The observation of a hedamycin–d(CACGTG)$_2$ covalent adduct by electrospray mass spectrometry

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Abstract Covalent binding of the antitumour antibiotic hedamycin to the self-complementary hexadeoxyribonucleotide 5′-CACGTG-3′ has been investigated by electrospray ionization mass spectrometry (ESI-MS). Ions due to double-stranded forms of the free 5′-CACGTG-3′ and the hedamycin–5′- CACGTG-3′ adduct have been observed in ESI mass spectra and their identity has been confirmed by resolution of individual charge states in ESI-MS spectra. Clear evidence that specific base-paired associations are being observed in ESI-MS is provided by the results of a titration experiment involving alkylated and non-alkylated complementary strands. This work demonstrates the potential of this powerful new tool for studying ligand–DNA binding.

Key words: Electrospray mass spectrometry; d(CACGTG)$_2$; Duplex formation; Hedamycin; DNA alkylation; Antitumour agent

1. Introduction

Electrospray ionization mass spectrometry (ESI-MS) is now extensively applied for the characterization of biomolecules, in particular proteins [1,2]. This is a very gentle ionization technique with multiply-charged ions from the species of interest and minimal fragmentation generally observed in ESI mass spectra. As a consequence, there is considerable interest in the applications of ESI-MS for the detection of non-covalent associations of biomolecules, and recently ions corresponding to double-stranded or duplex forms of oligonucleotides have been reported in ESI mass spectra [3,4]. Gale et al. [5] have also done a preliminary study of a non-covalent complex between distamycin and an oligonucleotide duplex. ESI-MS has considerable promise for the structural characterization of ligand binding to DNA, offering potential advantages of sensitivity and speed of analysis, compared with existing structural methods such as NMR and X-ray crystallography.

Hedamycin (Fig. 1) is a naturally occurring antitumour antibiotic that can both intercalate into and alkylate double-stranded DNA [6]. We have conducted extensive sequence analyses of hedamycin binding to purified plasmid and human DNAs [7], and also to DNA in intact human tumour cells [8]. These studies have demonstrated high levels of binding to isolated guanines, particularly those located in 5′-CGT sequences, and suggest that the N7 of guanine is the nucleophilic site on DNA. We have an on-going program aimed at elucidating the structural basis for this sequence selectivity, as well as for those of other naturally occurring and synthetic DNA intercalating/alkylating compounds. To date, these structural studies have involved the use of NMR spectroscopy, but this work is now complemented by our development of new mass spectral methods for assessing ligand binding to DNA.

In this paper, we demonstrate the use of ESI mass spectrometry to examine the covalent binding of hedamycin to the self-complementary hexadeoxyribonucleotide 5′-CACGTG-3′, which incorporates a single preferred binding site. Furthermore, we provide here the first clear evidence for the formation of specifically base-paired DNA duplex ions from a covalent ligand–DNA adduct in ESI mass spectra, thus opening the way for ESI-MS to be used as a sensitive structural probe for ligand binding to double-helical DNA.

2. Materials and methods

2.1. Preparation and purification of hedamycin–CACGTG adduct

The self-complementary deoxyribonucleotide 5′-CACGTG-3′ was synthesized 'trityl-on' with an Applied Biosystems Model 381A DNA synthesizer, using β-cyanoethylphosphoramidite chemistry. The crude trityl-on oligonucleotide was purified on a Waters Delta-Pak C_{18} reverse-phase HPLC cartridge system with a linear gradient of acetonitrile in 0.1 M aqueous ammonium acetate (pH 7.0). The purified oligonucleotide was detritylated with 80% acetic acid and re-chromatographed using the same stationary phase and solvent system.

A sample of hedamycin was generously donated by the Bristol-Myers Co. (USA) and was used without further purification. A stock solution of hedamycin was prepared by dissolving the solid in a minimum volume of acetonitrile and then diluting to the appropriate volume with 0.1 M aqueous ammonium acetate (pH 7.0). The stock solution (1.4 mM) was kept under nitrogen and was stored at −20°C in the dark.

Reactions between hedamycin and 5′-CACGTG-3′ were carried out at room temperature under a nitrogen atmosphere and in the dark. Ligand stock solution was added to the oligonucleotide dissolved in 0.1 M ammonium acetate (pH 7.0) in a mole ratio of 1:1 hedamycin/(oligonucleotide duplex). Reactions were monitored by reverse-phase HPLC, using the system described above. When reactions were judged to be complete, the major components in the mixtures were isolated by preparative HPLC, the solvent evaporated and each examined by ESI-MS as described below.

2.2. Electrospray mass spectrometry

The majority of ESI-MS spectra were acquired on a VG Biotech Quattro mass spectrometer equipped with an electrospray ionization source and a triple quadrupole mass analyser, which has a mass range for singly charged ions of 4000 Da. The resolution of the quadrupole was set to 1.5 Da (peak width at half-height). Under these conditions the isotopic species from multiply charged ions were not fully resolved, although quadrupole mass analysers can be used to resolve isotopes of triply charged ions in favourable cases. Oligonucleotide samples were dissolved in 50–100 μl of 10 mM aqueous ammonium acetate. The concentration of single-stranded oligonucleotide in these samples was 50–200 pmol/μl. An injection volume of 10 μl was used for each analysis. Samples were introduced into the source by a flow of solvent at 5 μl/min. A flow of warm, dry nitrogen (65°C, 1 atm) assisted evaporation of the solvent. The electrospray probe tip potential was 3.5 kV, with 0.5 kV on the chicane counter electrode. Spectra were acquired in negative ion mode over a range m/z 300–1700 scanned at a rate of

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100 mass units per second. The mass range in this mode was calibrated using a standard sugar mixture. A skimmer potential of 50 V was used for all oligonucleotide and oligonucleotide-adduct analyses.

Additional mass spectra were acquired on a Fisons Analytical MS Autospec magnetic sector mass spectrometer. Samples were approximately 150 pmol/μl in 10 mM aqueous ammonium acetate. A 20 μl aliquot was used for injection and the solvent flow was 40 μl/min. A resolution of 3000 was used for each analysis, which was sufficient to easily resolve individual isotopic species from triply charged oligonucleotides.

3. Results and discussion

Fig. 2 shows the ESI mass spectrum of d(CACGTG) following HPLC purification. There are two major peaks in the spectrum from the single-stranded oligonucleotide, namely the $[M-2H]^{2-}$ ion at m/z 895 and the $[M-3H]^{3-}$ at m/z 596. There is also a significant peak at m/z 1194 which we have attributed to the double-stranded oligonucleotide less three protons $[2M-3H]^{3-}$ (the charge of this peak has been confirmed by high resolution ESI-MS; see below). The intensity of this peak depends on the solvent composition. A wide range of solvents were tested and the optimum was found to be 10 mM ammonium acetate. This is consistent with earlier reports of duplex formation that noted that the presence of an appropriate buffer was necessary for these ions to be observed in ESI mass spectra [2,3]. This was also the evidence provided by these workers for the formation of duplex DNA stabilised by the presence of counter ions as opposed to non-specific gas-phase associations of these molecules.

Fig. 3a shows the ESI-MS of the hedamycin–d(CACGTG) adduct following HPLC purification. The two major peaks in the spectrum are both due to the single-stranded adduct, i.e. $[M-3H]^{3-}$ at m/z 845 and $[M-2H]^{2-}$ at m/z 1268. There are no duplex ions evident in this spectrum. That the mass spectrum of the purified hedamycin–DNA adduct shows no association between alkylated strands is understandable given that the hedamycin binding site is located in the centre of the sequence. In order for two adducts to form a fully basepaired duplex, the bulky hedamycin ligands would have to be placed in close proximity to each other, being attached to guanines that are on

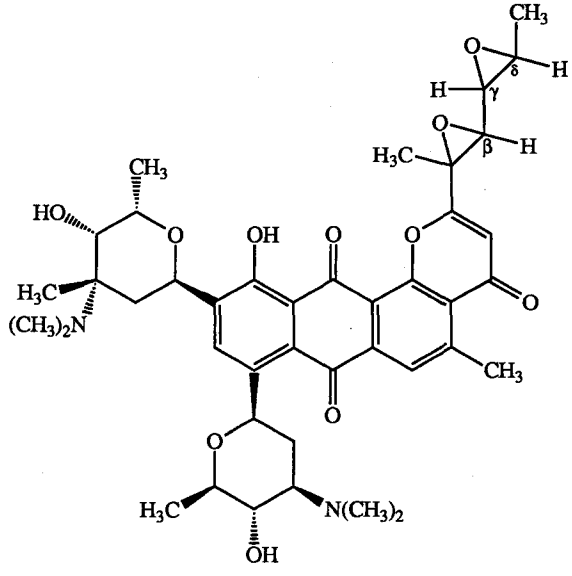

Fig. 1. Structure of hedamycin (M_r = 746.85).

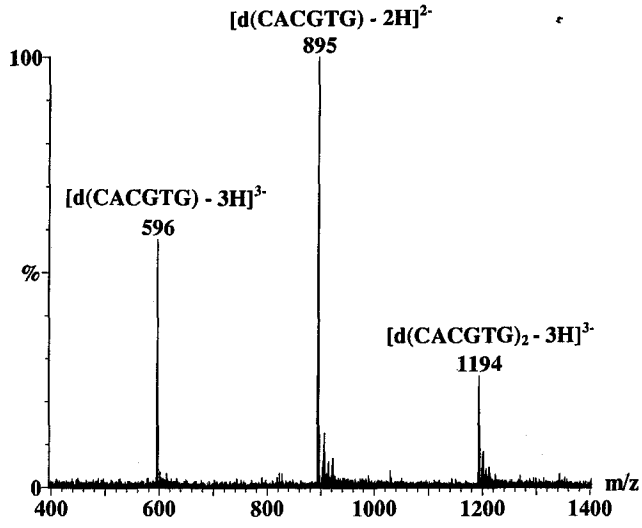

Fig. 2. Electrospray mass spectrum (ESI-MS) of d(CACGTG) (M_r = 1792.2).

opposite strands and in neighbouring basepairs, and this would lead to severe steric interactions.

Therefore, to generate a stable duplex from the purified adduct, we conducted a titration experiment where aliquots of free 5′-CACGTG-3′ were added to the purified, single-stranded hedamycin–5′-CACGTG-3′ adduct, with mass spectra being collected after each addition. After the first addition, a low intensity peak corresponding to hedamycin–$d(CACGTG)_2$ at m/z 1443 (due to the $[M-3H]^{3-}$ ion) appeared in the mass spectrum (not shown). With each subsequent addition, the intensity of this peak increased up until one equivalent was added, in which case the duplex ion was the major peak in the spectrum (Fig. 3b) and there was only a small peak due to the single-stranded adduct. A new peak (at m/z 895), due to free single-stranded 5′-CACGTG-3′, was also apparent. These results clearly indicate that the hedamycin–$d(CACGTG)_2$ adduct peak seen in the ESI mass spectra is due to specific basepairing between the hedamycin–5′-CACGTG-3′ adduct and the complementary 5′-CACGTG-3′ strand. This interpretation is consistent with both NMR spectroscopic evidence (Wickham et al., unpublished results) that hedamycin remains intercalated after alkylation of the guanine and earlier studies which show that hedamycin increases duplex stability [6]. In the ESI-MS spectrum of the free 5′-CACGTG-3′ (Fig. 2), even under the most favourable ionization conditions, the duplex form is only observed in ~25% abundance relative to the single-stranded oligonucleotide. In contrast, the hedamycin–$d(CACGTG)_2$ duplex is the most abundant species observed in Fig. 3b, which reflects the additional duplex stability arising from hedamycin intercalating between the two strands.

Finally, it should be noted that in ESI spectra containing multiply charged ions, the masses are derived using simple algorithms based on the mass difference between two multiply charged peaks with different charge states [9]. However, when there is only one peak, as is the case for many of the duplex peaks from small oligonucleotides and ligand–oligonucleotide adducts, the charge state of the peak, and hence the mass, can be determined from the spacing of individual isotopic species containing one and more ^{13}C-labeled isotopes, which differ in

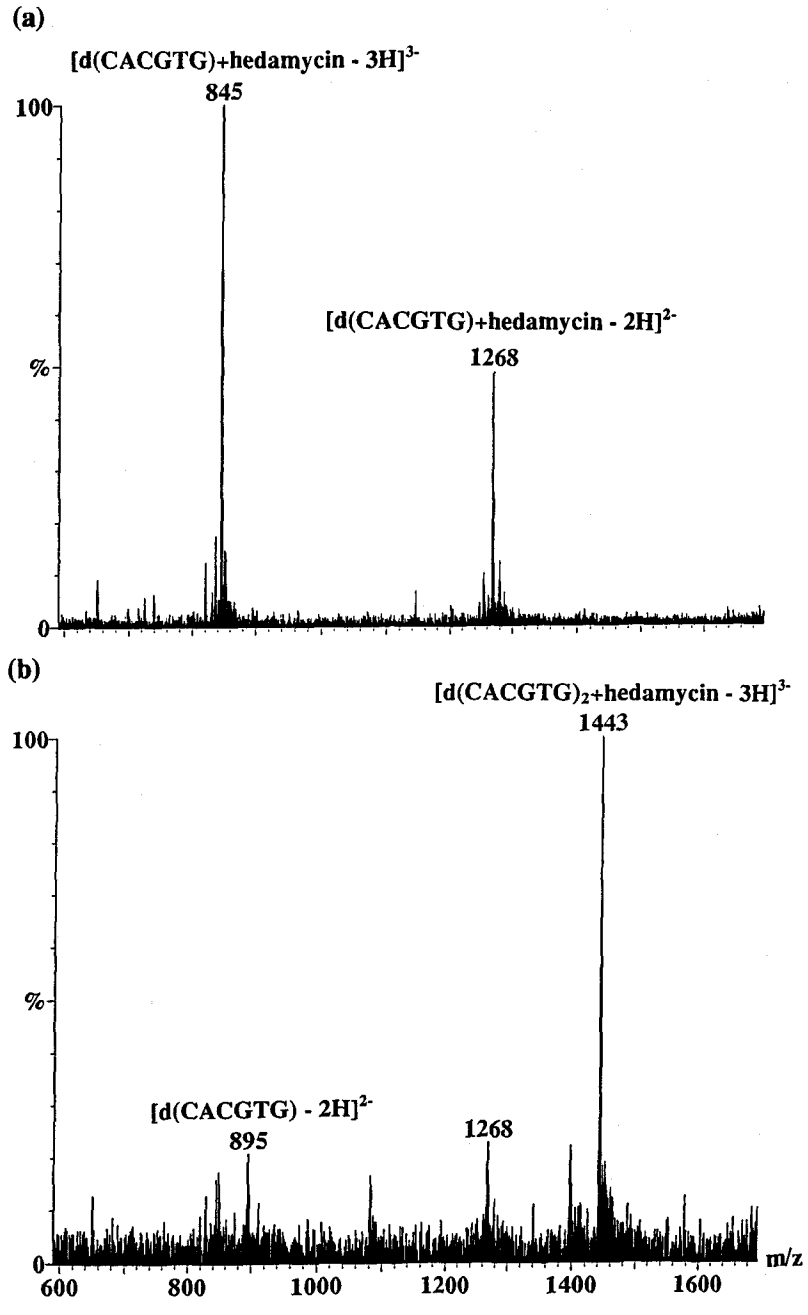

Fig. 3. ESI-MS of hedamycin–d(CACGTG) (M_r = 2539.1) (a) following HPLC purification, showing only single-stranded hedamycin–d(CACGTG) and (b) after titration with approximately one equivalent of free d(CACGTG), showing the formation of hedamycin–d(CACGTG)$_2$ (M_r = 4331.3)

mass by 1.003 Da. Fig. 4 shows two of the major ions from the spectra in Fig. 3 measured with a resolution of 3000. In the examples here, the m/z separation of 0.5 between adjacent peaks in Fig. 4a confirms that this ion of the single-stranded hedamycin–d(CACGTG) adduct has two charges, whereas in Fig. 4b the spacing of ~0.3 indicates that the observed hedamycin–d(CACGTG)$_2$ double-stranded adduct is a triply charged species.

In conclusion, therefore, while we and others have observed ions corresponding to duplex forms of DNA in ESI-mass spectra, the results described here are the first example of a covalent ligand–(duplex DNA) adduct. That no peaks corresponding to duplex DNA were observed for the sterically hindered alkylated oligonucleotide alone, but were readily observed when free oligonucleotide was added to the alkylated strand, provides strong evidence that stable, specifically basepaired duplexes are being observed in the mass spectrometer. Furthermore, the relative intensities of the ions observed in the ESI mass spectra reflect the greater stability of the hedamycin–d(CACGTG)$_2$ adduct compared to the free d(CACGTG)$_2$, consistent with other evidence that hedamycin both alkylates and intercalates into DNA. Thus, we anticipate that ESI-MS will prove to be an extremely valuable tool for the structural analysis of ligand binding to double-stranded DNA.

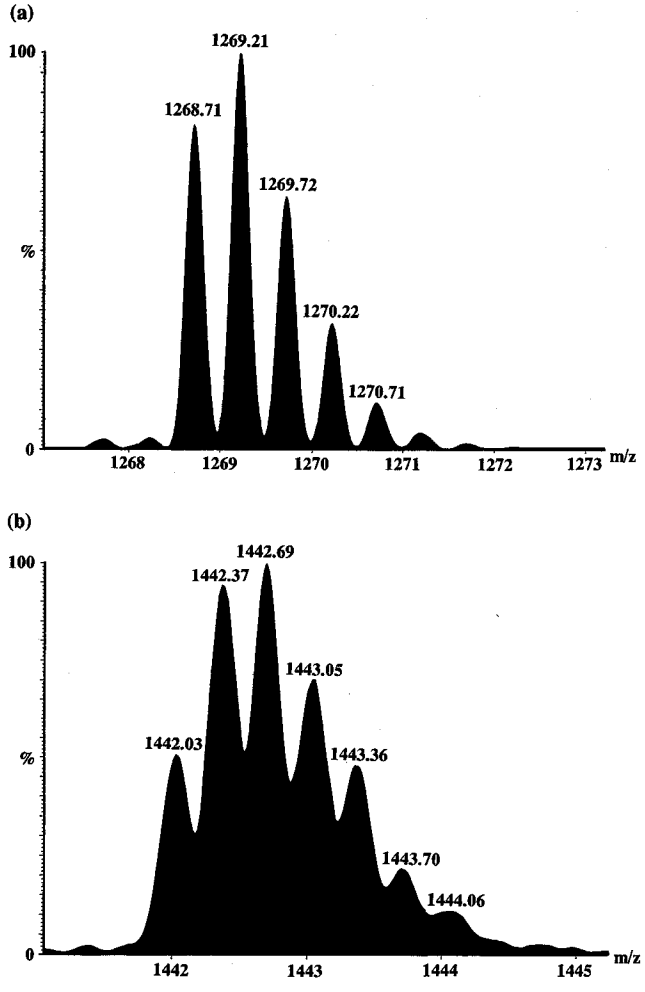

Fig. 4. ESI-MS obtained with a resolution of 3000 which is sufficient to resolve individual isotopic species of (a) [hedamycin–d(CACGTG)-2H]$^{2-}$. The peaks are separated by 0.5 confirming these are 2^- ions; and (b) [hedamycin–d(CACGTG)$_2$-3H]$^{3-}$, in which the spacing of ~0.3 indicates these are 3^- ions.

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