

A ^{31}P NMR study of the interaction of the antitumor active metallocene Cp_2MoCl_2 with calf thymus DNA

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Treatment of sonicated calf thymus DNA with the antitumor active metallocene Cp_2MoCl_2 afforded a metallocene-DNA complex which was characterized by ^{31}P NMR spectroscopy. In addition to the resonance for the phosphate backbone ($\delta -1.6$), the spectrum contained 2 signals assigned to a phosphate bound Mo-DNA complex(es) ($\delta 37.2, 36.5$) and a broad signal at $\delta 6.2$ ppm. This result suggests that covalent attachment of the metallocene Cp_2MoCl_2 occurs via phosphate(O) coordination and is accompanied by local distortion of the DNA backbone. This result supports recent ICP studies with Cp_2TiCl_2 that have DNA detected DNA-metalloocene adducts.

Metallocene; Calf thymus DNA; Antitumor; ^{31}P NMR

1. INTRODUCTION

The metallocene dichlorides, Cp_2TiCl_2 , Cp_2VCl_2 , Cp_2NbCl_2 and Cp_2MoCl_2 exhibit antitumor activity for a wide range of murine and human tumors [1]. Administration of Cp_2TiCl_2 or Cp_2VCl_2 inhibits cellular DNA synthesis [2], and it has been suggested [1,2] that the cytotoxicity of metallocenes is related to the formation of cisplatin-like adducts [3] with DNA. However, extensive studies on the aqueous chemistry and the binding sites of metallocenes with nucleobases and nucleotides show conclusively that cisplatin type adducts do not form [4]. Rapid hydrolysis of both the halide ligands and the cyclopentadienyl (Cp) rings in Cp_2MX_2 occurs in aqueous solution, the rate of both processes being dependent on the metal atom [4]. Nevertheless, inductively coupled plasma spectroscopy (ICP) measurements on the interaction of tritium labelled metallocenes with calf thymus DNA have detected the presence of a Cp_2Ti -DNA adduct at pH 5.2 and a CpTi -DNA adduct at pH 5.8 [5], confirming studies that implicate DNA as a site of interaction of metallocenes in cells.

Studies of the interactions of Cp_2MX_2 ($\text{M} = \text{Ti}, \text{V}, \text{Mo}$) with nucleotides suggest that the most likely coordination sites on DNA are the phosphate oxygens and/or the heterocyclic nitrogens in the nucleic bases [4]. The improved hydrolytic stability of the Mo-Cp bonds in Cp_2MoCl_2 compared to Cp_2TiCl_2 have allowed several adducts formed between Cp_2MoCl_2 and nucleotides to be identified [4] (Fig. 1). NMR studies of the interaction of Cp_2MoCl_2 with A-T and G-C base-pairs are consis-

tent with formation of phosphate centred complexes which do not disrupt the W-C base-pairs. It has not been established whether similar complexes are formed with bulk DNA. In duplex DNA there are steric constraints imposed by the stacked base pairs (Fig. 2) that need to be considered and that may lead to formation of different complexes than those formed with isolated base-pairs.

This paper reports a NMR spectroscopic study of the interactions of the metallocene Cp_2MoCl_2 with calf thymus DNA fragments. ^{31}P NMR spectroscopy was used to confirm the presence of isolable metallocene-DNA adducts, previously detected by ICP spectroscopy, and to provide information regarding the type of complexes formed.

2. EXPERIMENTAL

2.1. Calf thymus DNA

Calf thymus DNA (Sigma, sodium salt, Type 1, 160 mg/ml), was sonicated (50% duty cycle) at the maximum output power below cavitation by the use of a tapered probe of a Branson Model 250 sonicator. Solutions were 8–12 ml containing NaCl (0.2 M) and held at 0–2°C. After 6 h sonication, the samples were centrifuged at 14,000 for 1 h. The supernatant was dialyzed twice against a 1 l solution containing NaCl (0.2 M), cacodylate (10 mM) and Na_2EDTA at pH 7.0 for 24 h using a 12–14 kDa membrane. Further dialysis against 1 l of NaCl (0.1 M) was carried out for 24 h prior to lyophilisation.

2.2. DNA-metalloocene adducts

In a typical experiment, the DNA-metalloocene complexes were prepared in sodium perchlorate (110 mM) by addition of sonicated DNA solution (2 ml, 5 mM determined by UV absorbance at 260 nm, ϵ 6,600) under nitrogen to a septum-capped vial containing the metallocene (5 mg). The mixture was stirred at 37°C for 30 min prior to filtration through a glass wool plug. The complex was dialysed against 1 l of NaCl (0.1 M) for 12 h through a 12–14 kDa membrane to remove

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unreacted and hydrolyzed metallocene products, and was then lyophilized. NMR samples were prepared by addition of $\text{H}_2\text{O}/\text{D}_2\text{O}$ directly to these samples to give sample concentrations 0.1–1.0 M NaCl and at pH 7.0.

2.3. Molecular modelling

Modelling was carried out using MacroModel (version 3.1X) [6] on a Silicon Graphics 4D/30 personal workstation. A sequence of DNA was generated in the B-conformation and a metallocene Cp_2M^+ (built from X-ray coordinates), was manually docked at potential coordination sites along the helix, using the bumpcheck option to monitor the presence of non-bonded interactions.

3. RESULTS

Short fragments of calf thymus DNA (150–200 base pairs) were prepared by sonication [7] and were used in order to obtain NMR spectra that were sufficiently well resolved to allow partial assignment of the DNA and metallocene signals. The metallocene Cp_2MoCl_2 was studied due to the relative stability of the Cp rings toward protonolysis in aqueous solution and the data available on the nucleotide adducts formed with Cp_2MoCl_2 [4].

Fig. 3a shows the ^{31}P NMR spectrum of sonicated calf thymus DNA and this shows a single resonance for all the phosphate groups (δ –1.6 ppm). The spectrum of the same sample that has been treated with Cp_2MoCl_2 (Fig. 3b) contains, in addition to the resonance due to the backbone phosphates, three new signals – a broad signal at δ 6.2 ppm and two downfield singlets (δ 37.2, 36.5 ppm). The relative intensities of these 3 new signals, by integration, were estimated to be 2:1:1 respectively. The downfield singlets were assigned to a molybdenum phosphate (Mo–O–P) complex(es), as these resonances appear in the same region as the ^{31}P resonances in $\text{Cp}_2\text{Mo}(5'\text{-dAMP})$ (δ 35.8 ppm, Fig. 1a) [4]. For comparison, the signal due to uncomplexed 5'-dAMP appears at δ –0.04 ppm. The new broad signal (δ 6.2), adjacent to the backbone signal, was assigned to the phosphates near to the site of metal coordination. The appearance of a new signal slightly downfield (< 4 ppm) from the DNA backbone ^{31}P resonance has been observed in previous studies of drug–DNA complexes

[8], and reflects the local distortion of the backbone that occurs due to formation of the complex. In the present study, the new signal at δ 6.1 ppm is further downfield than the corresponding signal reported for intercalator and groove-binding DNA complexes. This larger change in chemical shift may partly be attributed to deshielding effects present due to the Cp rings in the adduct, but overall, suggests that the structure of the metallocene–DNA complex is significantly different from those drug–DNA complexes previously characterized by ^{31}P NMR spectroscopy.

4. DISCUSSION

While the antitumor properties of the metallocene dihalides are well established, the mechanism of action of this class of compounds is poorly understood [1,2,9]. Interaction of metallocenes with DNA is assumed to play a significant role in this process since the metallocenes inhibit nucleic acid synthesis and the metals derived from these compounds accumulate in nucleic acid rich regions of tumor cells [9].

Both the shape and size of the metallocenes are markedly different from other known organometallic agents that interact with DNA, notably cisplatin. ICP experiments [5], which indicate that the cyclopentadiene rings remain coordinated to the metal in metallocene–DNA complexes formed with Cp_2TiCl_2 , suggest that the halide ligands are replaced by other coordinating ligands present in DNA. In B-DNA, the most likely competitive ligands are nitrogen (present in the nucleobases) and oxygen (present in the phosphate groups) and metallocene complexes involving both these groups have been detected by Kuo et al. (Fig. 1) [4].

Molecular modelling studies, in which a metallocene Cp_2MX_2 was manually docked into both the major and minor grooves of DNA and the halide ligands were replaced with N donor or phosphate(O) ligands, were carried out. Due to the tetrahedral coordination of the central metal, formation of a complex using a nitrogen from one of the base pairs (as in Fig. 1) pointing into either the major or the minor groove results in severe

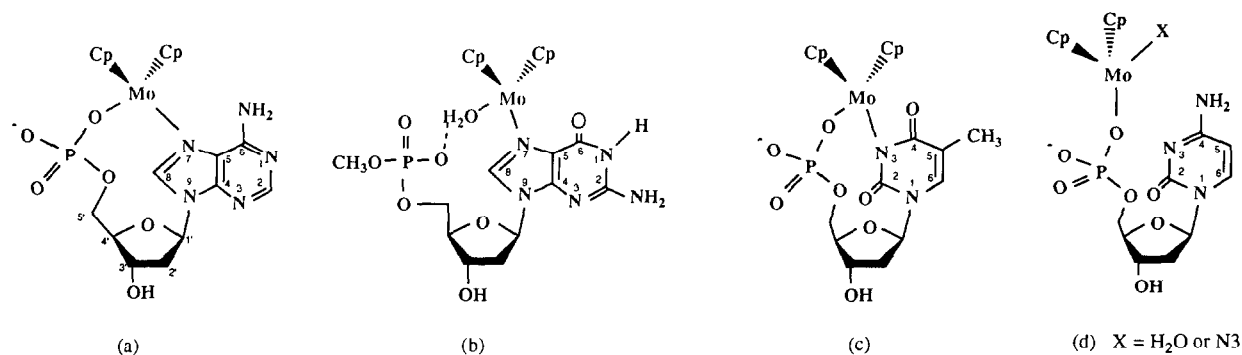


Fig. 1. Complexes formed in aqueous solution between Cp_2MoCl_2 and (a) 5'-dAMP, (b) methyl ester of 5'-dGMP, (c) 5'-dTTP, (d) 5'-dCMP (from [4]).

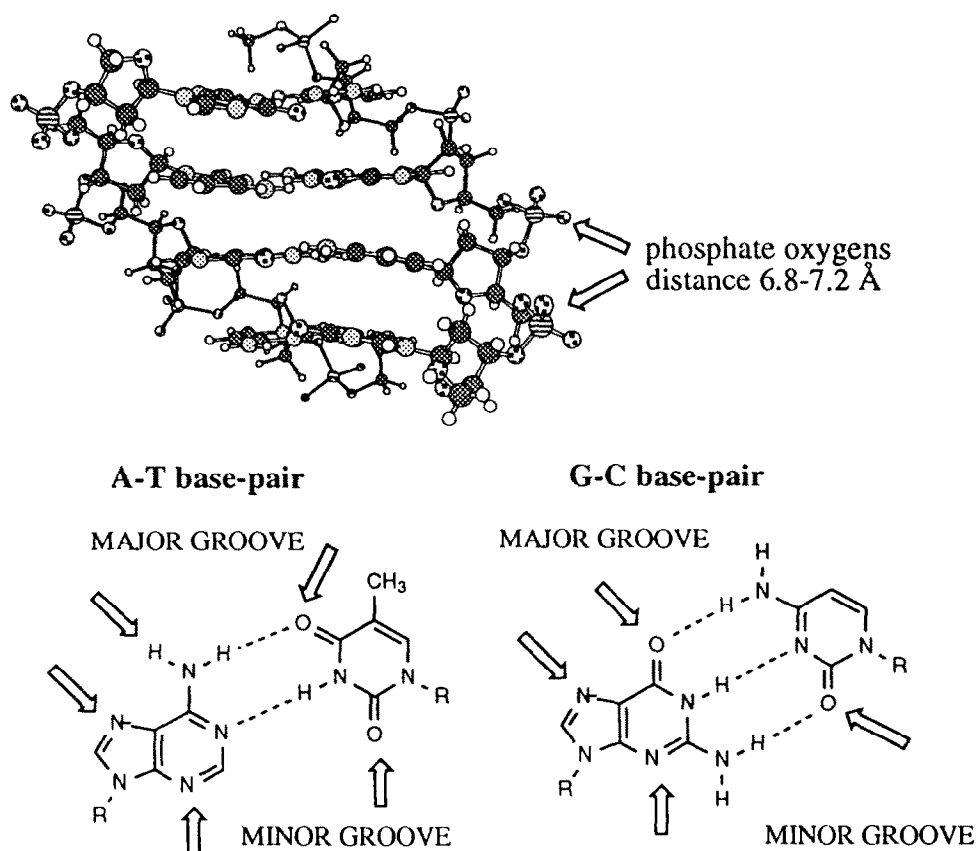


Fig. 2. Section of B-DNA and Watson-Crick base pairs. Arrows indicate potential nitrogen and oxygen coordination sites.

steric interactions between the coordinated Cp rings with the base-pairs above and below the coordination site. While DNA is conformationally flexible and can distort its structure on binding, modelling suggests that very severe distortion of regular DNA structure would be required to allow formation of such a complex. A similar conclusion has been reached by Kuo et al. [4] in attempts to model possible cisplatin type adducts. In contrast, phosphate coordination on the DNA backbone can occur with no (or little) steric impediment. It is possible for two phosphate(O) ligands on adjacent nucleotides to coordinate to the molybdenum centre. The distance between the oxygen donor atoms is ~ 7 Å (Fig. 2) while the 'bite' of metallocenes is ~ 3 –4 Å [1] and hence formation of such a complex would be expected to be accompanied by local distortion of the DNA backbone. Alternatively, the Mo coordinate sphere could be satisfied by one phosphate(O) and a solvent molecule or by a solvent mediated bridge to a second ligand (see Fig. 1b,d).

Numerous NMR studies of the interactions of drugs and small molecules with short oligonucleotides have been reported [10]. In the present study, long DNA sequences were used in preference to short oligonucleotides in order to eliminate possible formation of complexes involving the sterically accessible terminal base

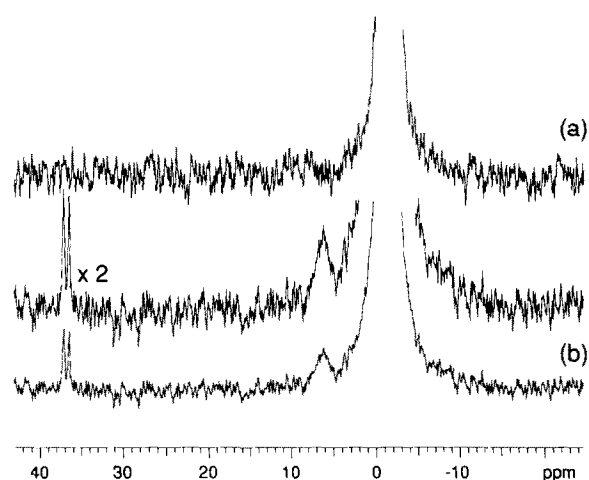


Fig. 3. ^{31}P NMR spectra of (a) calf thymus DNA, (b) calf thymus DNA incubated with Cp_2MoCl_2 . Spectra were recorded on a Bruker AMX400 spectrometer ($^{31}\text{P}\{^1\text{H}\}$ 161.98 MHz) at 300K, with quadrature detection employed throughout. Chemical shifts are in parts per million (ppm) referenced to external neat trimethyl phosphite (140.85 ppm). Spectra were recorded over 16K data points, SW 200pm, spectrum (a) 10,000 scans, (b) 20,000 scans. An exponential line-broadening factor of 30 was applied to both FID's prior to Fourier transformation.

pairs and terminal phosphate groups. In addition, as discussed above, modelling suggested that should phosphate coordination occur, this could be accompanied by substantial distortion of the backbone, which would be favoured by the extra stability present in longer sequences of DNA. Several NMR studies of the interaction of drugs with calf thymus DNA have been reported [7,11,12]. From the systems studied to date, ^{31}P NMR provides a convenient monitor of the phosphate ester backbone conformational changes upon binding of intercalating drugs, and chemical shift changes and have been used to assess differences in duplex unwinding angles of DNA in the presence of several intercalating drugs [7,8].

The interaction of Cp_2MoCl_2 with DNA appears to be totally different from other 'classic' DNA binders (intercalators, groove binders, charged compounds) and cisplatin and related compounds. The new resonances observed in the ^{31}P spectrum obtained after interaction of Cp_2MoCl_2 with DNA (Fig. 3b) are consistent with coordination of Cp_2MoCl_2 to phosphate groups on the DNA backbone. In aqueous solutions of Cp_2MoCl_2 , the major species present is believed to be $\text{Cp}_2\text{Mo}(\text{H}_2\text{O})(\text{H}_3\text{O})^+$ [4]. This cation may lose one or both ancillary ligands to become covalently attached to the DNA backbone via either one or two phosphate(O) bonds giving rise to one or two new phosphorus resonances as observed at δ 37.2, 36.5. While the exact structure of these complexes remains to be determined, the simultaneous appearance of a new resonance at δ 6.2 suggests that formation of the complex(es) is accompanied by local distortion of the DNA backbone. This distortion could arise if there is simultaneous coordination of Cp_2Mo^+ to two phosphate(O) atoms on adjacent nucleotides. Formation of such a complex would require the distance between the phosphate(O) groups to be reduced, unlike the process that occurs on intercalation where gradual unwinding of the helix occurs. Narrowing of the O-P-O bond angle is expected to produce a downfield shift of the ^{31}P resonance [13] and such bridging of neighbouring nucleotides may explain the

unusual chemical shift of the signal at δ 6.2 ppm. Clearly further data obtained with short oligonucleotides is required to conclusively establish the nature of these adducts.

To our knowledge this is the first example of formation of a drug-DNA complex which appears to be centred on the phosphate backbone. Whether this property is related to the antitumor activity of Cp_2MoCl_2 remains to be investigated. Experiments are underway with short DNA duplexes in order to fully characterize metallocene-DNA interactions and establish their role in the antineoplastic properties of this class of compounds.

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