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Structural studies on metallobleomycins: The interaction of Pt(II) and Pd(II) with bleomycin*

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Abstract: Two of the most successful chemotherapeutic agents used in the treatment of several neoplasias are bleomycin and cisplatin. Both drugs attack the DNA leading to the cancer cells death *via* different mechanisms. In view of the fact that the combination with each other leads to enhanced activity with less sever side effects, we have undertaken NMR studies on the complexes formed between bleomycin and Pt^{II}, Pd^{II}, cisplatin and transplatin. Herein we present a brief review of the studies on metallobleomycins which were carried out by our lab and others, as an outline of the results obtained using NMR in combination to circular dichroism spectroscopy. Our data indicate that in most cases and under several conditions studied, both metal ions form similar complexes with BLM, while more than one species are present in the solution. Structural implications and comparisons with other metallobleomycins are being discussed.

Keywords: bleomycin, platinum, palladium, cisplatin, NMR.

INTRODUCTION

The bleomycins (BLMs) are a family of glycopeptide antibiotics, isolated from the culture medium of *Streptomyces verticillus* as their copper chelates. The administered form of the drug (Blenoxane or Bleocin) consists mainly of BLM A2 (Fig. 1) and is clinically used in the treatment of several neoplastic diseases.[1–3] In the presence of the cofactors Fe^{II} and O_2, BLMs have the ability to mediate DNA single and double-strand scissions, with the latter being recognized to be the principal locus of their cytotoxicity.[4–7] DNA strand scission is initiated by the selective abstraction of a C4'–H on pyrimidine residues, mainly at 5'–GC or 5'–GT sequences.[8] Additionally, RNA cleavage and damage to other cell particles may contribute to their toxicity.[9,10]

BLMs were first isolated and characterized by Umezawa and co-workers in the late 1960s.[11,12] Since then, numerous studies have been focused on their mode of action[13] and the role of metal ions as cofactors.[14] Seeing that the biological active species produced by

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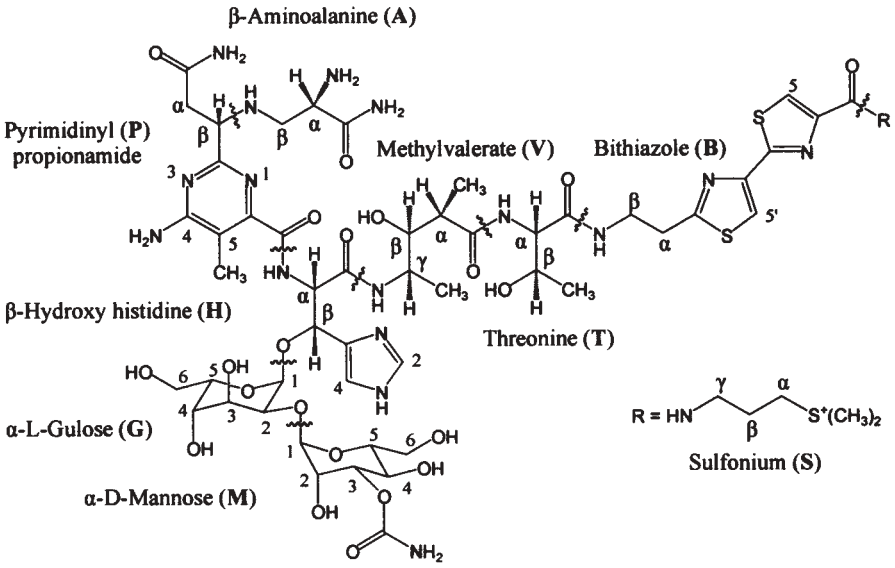

Fig. 1. Structue of bleomycin (BLM) A2. Bold atoms indicate the proposed metal binding ligands.

the reaction of the ferrous BLM complex with oxygen, designated as "activated bleomycin" [HOO–Fe^{III}–BLM], is short-lived and unsuitable for X-ray crystallography or NMR studies[15–18] a number of other models of metallo-BLMs have been employed in detailed structural characterization. So far, the crystal structures of severl BLM analogues (in all cases lacking the two sugars) complexed with cobalt,[19,20] copper and zinc[21–26] have revealed that BLM binds metal ions through the secondary amine of β-aminoalanine, the N1 of pyrimidine, the histidine amide and the amidazole nitrogen (Fig. 1). Only very recently Sugiyama and co-workers have managed to obtain crystals of both copper-bound and metal-free BLM A2 complexed with a BLM-binding protein.[27] The X-ray crystal structure of Cu^{II}–BLM is a five-coordinated complex with the N-donors arranged in a distorted square pyramide. The equatorial plane is defined by the above-mentioned ligands, while the primary amine of β-aminoalanine occupies the axial position. Interestingly, the metal-free BLM conformation did not differ much from the copper-bound one, whereas the disaccharide moiety was located at the same side with the axial amine ligand in both structures.

On the other hand, NMR studies have been proved to be imperative in understanding the DNA recognition and cleavage molecular basis of BLMs. In combination with molecular dynamics MD calculations several investigators have provided models for the solution structures of diamagnetic complexes with zinc,[28] cadmium,[29] Fe^{II}–CO,[30] cobalt,[31–33] as well as the paramagnetic Fe^{II} and Co^{II}.[34,35] These studies have introduced a controversy regarding the implication of the carbamoyl moiety of mannose to the coordination sphere of the metal ions. With the exception of the studies on the Co^{III}–BLM complexes carried out by the Stubbe lab, all others have proposed metallo–BLM models with the carbamoyl nitrogen of mannose (Fig. 1) as an axial ligand. Both HOO–Co^{III}–BLM (CoBLM

"green") and Co^{III}–BLM (CoBLM "brown") forms[36] were found to bear a strong resemblance to the crystal structure of Cu^{II}–BLM.[27] Since the former has been shown to mediate DNA cleavage upon photoirradiation in a similar fashion to that of the therapeutic relevant iron complex[37,38] extensive NMR studies have been utilized so as to examine its sequence-specific interaction with oligonucleotides[39–45] (Fig. 2).

Fig. 2. Model of the DNA-metallobleomycin complex after a single-strand scission. PDB access code 1GJ2.[44]

Ruthenium(II) and (III) compounds are considered to be very potent antitumor agents. We have previously studied the interaction of the two neutral octahedral Ru^{II}–dimethylsulfoxide compounds, *cis*- and *trans*-$RuCl_2(DMSO)_4$, that exhibit antineoplastic and mainly antimetastatic activity, with oligonucleotides model-system.[46] In an effort to produce a new generation of potent anticancer agents, we have studied the interaction of BLM with *cis*- and *trans*-$RuCl_2(DMSO)_4$. Using a series of spectroscopic techniques including UV-Vis absorption, circular dichroism, resonance Raman, ^{1}H-NMR, as well as electrospray mass spectrometry we have shown that Ru^{II} ions bind to bleomycin, forming an equimolar complex, similarly to Fe^{II}. The reaction of Ru^{II}– BLM with O_2, H_2O_2, or PhIO leads to formation of the oxy species in which only one oxygen atom is bound to metal ion. According to our data, the reaction of Ru^{II}–BLM complex with oxygen species leads to different product than that suggested for Fe^{II}–BLM. The formation of the BLM–Ru–O–Ru–BLM dimeric unit, similar to that found for sterically unhidered Ru–porphyrins, seems to be the most likely.[47]

Based on an early study by Lenkinski *et al.*[48] which reported that Ga^{III} forms stable 1:1 complexes with BLM A2 and B2 we have carried out a detailed structural characterization of the Ga^{III}–BLM A2 complex. The diamagnetic nature of Ga^{III}–BLM is ex-

ploited as a probe for Fe^{III}–BLM using NMR, owing to the fact that both metal ions have similar ionic radii, ionization potentials and coordination numbers[49–51] (Ga^{III} has a filled 3d orbital compared to Fe^{III} which has a half-filled 3d orbital). The stability and homogeneity of Ga^{III}–BLM allowed us to obtain a wealth of structural data from the 2D NMR experiments.[52] In combination with molecular dynamics calculations we have managed to define the solution structure of the penta-coordinated complex which resembles those of Cu^{II}–BLM and Co^{III}–BLM (Fig. 3). We have also reported the NMR studies on the interaction of Ga^{III}–BLM with the decameric oligonucleotide d(CCAGGCCTGG) which results in the formation of only one 1:1 complex in slow exchange on the NMR time scale. Our data indicate the partial intercalation of the bithiazole moiety between C6 and C7 in a similar mode to that reported by earlier studies.[39–45]

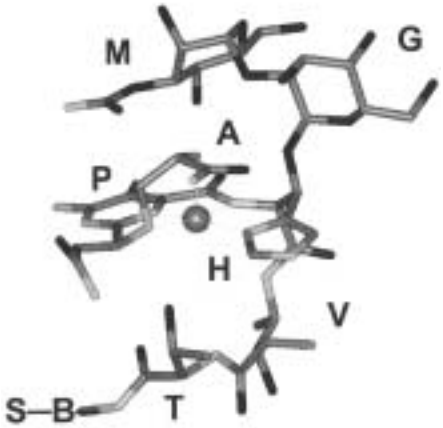

Fig. 3. Metal binding domain of the Ga^{III}–BLM A2 complex excluding bithiazole and sulfonium tail.

A veriety of metal ions have been used as radiolabeling imaging agents in combination with BLM, such as Co-57, Ga-67, Tc-99m and In-111. Most of them have failed either because of lack *in vivo* stability or tumor affinity. However, In-111 has show to form a stable complex with BLM at low pH which did not demonstrate any affinity for transferring and exhibited very high sensitivity and specificity in head and neck cancer patients.[53] By means of NMR studies we have found that In^{III}–BLM complex formed at low pH is also stable under physiological conditions (submitted). Employing 2D NMR experiments we are in progress of the structural characterization of the complex formed, in combination with molecular dynamics calculation carried out with the Amber force field.[54]

The interaction of Pt^{II} and Pd^{II} with BLM has been investigated previously by means of circular dichroism (CD) and fluorescence spectroscopy.[55] It was proposed that both metal ions form equimolar complexes with BLM in a three-stage process, but no effort has been made to characterize the different species. In order to gain a more detailed scope of this system we have studied the interaction of both metal ions with BLM using high resolution NMR techniques. Cisplatin and transplatin were also employed to this study, so as to make a direct comparison of the complexes formed. All the reactions were performed in aqueous solutions and were also monitored by CD and UV-Vis spectroscopy.

MATERIALS AND METHODS

Bleocin® was a generous gift from Nippon Kayaku Co., Ltd and was used without further purification. Both *cis*- and *trans*-platinum(II) diammine dichloride were purchased from Sigma while potassium tetrachloroplatinate(II) and tetrachloropaladate(II) 99.9 % were purchased from Alfa Aesar of the Johnson Mathey company. For the NMR experiments D_2O 99.95 %, DCl 38 % and NaOD in D_2O used were purchased from Deutero GmbH and Stohler Isotope Chemicals.

The samples were prepared at room temperature in 5 mm NMR tubes at final concentrations ranging from 1 to 5 mM. The pH (uncorrected for the isotope effect) was measured with a Philips Scientific PW 9420 pH-meter equipped with a 3.7 mm CMAW 711 electrode. For the adjustment to the desired value aliquots from 1 or 0.1 M solutions of DCl or NaOD were added. The reactions were carried out in a heat bath at either 40 of 60 ºC.

The CD and UV-Vis spectroscopy was performed at a Jasco V-715 dichograph. Spectra were recorded at 250–750 nm with a 0.5 nm step at 200 nm/min, 1.0 nm bandwidth, 1.0 s response and 4–10 acquisitions. Temperature was controlled by an external thermostat to 25 ºC and the solutions were dissolved to concentrations ranging from 0.1 to 0.5 mM.

The NMR spectra were recorded at a Bruker Avance 500 MHz instrument and were processed using X-WIN NMR 2.6 (Bruker Analytic GmbH). Data sets acquired at 298 K with a time domain of 32 K and 64 scans. During the relaxation delay of 2.0 s a presaturation pulse was used to suppress the solvent signal, while the FIDs were processed with an exponential weighting function.

RESULTS AND DISCUSSION

Pt(II) and Pd(II)–BLM complexes

The addition of Pt^{II} and Pd^{II} to solutions of BLM is followed by a drop of pH. Titration of BLM with Pd^{II} resulted in precipitation whenever excess of the metal ion was reached, even at very acidic solutions (pH < 2). The insoluble product formed is probably a poly-hydroxo palladate complex of BLM, in view of the fact that no spectra of BLM could be obtained from the solution. This has also been observed previously[55] as the reaction performed in buffer at neutral pH. Using equimolar amounts of Pd^{II} and BLM under acidic conditions we have followed their reaction by CD spectroscopy after heating at 40 ºC. A tree-stage course in which BLM coordinates to Pd^{II} was identified as is also evident by the NMR spectra.

As shown in Fig. 4, at the region where the pyrimidine methyl group signal is detected the formation of complex I is readily observed. After 1 h complex II is then formed which is completed at 4 h. Finally complex III formation occurs which is rather slower and is completed within 24 h. Taking into account the changes of the chemical shifts of the histidine and the pyrimidine methyl protons, we can conclude that BLM is initially coordinating to the metal ion through the N1 atoms of the rings. Seeing that the histidine aromatic protons are immediately shifted to lower fields (≈ 0.5 – 1.0 ppm) we can assume that complex I represents the coordinated BLM through the histidine. At the next step, BLM also binds Pd^{II} through the N1 of the pyrimidine ring (complex II), as the upfield shift of the P_{CH_3} signal indicates (Fig. 4b). Finally, following the dissociation of the remaining chlorides from the metal ion, BLM also binds to Pd^{II} *via* the secondary amine of aminoalanine and the deprotonated histidine amide (Fig. 1). The last is rather slower and results in the formation of complex III, which is very stable in a wide range of temperature and pH for several days. As in all cases of metallobleomycins studied, the equatorial plane is defined

by the above mentioned ligands, which in our case shall form a distorted square planar conformation around the metal ion.

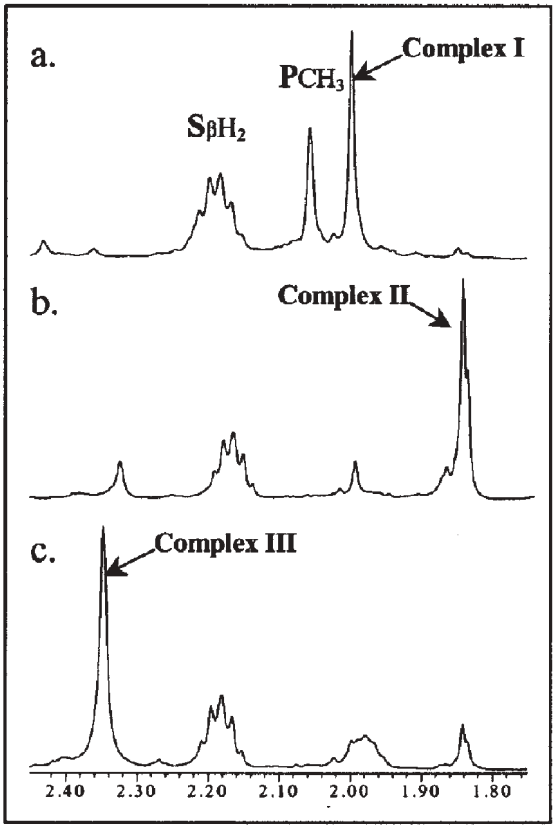

Fig. 4. Region of the ^{1}H-NMR spectra of BLM 3 mM in the presence of equimolar amount of Pd(II) at 4.5. Spectra recorded 30 min (a), 4 h (b) and 24 (c) after heating at 40 ºC.

In the case of platinum, the reaction with BLM is not following the same pathway as with palladium. Formation of more than one species is clearly evident by the ^{1}H-NMR spectra recorded since the addition of Pt^{II} to the BLM solutions. Additionally, the reaction rate is lower, as even after 24 h free BLM signals are still observable. Nonetheless, we were able to recognize the same species as in the case of Pd^{II}–BLM from their chemical shift values. We can thus suppose that Pt^{II} forms the same complexes with BLM as Pd^{II}, which is expected due to their structural similarity in most of the compounds present. This hypothesis is also supported by the identical CD spectra recorded for both reactions.

Cisplatin and transplatin–BLM complexes

The addition of an equimolar amount of solid cisplatin or transplatin to an aqueous solution of BLM does not result in changes to the CD spectra acquired even after a couple of days. For this reason we have used a threefold excess of the metal complex added to an 1

mM solution of BLM at pH 4. The solutions prepared were heated to 60 ºC while their CD and NMR spectra were recorder periodically. As illustrated in Fig. 5, the reaction is completed after 5 days, where no more changes at the CD spectra are observed. Similar bands were also observed at pH 7 but with rather faster kinetics, since the complex formation is completed in 3 days. No differences were observed between the two isomers of platinum.

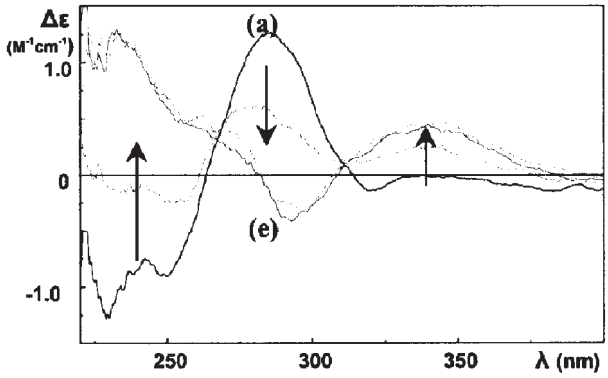

Fig. 5. CD spectra of BLM with a threefold excess of cisplatin at pH 4.0 after heating at 60 ºC for 6 h (a) up to 5 days (e).

NMR spectra on the other hand have been more informative concerning the several complexes formed. At least four different species are present in the solutions, even after prolonged heating, which possibly accounts for complexes formed between BLM and Pt^{II} having lost one or two of its amine and chloride ligands. A striking difference observed between cis and transplatin which could not be detected at the CD spectra is that the latter reacts with BLM in a roughly speaking two-stage process. As evident from the ^{1}H-NMR spectra, two major species are present when BLM is mixed with transplatin after a couple of days at 60 ºC which could be recognized as the complexes **I** and **II** shown in Fig. 6. The two chloride anions of platinum are being dissociated faster than the amine groups. After leaving the two trans positions of Pt^{II} free, BLM can then coordinate through the aromatic rings (pyrimidine and histidine). This trans effect probably supports the formation of complex **I**, which is then followed by the slow dissociation of the two amines to finally form complex **II** (Fig. 6). On the other hand, cisplatin not having the two trans positions readily available cannot coordinate to both aromatic rings of BLM at once. This results in the slow formation of species with either histidine or pyrimidine ring coordinated to the metal ion.

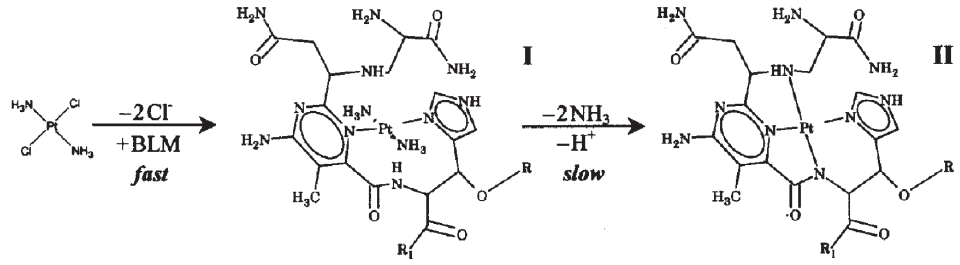

Fig. 6. Proposed structures of the major square planar Pt^{II}–BLM complexes **I** and **II** formed between BLM and transplatin. R and R_1 represent the disaccharide and the peptide linker domains of BLM, respectively.

After several hours heating at 60 ºC, cisplatin can also form complex **II**, as indicated by the similarities in their chemical shifts with transplatin.

The final species formed between cis and transplatin, designated as complex **II** (Fig. 6), is similar to that Pd^{II}–BLM complex III. Although their CD bands and the proton chemical shifts are not identical, their similarity is evident. Their differences can be attributed to variations in the electronic arrangement and distribution around the two metal ions. All the species formed are quite stable in solution even after several months at room temperature.

CONCLUSIONS

Palladium and platinum (II) metal ions react with the anticancer drug BLM forming square planar complexes. While Pd^{II} coordination is characterized by a tree-stage process, Pt^{II} forms several species with BLM at the same time. As indicated by the ^{1}H-NMR spectra, BLM binds to the metal ions initially through the aromatic rings, followed by coordination of the aminoalanine secondary amine and the histidine amide. Accordingly, both cisplatin and transplatin complexes are able to react with BLM but with a lower rate. This is attributed to the slower dissociation of the two amine ligands. As in the case of Pt^{II} a mixture of species are present in the solutions, while transplatin favors the formation of two major species with BLM owing to a trans effect of its chloride ligands.

ИЗВОД

ПРОУЧАВАЊЕ СТРУКТУРЕ МЕТАЛОБЛЕОМИЦИНА: ИНТЕРАКЦИЈА Pt(II) И Pd(II) СА БЛЕОМИЦИНОМ

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У овом раду дат је кратки преглед NMR студија и спектроскопије циркуларног дихроизма комплекса блеомицина (BLM) и Pt^{II}, Pd^{II}, цисплатине и трансплатине. Наши подаци указују да у највећем број случајева, и под различитим експерименталним условима, оба јона метала граде сличне комплексе са BLM, уз истовремено присуство више од једне реакционе врсте у раствору. Дискутоване су структурне импликације детектованих комплекса, а упоређени су и са другим металоблеомицинима.

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