ODN II was digested by BSP and SVP, respectively, followed by LC-MS analysis of the exonuclease ladders. The results (Figure 2a) showed that two mono-ruthenated ladders F 5′+1’ (obs. m/z 1024.19, calc. m/z 1024.18) and F 5′+2’ (obs. m/z 879.66, calc. m/z 879.66), where F 5′ = 5′-G6G5CCC-3′ and F 5′ = 5′-G6G5CCC-3′, and their sodium adducts were detected in the BSP digest (Figure S5), and that only one ruthenium-containing ladder F 5′+1’
These results suggest that complex 1 may bind to either C3 or G4 in the mono-ruthenated fragments. These 5' terminus [a−B] fragments up to [a−G4] were detected to be Ru-free, whereas mono-ruthenated fragment [a−C3] was observed at m/z 929.61 (calc. m/z 929.62 for [a+C3]− en)15, accompanied by the detection of larger mono-ruthenated [a+C3] and [K−C3] fragments. These complementary [a−B] or [a−C3] fragments (Figure 3c) allow unambiguous identification of G4 in I as the sole binding site for complex 1.

The binding sites of complex I on strand II were analogically identified by ESI-MS/MS, and the results are shown in Figure 4 and Table S2. From 3' terminus until w4, no ruthenated fragments were detected. Then both unruthenated and ruthenated w4 fragments were observed at m/z 606.09 (calc. m/z 606.06 for w4) and 656.04 (calc. m/z 656.04 for w4−en)15, respectively, accompanied by the detection of larger mono-ruthenated fragments w5, w6, and w7 with losing en or both bip and en ligands in complex 1 (Figure 3b, Figure S7 and Table S1). Meanwhile, the 5' terminus [a−B] fragments up to [a−G4] were detected to be Ru-free, whereas mono-ruthenated fragment [a−C3] was observed at m/z 929.61 (calc. m/z 929.62 for [a+C3]− en)15, accompanied by the detection of larger mono-ruthenated [a+C3] and [K−C3] fragments. These complementary [a−B] or [a−C3] fragments (Figure 3c) allow unambiguous identification of G4 in I as the sole binding site for complex 1.

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Thus, we speculate that a similar \( 5' \) end happened to platinated DNA by cisplatin.\(^{22}\) Such arresting of exonuclease digestion at \( 5' \) -side orientation upon the binding of complex \( \text{Ru} \text{(en)Cl}\) and the phosphodiester bond between \( \text{G} 4 \) and \( \text{G} 5 \). The hydrocarbon portion of the \( \text{R}-\text{anti-BPDE} \text{ at } \text{dG adducts} \) orients towards the 3'-end, inhibiting the spleen phosphodiesterase (SPD) cleavage of the 5'-side phosphodiester bond of the modified guanine site.\(^{28}\) The adenine-modified oligonucleotides (dA adducts) with benz[a]anthracene and benz[c]phenanthrene dinucleotides also showed diastereomer-dependent stalling for both SVP and SPD digestion.\(^{23,24,25}\) However, the patterns of resistance to exonuclease digestion of the dA adducts are different from those of dG adducts. The SVP hydrolysis of both R- and S-adducts at dA was blocked at the 5'-side phosphodiester bond adjacent to the modified adenine base, and then jumped to the 5'-side phosphodiester bond adjacent to the N(−1) nucleotide, producing a dinucleotide adduct dN(−1)dA, while the SPD cleavage of the 5'-side phosphodiester bond adjacent to the modified adenine base by R- or S-PAHs was found to be retarded.\(^{32}\)

For MS/MS analysis, the dissociation of phosphodiester bonds in the backbone of ODNs occurs in gas phase, and the ruthenium complex \( \text{I} \) with the bulky biphenyl ligand binding to guanine base in ODNs \( \text{I} \) and \( \text{II} \) has little steric effect on the fragmentation of the phosphate backbones. Therefore, the fragmentation of the ruthenated strands \( \text{I} \) and \( \text{II} \) was similar to the fragmentation of ODNs and the resulting sequential and complementary fragments provide sufficient structural information for discrimination of the binding sites of the ruthenium complex on ODN strands (Figures 3 and 4).

**Conclusions**

In the present work, our mass spectrometric studies showed that the reactions of the ruthenium anticancer complex \([([\eta^-\text{biphenyl}]\text{Ru}(en)Cl)]^+ \) with single-stranded oligonucleotide (ODNs), \( 5'\text{-CCCA}_4\text{G}_4\text{C}_4\text{C}-3' \) (\( \text{I} \)) and \( 5'\text{-CCCC}_4\text{G}_4\text{A}_4\text{C}-3' \) (\( \text{II} \)), at a low molar ratio of \([\text{I}]/[\text{ODN}] \) afforded two mono-ruthenated ODNs. The bulky arene ligand in complex \( \text{I} \) appears to orient towards the 5'-side of the ruthenation sites via interactions with adjacent bases, blocking the 5' and 3'-exonuclease hydrolysis of the 5'-side phosphodiester bonds adjacent to the ruthenated guanosine and leading to formation of ruthenated 5'-NG- and sNG'-3' ladders, respectively. These results cannot allow unambiguous identification of the binding sites of complex \( \text{I} \) on the ODN adducts. In contrast, due to little effect on the gaseous dissociation of phosphodiester bonds of ODNs arising from the ruthenation, tandem mass spectrometry
analysis of the ruthenated ODN adducts provided sequential 
and complementary fragments, clearly indicating that complex 
I selectively bound to G₃ in I and G₄ in II. These findings 
imply that much attention should be paid to the steric effects of 
bulky ligands in metal complexes on the exonuclease digestion, 
which may interfere with the correct identification of 
mollad materials on DNA by MS analysis of exonuclease 
digestion of the metallated DNA adducts.

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Notes

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(Tables S1 - S2, Figures S1 - S8). See DOI: 10.1039/b0000000x/

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