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Non-covalent Complexes Between DNA-binding Drugs and Double-stranded Deoxyoligonucleotides: a Study by Ionspray Mass Spectrometry

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The non-covalent complexes between some DNA-binding drugs and duplex oligodeoxynucleotides were studied by ionspray mass spectrometry, with the aim of evaluating the suitability of this technique to screen rapidly a series of drugs exerting their activity through non-covalent binding to specific base sequences of DNA. Two classes of drugs were considered, distamycins (which show affinity for the minor groove of DNA) and anthracyclines (which interact through intercalation between bases). For the former, d(CGCGAATTCGCG)$_2$ was chosen as the model oligodeoxynucleotide. Following optimization of sample preparation and instrumental conditions, the complexes of different distamycins were observed; depending on the ligand considered, 1:1 or 2:1 complexes were formed preferentially. A semi-quantitative evaluation of the relative affinities was made by measuring the ratio of the complexes signals to those of the duplex, and also by competitive binding with equimolar amounts of distamycin. For anthracyclines, the daunorubicin–d(CGATCG)$_2$ complex was chosen as the model for a preliminary mass spectrometric study; however, the signals of the duplex and the complex were very low compared with the monomer signal. Since the complex was known to be stable in solution, this was ascribed to gas-phase instability, probably caused by electrostatic repulsion between negatively charged phosphate groups. © 1997 John Wiley & Sons, Ltd.

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INTRODUCTION

Specific non-covalent interactions with DNA provide the molecular basis for the activity of many anti-tumour, anti-viral, anti-bacterial and anti-protozoarian drugs. The increasing interest in the discovery of new DNA-binding drugs led to the development of physico-chemical methods for the analysis of these drug–DNA (or drug–model deoxyoligonucleotide) complexes, aiming at the elucidation of their structures and the evaluation of the binding constants. A wide variety of techniques have been used, such as IR spectrometry,[1,2] IR linear dichroism,[1,3] circular dichroism,[1,4] fluorimetry,[1,5] gel footprinting,[6–8] X-ray crystallography[9–11] and NMR spectroscopy.[12–16]

In recent years, mass spectrometry (MS) has also been introduced as a sensitive and specific tool for the analysis of specific non-covalent complexes involving biopolymers,[17–23] thanks to the development of extremely mild ionization techniques such as electrospray ionization[24] (ESI) and pneumatically assisted ESI or ionspray[25] (IS), which allow the preservation of even very weak interactions during the transfer of ions from the liquid to gas phase, and the subsequent mass analysis of the whole complex.[26,27] A significant advantage of ESI over other soft ionization techniques is that samples can be analysed directly in aqueous solutions, that is, under conditions very similar to those occurring in biological fluids, without any need for alien additions such as a viscous matrix (as in fast atom bombardment) or a solid matrix (as in matrix-assisted laser desorption/ionization). ESI has been successfully employed for the analysis of specific associations involving dimer[28–30] and tetramer[31] oligonucleotides, in addition to non-covalent complexes of single-[32] and double-stranded[33,34] oligonucleotides with small organic molecules.

The aim of this work was the evaluation of the potential of MS to screen rapidly a series of drugs exerting their activity through non-covalent binding to specific base sequences of DNA. Two classes of DNA-binding drugs were considered, i.e. distamycins and anthracyclines. The former are classified as minor groove binders:[35] they interact externally with the minor groove of the B DNA double helix displaying sequence specificity for consecutive A–T base pairs. The self-complementary oligodeoxynucleotide d(CGCGAATTCGCG)$_2$ or Dickerson dodecamer was chosen as a model to study the distamycin interactions with DNA. The 1:1 complex with distamycin A (**1**) (Fig. 1) was studied by NMR and molecular mechanics,[36–38]

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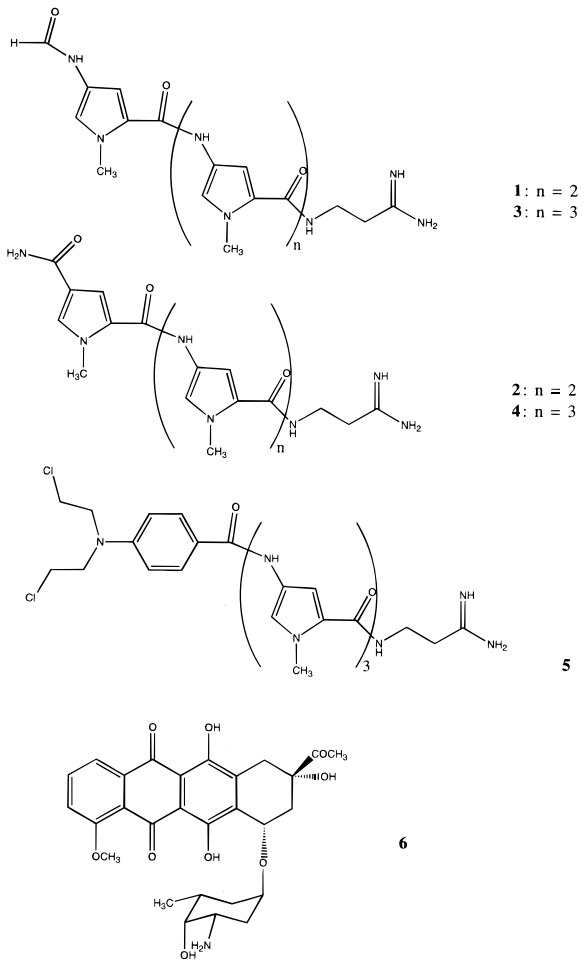

Figure 1. Structural formulae of the ligands considered.

allowing the definition of the molecular interactions at the binding site; the binding constant of distamycin A is $\sim 10^7$ M^{-1}.[39] No previous study by MS has been performed with this complex; however, the analysis by ESI of the 1:1 and 2:1 complexes of distamycin A with the dodecamer d(CGCGAAATTTGCG)$_2$ was extensively described by Gale and Smith.[34] For the purpose of our study, we were interested in an oligonucleotide with a shorter binding site (four consecutive A–T base pairs instead of six); here we report the behaviour of distamycin A and some synthetic analogues with this double-stranded dodecamer, and add some instrumental and sample preparation considerations.

The second class of DNA-binding drugs considered was of anthracyclines;[40] they interact with DNA through intercalation between bases, and display affinity for C- and G-rich base sequences (especially for the CGT sequence).[41] The complex between daunorubicin (**6**), the precursor of this class of drugs, and the self-complementary hexadeoxynucleotide d(CGATCG)$_2$, was used as the model in this case; the crystal structure of this complex is known to 1.2 Å resolution.[9,11,42] Preliminary results of the analysis of the d(CGATCG)$_2$–doxorubicin (an anti-neoplastic drug structurally related to daunorubicin) complex by MS were reported by Gentil and Banoub.[43]

EXPERIMENTAL

Materials

Distamycin A and daunorubicin were purchased from Sigma Chemical (St Louis, MO, USA). Published methods were used to synthesize MEN 10400[44] (**2**), MEN 10567[44] (**3**), MEN 10706[44] (**4**) and FCE 24517[45] (**5**). d(CGCGAATTCGCG) was synthesized using phosphoramidite chemistry and purified by reversed-phase high-performance liquid chromatography. d(CGATCG) was purchased from Biotec Italia (Rome, Italy) and used without further purification.

Sample preparation

A 0.8 mM solution of oligonucleotide in 1 M ammonium acetate was annealed by heating for 10 min at 80 °C and slowly cooling at room temperature, to favour dimerization. A 20 µl volume of this solution was mixed with 0–100 µl of 0.2 mM ligand solution in water and the volume was made up to 200 µl with water.

Where specified, some samples were filtered through a 3000 Da cut-off membrane (Centricon 3; Amicon, Beverly, MA, USA) by centrifugation for 15 min at 6500*g*.

Mass spectrometry

All the mass spectra were acquired using a Sciex API III Plus mass spectrometer (Sciex, Thornhill, ON, Canada), equipped with an articulated ionspray interface operated in the negative ion mode. The nebulizer potential was set at 4000 V and the orifice potential (OR) was 80 V (100 V for the daunorubicin–d(CGATCG)$_2$ complex). Air was used as the nebulizing gas and dry nitrogen as the curtain gas.

The samples were introduced by infusion at 5 µl min^{-1} using a Model 22 syringe pump (Harvard Apparatus, South Natick, MA, USA).

Full-scan acquisitions were performed in the multi-channel mode, summing 40 scans, with a scanning interval in the m/z range 400–2200 (step 0.2 u, dwell time 1 ms). Multiple ion monitoring (MIM) acquisitions were also performed in the multi-channel mode, summing 20 scans and using windows of 5 u for each selected ion (step 0.1 u, dwell time 20 ms).

RESULTS AND DISCUSSION

The first part of our study was devoted to optimizing the sample preparation and instrumental parameters. A step-by-step procedure was followed, on the assumption that good conditions for observing oligonucleotide duplexes alone would have been the best starting point to find the optimum experimental conditions for the analysis of the complexes.

Observation of duplex oligodeoxynucleotides

The response of non-annealed oligonucleotides in different buffers was tested (ammonium citrate, carbonate, formate and acetate, 10 mM); preliminary data showed that the nature of the buffer influenced the absolute abundance of the peaks obtained, and that ammonium acetate and formate were the most suitable. The buffer did not influence significantly the appearance of the spectra with respect to charge state distribution or the sensitivity, leading to differences by a factor of two between the best and the worst.

The buffer concentration also played a role in the appearance of the spectra, higher sensitivity being obtained with 10 mM buffer. On going from 10–100 mM, the oligonucleotide ion abundances diminished by about half, whereas higher concentrations were of no use: in 200 mM buffer the signals were strongly disturbed by the background noise.

The 1:1 addition of an organic modifier such as acetonitrile gave, as expected from literature data,[46] a significantly higher response. However, not surprisingly, it destroyed the duplex signals, which under certain conditions where observable at very low relative abundances in non-annealed solutions, as also reported by others.[47]

A great influence on the appearance of the spectra occurred on varying OR in the range 35–120 V. In fact, in agreement with several literature reports for peptides and oligonucleotides,[21,48] a low OR markedly shifted the charge state distribution towards lower *m/z* values, and the opposite happened for high OR settings. For example, the spectrum of d(CGCGAATTCGCG) recorded at 50 V showed the five-charged ion as the base peak,[49] whereas at 100 V the base peak was the three-charged ion. Moreover, the dimer oligonucleotide ions (see later) were clearly observable only at high OR values, the optimum voltages being 80 V for d(CGCGAATTCGCG) and 100 V for d(CGATCG), that is, under conditions which usually favour collision-induced fragmentation of the ions being analysed. This paradoxical finding suggested a good gas-phase stability for the duplex ions, and was in contrast with some literature data reporting that oligonucleotide duplexes, and generally non-covalent complexes, are best observed under low declustering potentials and mild ion source conditions.[27,34]

Most probably, this discrepancy is due to the different designs of the instruments used. On the other hand, others have reported the need for a high declustering potential for oligonucleotide duplex analysis,[30,47] and also for other kinds of non-covalent complexes.[50] However, analysis of an annealed solution of d(CGCGAATTCGCG) in 10 mM buffer under optimized instrumental conditions resulted in very low, if any, duplex signals, as shown in Fig. 2(a), in which only the three- and two-charged ions of the monomer at *m/z* 1214 and 1822, respectively were present.

As also described by others,[34] the interpretation of homodimer mass spectra needs particular care with regard to the ambiguities between the *m/z* values observed for the monomer ions having *n* charges and dimer ions having 2*n* charges, while obviously this problem does not arise for odd-charged dimer ions. In cases where sufficient resolution to assign the charge

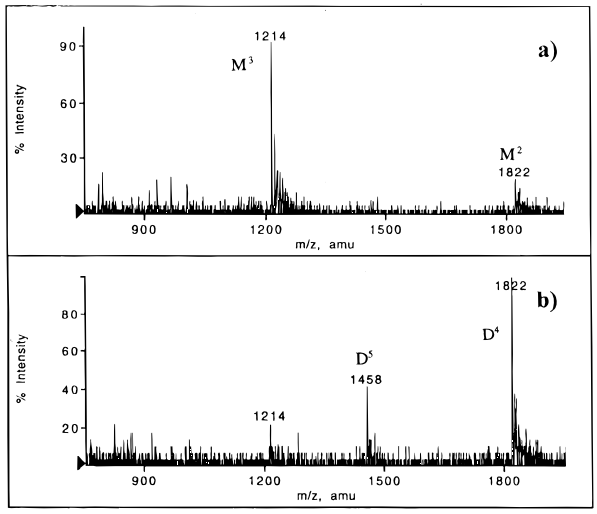

Figure 2. Ionspray mass spectra of d(CGCGAATTCGCG), after annealing in (a) 10 mM and (b) 1 M ammonium acetate. $M^n = n$-charged monomer oligonucleotide; $D^n = n$-charged double-stranded oligonucleotide.

state from the isotopic pattern is not available, such as often happens with linear quadrupole analysers, the correct assignment can be made by measuring the distance between the signal and known adducts with some components of the mobile phase, either occurring incidentally such as sodium and/or potassium ions, or added on purpose such as crown ethers.[51] In the present case, the correct assignment to the monomer is possible by looking at the sodium adducts of *m/z* 1214 (differing from the deprotonated ion by 22/3 u) and *m/z* 1822 (differing by 22/2 u). Incidentally, the term 'adduct' does not appear to be strictly correct to describe such ions, since effectively here a sodium ion has replaced one mobile hydrogen in the molecule, and is not simply added to it; however, it is generally accepted in similar cases, and hence it will be used also in this paper.

The initial failure to obtain satisfactory duplexes signals was ascribed to low stabilization of the duplex in solution, due to insufficient ionic force of the buffer. In fact, it is well known that an important stabilizing factor for double helixes, in addition to hydrogen bonding between complementary adjacent bases and hydrophobic interactions between consecutive bases (base stacking), consists in the electrostatic interactions between the cations in solution and the negatively charged phosphate groups of the polynucleotide chain, attenuating the repulsion between the negative charges of opposite strands.[30,52] Thus, d(CGCGAATTCGCG) was annealed in 1 M buffer[30] and then diluted tenfold prior to MS analysis. The resulting concentration of ammonium acetate, 100 mM, was a compromise between duplex stabilization and sensitivity of measurement; in addition, this buffer concentration gave a significant reduction in the relative abundances of adduct ions as compared with lower concentrations, giving simpler spectra.[49]

The result is shown in Fig. 2(b): after annealing in concentrated buffer, a new peak at *m/z* 1458 can be seen, which is unambiguously assigned to the five-

charged duplex. Furthermore, by looking at the distance of sodium adducts, the small m/z 1214 ion is still assignable to the three-charged monomer, whereas the m/z 1822 ion corresponds mainly to the four-charged duplex. In this last case, a small contribution from the doubly charged monomer cannot be excluded, since its first sodium adduct would have the same m/z value as the second sodium adduct of the four-charged duplex; anyway, this contribution is negligible, since in the corresponding mass spectrum of the monomer in Fig. 2(a) the abundance of the m/z 1822 ion is about one-quarter of that of the m/z 1214 ion.

In the case of $d(CGATCG)_2$, only very low duplex signals were observed, even after optimization of the ion source parameters. The spectrum of annealed d(CGATCG) showed mainly the doubly charged single-stranded oligonucleotide and two very low-abundance peaks at higher m/z values, corresponding to the triply and doubly charged duplex, respectively.[49] The poor abundance of the duplex signals is probably due to its instability in the gas phase, since literature data indicate stability of the duplex in buffer solutions of similar concentrations.[5]

Observation of $d(CGCGAATTCGCG)_2$–distamycin A complexes

Figure 3 shows the mass spectrum of a solution containing 40 μM $d(CGCGAATTCGCG)_2$ and a slight molar excess of distamycin A (60 μM): the peaks corresponding to the free duplex and monomer have virtually disappeared, and five newer peaks are present. The peak at m/z 480 corresponds to the distamycin quasi-molecular anion. Among the other peaks, those at m/z 1554 and 1942 correspond, respectively, to the five- and four-charged 1:1 complex, having a relative molecular mass of 7774.4 Da (experimental value 7772.9), whereas those at m/z 1650 and 2062 are assignable to the five- and four-charged 2:1 complex, having a relative molecular mass of 8255.9 (experimental value 8255.4). The complex was stable for at least 48 h at room temperature.

An evaluation of the species occurring in solution with increasing amounts of distamycin A (at concentrations in the range 10–100 μM) was also made, always keeping a 40 μM duplex concentration. For each point, a full-scan acquisition was made in order to check the overall appearance of the spectrum; then, in order to obtain more accurate abundance measurements, representative ions of the species occurring in solution were acquired in the MIM mode. Thus, ions at m/z 480, 1214 and 1457 were acquired for distamycin monomer and dimer oligonucleotide, respectively, whereas for the complexes both of the ions representing each of them were monitored. The amounts of the species in solution were expressed as peak heights (or sum of peak heights in the case of complexes). The full-scan experiments showed that no variation in the charge state distribution occurred during the titration; furthermore, the complexes and free oligonucleotides were represented only by the ions just described, and no by other higher m/z ions, as assessed by making some measurements with an extended mass range instrument.[53]

The amounts of the species in solution versus the concentration of added distamycin are reported in Fig. 4. As can be seen, at low concentrations there is no appreciable free distamycin signal; given that the 1:1 complex signals are readily evident at those concentrations, this means that distamycin is not a fragment ion of the complex. If we assume the same also for the 2:1 complex (which is reasonable given the lack of evident fragment ions in the spectra), then the distamycin signal is only generated by the free ligand. Hence, it could be theoretically possible to measure the concentration of free ligand by using an external standard and calculate the binding constants of these complexes by MS, as already reported by Greig *et al.*[54] for the determination

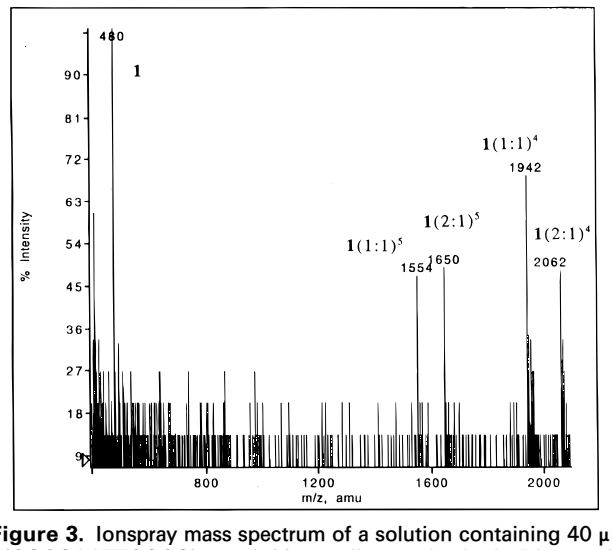

Figure 3. Ionspray mass spectrum of a solution containing 40 μM $d(CGCGAATTCGCG)_2$ and 60 μM distamycin A. **1**, Distamycin quasi-molecular anion; **1**(1:1)^n and **1**(2:1)^n, n-charged 1:1 and 2:1 distamycin complex, respectively.

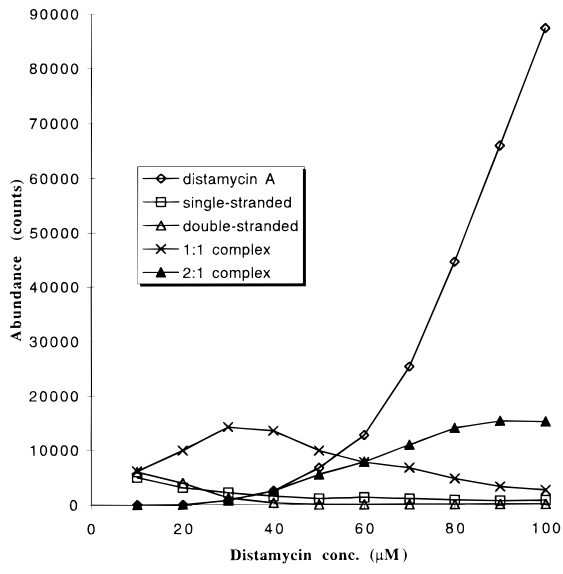

Figure 4. Amounts of the species in solution versus the concentration of added distamycin A.

of dissociation constants of single-stranded oligonucleotide–serum albumin complexes.

The formation of duplex oligonucleotide complexes with two distamycins binding at the minor groove is a well known phenomenon. For example, NMR studies showed that d(CGCAAATTGCG)$_2$ and d(CGCAAATTTGCG)$_2$ form 2:1 complexes in which two distamycins bind head to tail and side by side in the same region of the minor groove, which widens itself by about 0.35 nm to accommodate the second ligand.[11,14,55] The 2:1 d(CGCAAATTTGCG)$_2$–distamycin complex was also observed by ESI-MS,[33,34] as reported earlier, whereas an analogous 2:1 complex with d(CGCGAATTGCG)$_2$, which carries a shorter binding site of four A–T consecutive base pairs, to the best of our knowledge has never been observed by any spectroscopic methods, and is shown here for the first time.

Although care must be taken in assessing the real occurrence and the specificity of such a non-covalent complex, and confirmations are still to be made with other methods, the 2:1 d(CGCGAATTCGCG)$_2$–distamycin complex appears to be really specific. In fact, it has a high intensity relative to non-complexed oligonucleotide peaks, and at appropriate drug–binder ratios it is observed preferentially with respect to the 1:1 complex. In addition, the complex is not observed following modifications of the solution which are known to destabilize it and/or the duplex (addition of acetonitrile, use of very dilute buffers), and the substitution of distamycin with analogues leads to significant differences in the appearance of the spectra, which can at least in part be explained on the basis of known affinity scales (see the next section). In other words, the complex meets some important criteria discussed by Smith and Light-Wahl[27] for distinguishing specific from non-specific non-covalent associations by MS. In addition, the 2:1 complex remained by far the most abundant one even in presence of a 20-fold excess of distamycin. Under these conditions, which should greatly favour the formation of higher order non-specific complexes, no 1:1 complex was present, and only low percentages of 3:1 and 4:1 associations were observed,[49] the latter having to be ascribed to electrostatic interactions due to the high ligand concentration.

Observation of d(CGCGAATTCGCG)$_2$ complexes with distamycin analogues

In order to investigate the capability of ESI-MS to detect differences in binding modes and affinities among different ligands, the mass spectra of solutions containing duplex d(CGCGAATTCGCG) and distamycin-related molecules, both 40 μM, were registered.

The structures of the compounds investigated are depicted in Fig. 1; compounds **2** and **5** contain three *N*-methylpyrrole units like distamycin, and a carboxamide or an *N*,*N*-di(2-chloroethyl)aminobenzoylamide at the place of the formylamino moiety, respectively, whereas compounds **3** and **4** both contain a longer chain of four pyrrole rings, and a formylamino and a carboxamido group, respectively.

^{1}H-NMR studies showed that **2** binds to d(CGCGAATTCGCG)$_2$ very similarly to distamycin A, but with decreased affinity.[56] The structures of the 2:1 complexes of **3** and **4** with d(CGCAAATTTCGC)$_2$ have also been elucidated by NMR and molecular modeling.[44]

Compound **5** displays a similar Cotton effect to distamycin A when complexed with calf-thymus DNA in circular dichroism experiments, which is a measure of the binding affinity for this class of molecules;[45] its binding was shown to be essentially non-covalent and of electrostatic nature, although the molecule carries a nitrogen mustard alkylating moiety.[45] In addition, some selective alkylations to *N*-3-adenine in a small number of sites in the minor groove of DNA were described.[57] The structure of its complex with d(CGTATACG)$_2$ was investigated by NMR.[58]

The mass spectra of the equimolar solutions of d(CGCGAATTCGCG)$_2$ with **2**–**5** showed different preferential stoichiometries and affinities. For example, the spectrum of the complex with **3** is shown in Fig. 5. The highest peak corresponds to the five-charged 2:1 complex (*m*/*z* 1699); the signal of the five-charged 1:1 complex (*m*/*z* 1578) has a much lower intensity, indicating a clear preference for the 2:1 complex. In other words, the binding of this molecule to d(CGCGAATTCGCG)$_2$ is a co-operative one, since the first bound ligand increases the affinity of the site for a second ligand.

An overall view of the species occurring in solutions for each of the compounds considered is given in Fig. 6, showing in a single histogram the abundances of free single- and double-stranded oligonucleotide, 1:1 and 2:1 complex. It can be seen that there are ligands with great differences in behaviour, compounds **1**, **3** and **4** being endowed with greater affinities than **2** and **5**. Moreover, compounds **3** and **4**, characterized by four *N*-methylpyrrole units instead of three, have a stronger tendency to form 2:1 complexes; as a first hypothesis, this may be due to a greater distortion of the oligonucleotide double helix caused by the increased length of

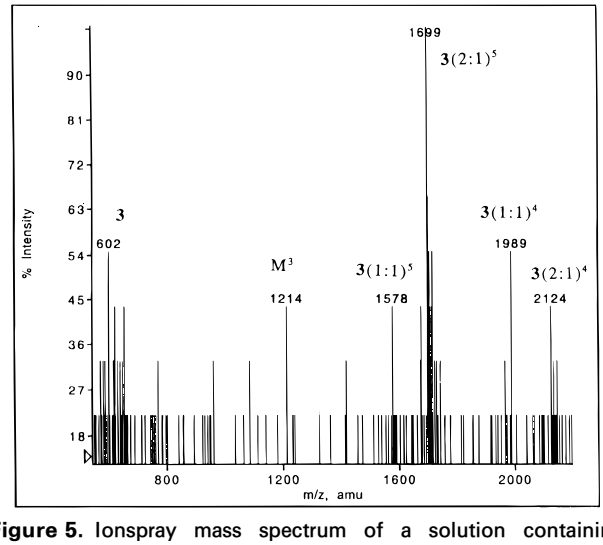

Figure 5. Ionspray mass spectrum of a solution containing d(CGCGAATTCGCG)$_2$ and **3**, each 40 μM. **3**, Quasi-molecular anion of **3**; **3**(1:1)^n and **3**(2:1)^n, *n*-charged 1:1 and 2:1 complex of **3**, respectively; M^3, three-charged oligonucleotide monomer.

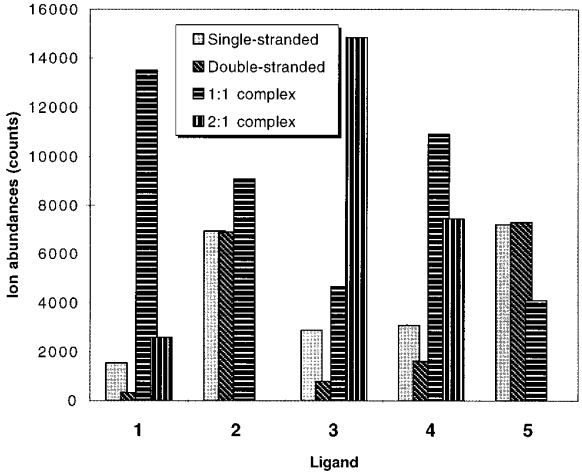

Figure 6. Absolute abundances of single- and double-stranded d(CGCGAATTCGCG)$_2$ and 1:1 and 2:1 complexes for each of the ligands considered.

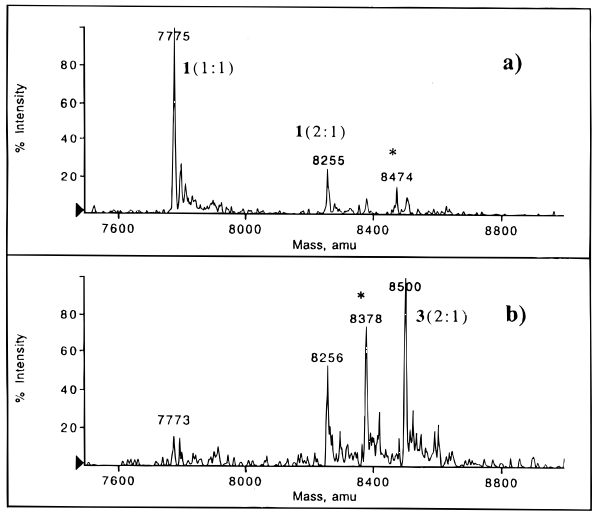

Figure 7. Deconvoluted mass spectra of d(CGCGAATCGCG)$_2$ in presence of (a) **1** and **5** and (b) **1** and **3**. **1**(1:1) and **1**(2:1), complexes of **1**; **3**(2:1), complex of **3**; asterisks, mixed complex.

the ligand, which in this case facilitates the binding of a second molecule. This finding has, of course, to be confirmed with studies by more traditional techniques such as NMR, which are able to elucidate in detail the molecular interactions involved; nevertheless, ESI-MS can be considered as a simple and rapid method to screen candidate new drugs before obtaining further detailed information.

Competitive binding of distamycins

Another possible way to estimate the affinity of a potential new drug for a receptor can be from the competition with a reference compound for the same binding site. For this purpose, equimolar amounts (40 μM) of duplex, **1** and in turn **3**, **4** and **5** were mixed. The comparison with **2** was not performed, as it has a molecular mass as identical with that of **1**. The study of competitive binding by ESI-MS appears to be very promising in terms of sensitivity and specificity, opening up new perspectives for the study of complicated mixtures.[59–61]

In the simplest case, the mass spectra indicated the preferential binding of the ligand having greater affinity; as an example, Fig. 7(a) reports the deconvoluted spectrum of a solution containing **1** and **5**, that is, the reference ligand and a molecule having low affinity: only the 1:1 and the 2:1 complexes of **1** are evident, strongly suggesting that the signal of the complexes arise from a specific interaction. Another peak at 8474 Da is present, and is assignable to a mixed complex in which one molecule of **1** and one of **5** are binding to a duplex.

Another example is depicted in Fig. 7(b), showing the deconvoluted mass spectrum obtained from the competition of **1** with **3**, which had been shown in the previous section to have a strong tendency to form a 2:1 complex with d(CGCGAATTCGCG)$_2$. In this case, only a small amount of the 1:1 complex of **1** is observed (7773 Da), whereas much greater amounts of the 2:1 complexes of both ligands are present at 8256 and 8500 Da, respectively, the latter being about two times more abundant than the former. Also in this case a further peak, having intermediate abundance, is present at 8378 Da, and corresponds to a mixed complex.

The real occurrence of specific mixed complexes in solution has to be assessed with caution, since the possibility of observing some false positives must always be considered. For example, Cunniff and Vouros[62] described the observation of non-specific electrostatic adducts of amino acids and peptides with cyclodextrines and Aplin *et al.*[63] interestingly reported that porcine pancreatic elastase binds with some peptide substrates and their enantiomers, but not with randomly chosen peptides. In Fig. 7(a), a non-specific interaction cannot be ruled out, although mixed complexes were not always observed by us during competition experiments with other compounds, suggesting that they could not be merely random aggregates. In Fig. 7(b), a purely statistical aggregation of the 2:1 complexes would have given a twofold probability for the mixed complex to be formed, and its relative abundance would have been predictable on the basis of binomial expansion, assuming equal ionization efficiencies.[47] As a consequence, the mixed complex would have been more abundant than the two homogeneous 2:1 complexes, which was never observed by us.

Observation of the d(CGATCG)$_2$–daunorubicin complex

In the spectrum of the complex between duplex d(CGATCG) and daunorubicin, similarly to the oligonucleotide alone, the only relevant peak was the one corresponding to the doubly charged single-stranded oligonucleotide. Two other very low-intensity peaks were observed at higher masses, assignable to the triply charged duplex and to the triply charged duplex/daunorubicin complex.[49] Also in this case, this was ascribed to gas-phase instability of the duplex, since the

complex was stable in solution,[5] as shown by ultrafiltration through a 3000 Da cut-off membrane; in this case, the supernatant visibly still contained the ligand, which is colored red, whereas after ultrafiltration of a solution of daunorubicin and non-annealed oligonucleotide the red colour passed to the ultrafiltrate. Very low signals of non-covalent complexes involving d(CGATCG) were also observed by Gentil and Banoub[43,64] for the complex with doxorubicin, which is structurally related to daunorubicin.

The poor $d(CGATCG)_2$ duplex gas-phase stability can be explained in terms of shorter chain length compared with $d(CGCGAATTCGCG)_2$. This is true also for the solution phase; for example, calculated duplex dissociation free energy values according to Breslauer *et al.*[65] are 6.5 kcal mol^{-1} for $d(CGATCG)_2$ and 23.7 kcal mol^{-1} for $d(CGCGAATTCGCG)_2$ (1 kcal = 4.184 kJ), suggesting that a correlation exists between solution and gas-phase stability, at least qualitatively. For example, lower duplex signals in the case of shorter interacting strands were reported by Bayer *et al.*[30] in studies of the interactions of a fixed-length oligonucleotide with complementary strands of increasing lengths, and Gale *et al.*[66] showed a correlation between duplex melting points and their relative abundances under ESI-MS and ESI-MS/MS conditions. However, stability in the gas phase should be significantly lower, mainly because of the repulsion between opposite negative phosphates which are no longer masked by counter ions in solution. This should be especially true for shorter duplexes, for which stabilization via hydrogen bonding and base stacking might not be sufficient to compensate for electrostatic repulsion. According to the above observations, d(CGATCG) is not a very suitable model to study non-covalent interactions between DNA and anthracyclines by MS, although the complex is stable in the solution being analysed.

This can also explain why oligonucleotide duplexes and complexes are best observed at high OR voltages, even in more severe collisional activation conditions: in fact, at low OR the charge state distribution is shifted towards higher values, and a greater neat negative charge increases the repulsion between the strands. The opposite happens for high OR values, because charge-stripping phenomena occurring in the declustering region of the ion source[21] cause the charge state distribution to shift towards lower values, thus reducing repulsive forces between strands.

CONCLUSIONS

Non-covalent complexes between duplex d(CGCGAATTCGCG) and several minor groove-binding ligands of the distamycin family were reproducibly observed by ionspray MS following optimization of sample preparation and instrumental conditions. Depending on the ligand considered, the preferential formation of 1:1 or 2:1 complexes was observed; generally, distamycins with three pyrrole units had a lower tendency to form 2:1 complexes than those with four pyrrole units, probably because of a greater distortion of the minor groove in the latter case, rendering the duplex more prone to accommodate a second molecule of ligand. The formation of 2:1 complexes between distamycins and a duplex such as $d(CGCGAATTCGCG)_2$, with a binding site of only four AT consecutive base pairs, had never been observed before, and needs confirmation by other spectroscopic methods such as NMR, which should also describe the interactions at the molecular level.

A semi-quantitative evaluation of the relative affinities was made by measuring the ratio of the complex signals to those of the duplex, and also by competitive binding with equimolar amounts of distamycin.

The model daunorubicin–$d(CGATCG)_2$ is not the most suitable for the observation of non-covalent interactions between DNA and anthracyclines by MS, although it was extensively analysed by different techniques; in MS analysis the signals of the duplex and the complex are very low compared with the monomer signal. This is probably due to the low gas-phase stability of the complex, caused by electrostatic repulsion between negatively charged phosphate groups of opposite strands. This is not observed in the case of $d(CGCGAATTCGCG)_2$, probably because repulsion is counterbalanced by a greater number of complementary base pairs interacting on opposite filaments, provided that a high declustering potential, shifting the charge state distribution towards lower values, is used.

When a suitable model for DNA–drug interactions is available, ESI-MS can be considered a good technique for observing the non-covalent complexes obtained. One of the main advantages with respect to more traditional techniques is easy data interpretation, since the complexes are identified by their molecular mass, which can be accurately measured. In addition, short analysis time, relatively simple operation and high sensitivity of detection are very useful, especially at an early phase of investigation, when a rapid preliminary screening with low sample consumption is needed. The possibility of observing with great specificity each of the species in solution is another remarkable feature of MS, which can be used conveniently in experiments where the complexes are present in mixtures. Also, the easy interfaceability with liquid chromatography and capillary electrophoresis can be very useful, provided that the composition of the mobile phase used does not affect the stability or the detectability of the complexes.[67]

Among the disadvantages of an ESI-MS approach are that complexes with low gas-phase stability are not simply observable, and that no structural information about the site of binding and the interacting groups involved can be obtained; in fact, mass-selected complex ions under collision-induced decomposition (CID) usually fragment into their constituents.[29,34] CID tandem MS has instead been used to assess the non-covalent nature of the observed complexes[27,29] and to give an indication of their stability.[34]

Future developments of this technique will involve rapid and sensitive screening for candidate new DNA-binding drugs, through the determination of the binding stoichiometry and the evaluation of binding affinities, and the determination of the sequence specificity of selected ligands, through complexation with known-sequence oligonucleotides.

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