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Electrospray ionisation mass spectrometric detection of weak non-covalent interactions in nogalamycin–DNA complexes

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We demonstrate the use of electrospray ionisation mass spectrometry (ESI-MS) in high salt solutions for the analysis of weak non-covalent complexes of the anthracycline antibiotic nogalamycin with novel DNA hairpin structures; high signal-to-noise ratios for the complexes in the absence of bound Na^+ ions permits relative binding affinities to be estimated.

Nucleic acids adopts a variety of conformations.[1] Hairpins are a common secondary structural feature in RNA,[2] but are less common in DNA.[3] However, there have been a number of recent reports exploring DNA sequences that form hairpin structures that are extraordinarily stable.[2,4,5] Hirao *et al*.[4] have shown by NMR spectroscopy that 5′-GCGAAGC forms a hairpin stabilised by a 5′-GAA loop involving a non-Watson–Crick sheared G–A base pair linked by the bridging adenine (see Fig. 1). Using this novel hairpin structure as a stabilising template, we have designed a number of DNA structures for drug binding studies that contain bulged bases, and a novel double hairpin structure for which co-axial stacking of the two hairpin components creates a single strand break at the centre of the duplex stem region (Fig. 1).[6] We have investigated by NMR and ESI-MS whether intercalating drug molecules are able to recognise these unusual structures, and have determined the binding stoichiometry and relative affinities for these sequences using ESI-MS.

Nogalamycin (Fig. 1) forms a stable non-covalent intercalation complex by threading through the DNA helix making stabilising interactions through sugar residues that reside in both the major and minor groove.[7,8] The antibiotic inhibits transcription factor binding and interferes with topoisomerase activity,[9] showing a sequence binding preference for 5′-pyrimidine–purine sites (5′-CG and 5′-TG); X-ray[10] and NMR[11] structures of drug–DNA complexes have been reported. In the novel DNA hairpin structures shown in Fig. 1, a single 5′-TG binding site was incorporated within the stem region: in (b) a break in the DNA backbone occurs across the 5′-TG step as a consequence of coaxial stacking of the two hairpin components,[6] whereas in the bulged/mispaired structures in (c) and (d) a thymine base is inserted into the 5′-CA step on the opposing strand (5′-C^{T}A). Many of the structural studies so far reported for nogalamycin bound to DNA highlight the need for an intact G–C base pair at the intercalation site.[10,11] Thus, in (c) we postulate that the extra thymine would adopt a bulged-out conformation to satisfy this requirement, while in (d) both the mis-aligned sequence or a bulged-T structure was a possibility. Preliminary NMR studies of these complexes confirm that conformations shown in (b) and (c) are those preferred, although in (d) the structure is more dynamic suggesting the possibility of an equilibrium between the bulged-T structure and the mis-paired conformation. The relative affinities for these different sites (b)–(d) are difficult to assess by NMR.

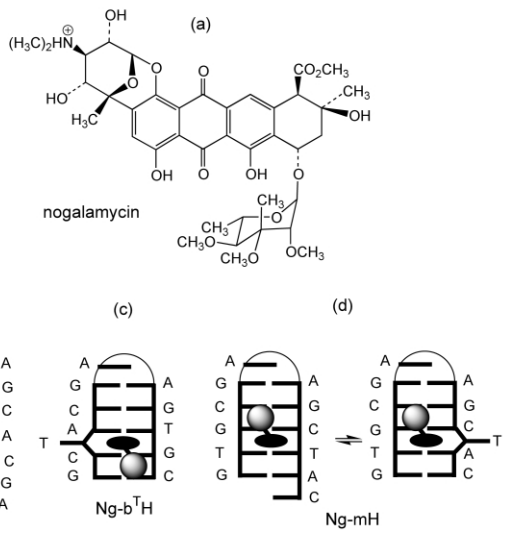

Fig. 1 Structure of nogalamycin (a); drug–DNA complexes, (b) double hairpin (**Ng-dH**), (c) T-bulged hairpin (**Ng-b^{T}H**), and (d) mis-paired hairpin (**Ng-mH**) in equilibrium with T-bulge.

Electrospray ionisation mass spectrometry (ESI-MS) has been applied to the study of a number of non-covalent complexes of drug molecules with double-stranded DNA.[12,13] In earlier studies using gentle ionisation conditions for the detection of non-covalent interactions, we examined the binding of nogalamycin to a range of oligonucleotides forming 'normal' duplex DNA.[14] Recently, the binding of the neocarzinostatin chromophore to unusual DNA structures including bulged DNA and RNA/DNA hybrids has been explored by ESI-MS.[15]

The complexes formed between nogalamycin and the oligonucleotides d(GCGAAGCACGAAGT) (double hairpin, **dH**), d(GTGCGAAGCTAC) (T-bulged hairpin, **b^{T}H**) and d(GCTACGAAGTGC) (mispaired hairpin, **mH**) were prepared by titration of the ligand into a solution of the oligonucleotide; the formation of the complexes was monitored by ^{1}H-NMR spectroscopy. NMR solutions initially contained salt concentrations of 100 mM NaCl/10 mM NaH_2PO_4 in order to stabilise these non-standard conformations. Previously, samples containing high salt have not been amenable to analysis by ESI-MS since the presence of sodium both reduces ionisation efficiency and limits mass accuracy owing to the presence of multiple species with different numbers of sodium ions bound.[16]

The use of newly introduced ESI mass spectrometers with the ion source in the Z-spray configuration, designed to minimise contamination,[17] allowed us to analyse protein–DNA complexes in solutions containing high salt concentrations.[18] Hence, we have also explored the possibility of using solutions directly from NMR without further purification or desalting. The NMR samples were diluted using 100 mM ammonium acetate (pH 7.0) to give a final concentration of the complex of 10 pmol μL^{-1} and 5 mM NaCl/0.5 mM NaH_2PO_4. An electrospray probe tip potential of ~2 kV and a source temperature of 40 °C were used. Electrospray analysis was performed in negative ion mode using a Micromass Q-TOF 2. Other conditions were similar to those described recently for protein–DNA complexes.[18]

The ESI mass spectrum of the unbound **dH** is shown in Fig. 2. There are a large number of peaks owing to multiple sodium adducts making interpretation of the spectrum difficult. In

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contrast, in the ESI mass spectrum of the complex formed with nogalamycin (**Ng-dH**), much stronger peaks are observed for the complex with little evidence for sodium adduct formation, confirming a drug : DNA binding stoichiometry of 1 : 1. The stabilisation of the folded conformation by the drug precludes the need for stabilising alkali metal counter ions. Fig. 2 also shows the ESI mass spectrum of the T-bulge complex (**Ng-b^{T}H**). In this case, the ions from the drug–DNA complex were less abundant relative to the free oligonucleotide. Finally, we examined the binding of nogalamycin to the mis-paired hairpin (**mH**), however the intact adduct was not detected, only the unbound oligonucleotide, indicating that this was the least stable complex. This is consistent with the NMR data that suggest that the imino protons of G1 and T2 are exchange broadened even at 5 °C and that these nucleotides are not involved in stable base pairing or in significant stabilising drug–DNA interactions.

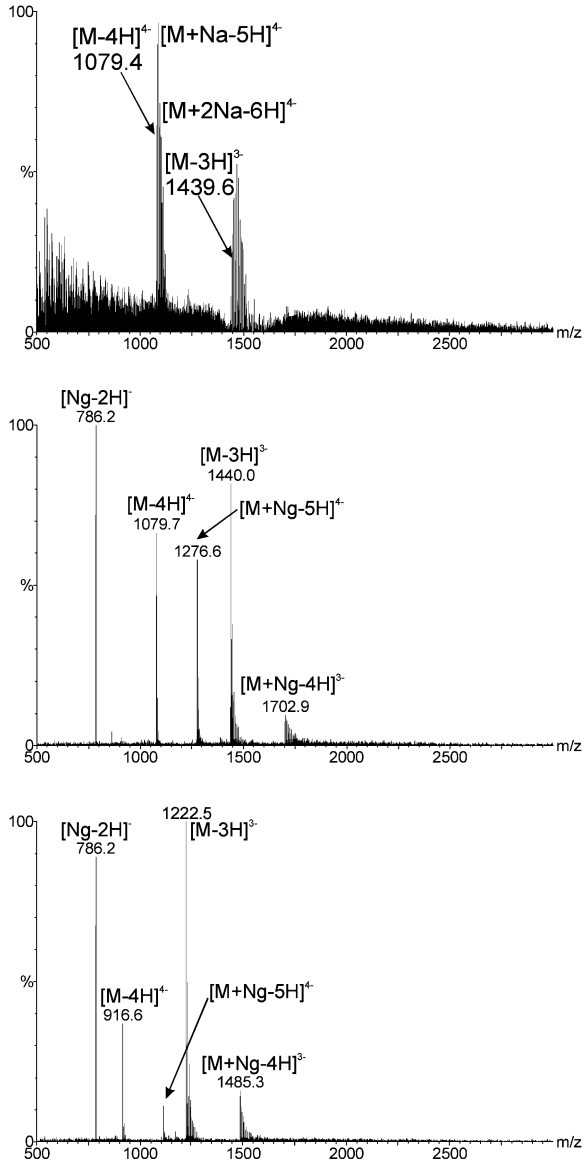

Fig. 2 ESI mass spectra of (top) unbound **dH**, (middle) **Ng-dH** complex, and (bottom) **Ng-b^{T}H** complex (see Fig. 1). The identity of the major species are as indicated. M is the neutral form of the oligonucleotide.

We have used the ESI-MS data to derive a semi-quantitative estimate of the relative stabilities of the complexes formed. The *m*/*z* cut-off used precludes the observation of doubly charged **Ng** species, however, all peaks due to the free and bound forms of the DNA should be detected. We have calculated the abundance of the free and bound forms of the DNA in each spectrum by summing the intensities of all peaks due to each species. Thus, for **Ng-dH**, **Ng-b^{T}H** and **Ng-mH,** we estimate relative binding affinities in the ratio $\sim 10 : 3 : < 1$. A rough estimate of the equilibrium binding constant for the **Ng-dH** complex based on the abundance of the free and bound forms, under the destabilising conditions of the ESI-MS experiment, suggests $K_{eq} \sim 0.7 \times 10^5$ M.

Overall, the ESI-MS data indicate that the relative stability of the complexes formed with nogalamycin is in the order: **Ng-dH** (double hairpin) > **Ng-b^{T}H** (T-bulge site) > **Ng-mH** (mis-paired hairpin). These results show that relatively weak binding interactions in non-covalent drug–DNA complexes involving destabilising effects from single strand breaks and bulged-bases are detectable under gentle ionisation conditions using salt-tolerant ESI source configurations. Perhaps most importantly, the spectra are observed with greatly enhanced signal-to-noise ratios in the presence of nogalamycin, with the stabilising effects of the drug precluding the need for stabilising Na^+ counter ions.

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