Non-Covalent Complexes between DNA-Binding Drugs and Double-Stranded Oligodeoxynucleotides: A Study by ESI Ion-Trap Mass Spectrometry

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Abstract: We developed an assay that utilizes electrospray ionization mass spectrometry (ESI-MS) to determine rapidly the noncovalent binding of drugs with oligodeoxynucleotides and to assess their relative affinities and stoichiometries. The method uses a set of self-complementary oligodeoxynucleotides that differ in length (6-mer to 12-mer), motif (GC-rich or AT-rich), and sequence, and these were annealed to form duplexes. To the oligodeoxynucleotides are bound a group of drugs (distamycin, Hoechst 33258, Hoechst 33342, berenil and actinomycin D abbreviated as D1, D2, D3, D4, and D5, respectively) that are classic minor-groove binders and intercalators. A second group (porphyrin H_2TMpyP-4, metalloporphyrin CuTMpyP-4, FeTMpyP-4 and MnTMpyP-4 and [Ru(II)12S4dppz]Cl_2 abbreviated as D6, D7, D8, D9, and D10) binds via mixed modes (i.e., groove binders and intercalators). The results confirm the binding stoichiometry and show preferred binding of minor-groove binders (distamycin, Hoechst 33258, Hoechst 33342, and berenil) to AT-rich oligomers and preferred interaction of the intercalator actinomycin D with GC-rich oligomers. The drugs H_2TMpyP-4 and CuTMpyP-4 bind via mixed modes, whereas FeTMpyP-4 and MnTMpyP-4 interact by minor groove-binding only. Competitive binding experiments show that group-I drugs with duplex 5′-CGCAAATTTGCG-3′ have binding affinities in the order D3 > D2 > D1 > D4. The order for group-II drugs with duplex 5′-ATATAT3-′ is D6 ≈ D7 > D8 ≈ D9.

Introduction

The specific, noncovalent interaction of small organic molecules with duplex DNA is the molecular basis of many antitumor, antiviral and antibiotic drugs. Compounds that bind to DNA with high affinity can influence gene expression and, therefore, affect cell proliferation and differentiation. More efficient DNA-binding drugs are those that have improved binding affinity and specificity toward target DNA. Small duplex oligonucleotides have served as appropriate models for assessing binding properties, and the results allow refinement of candidate structures. The "lead compound" or candidate is usually a natural product that can be modified by organic synthesis to prepare more effective candidates. For example, rational structure modifications of the antibiotics netropsin and distamycin led to the development of lexitropsins.[1]

DNA-binding drugs interact with duplex DNA in two principal ways: groove binding and intercalation.[2] The majority of small molecules bind to the minor groove of B-DNA, presumably because they find stronger van der Waals contacts in this region.[2] Many minor-groove ligands, especially those that are positively charged at physiological pH, prefer A and T sites because the electrostatic potential is negative in the minor groove of the AT-rich region[3,4] whereas many intercalators have a preference for G and C regions of DNA.[2]

Combinatorial chemistry has made available a large number of candidate DNA-binding agents. In parallel with this development, a wide variety of physical and chemical techniques have emerged to meet, in part, the demand for determining structure and binding stoichiometry, specificity, and affinity of these noncovalent complexes. These methods include NMR,[5−10] X-ray crystallography,[8,10−12] gel footprinting,[7,13,14] Fourier transform infrared,[15−19] circular dichroism,[20,21] electric linear dichroism,[22,23] viscosity, and fluorescence spectroscopy.[24]

Mass spectrometry has assumed a more active role in investigations of noncovalent complexes involving biopolymers,[25−28] owing to the gentle nature of the electrospray ionization process, which allows a wide range of noncovalent complexes to be introduced intact into the gas phase.[29] Electrospray ionization mass spectrometry (ESI-MS) can reveal binding stoichiometry for relatively small amounts (picomoles) of material. Nonspecific aggregation can also be reduced because the sensitivity of ESI-MS allows studies to be conducted for

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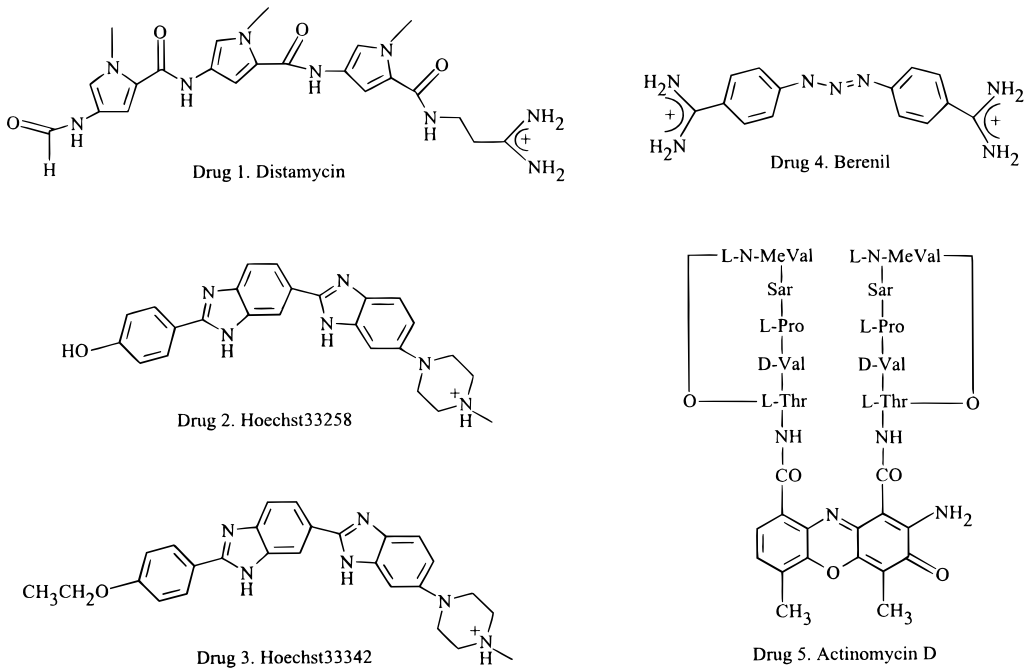

Figure 1. Structures of group-I drugs.

solutions at micromolar concentration. Competitive binding of various ligands is easy to evaluate, yielding relative binding affinities and specificities. The combination of ESI and tandem mass spectrometry can provide some structural detail about the complexes, and gas-phase stability can be compared with that in solution to seek correlations and insight on intrinsic binding. For example, ESI-MS has been successfully employed to study a wide variety of noncovalent interactions, including those of multimeric proteins,[30,31] enzyme−inhibitor complexes,[32,33] oligonucleotide duplexes,[34−37] and tetramers,[38] and noncovalent complexes of small organic molecules to single-[39] and double-stranded[40−43] oligonucleotides.

Using these mass-spectrometry precedents and the previously determined modes of drug/DNA binding as a foundation, we designed an assay that utilizes a series of self-complementary oligodeoxynucleotides to study the noncovalent interactions of two groups of model drugs. The goal is to develop a fast screening method to assess the binding properties of the candidate drugs. We began with a set of duplexes of different length (6- to 12-mer), motif (i.e., GC-rich or AT-rich), and sequence and the drugs to validate the method; that is, Group-I drugs (Figure 1), which contain the classic minor-groove binder distamycin and the intercalator actinomycin D, because they have been well characterized by various analytical methods including mass spectrometry.[40,41,43,44] Group-II drugs (Figure 2), which include prophyrins and metalloporphyrins, have more complicated binding modes. A competitive-binding study was carried out to assess relative binding affinities.

In a recent contribution to this journal[45] an ESI-MS-based assay method was described as a means to screen combinatorial libraries. The binding in that assay was to RNA, and the main goal was to locate the binding site, not to determine the nature of binding.

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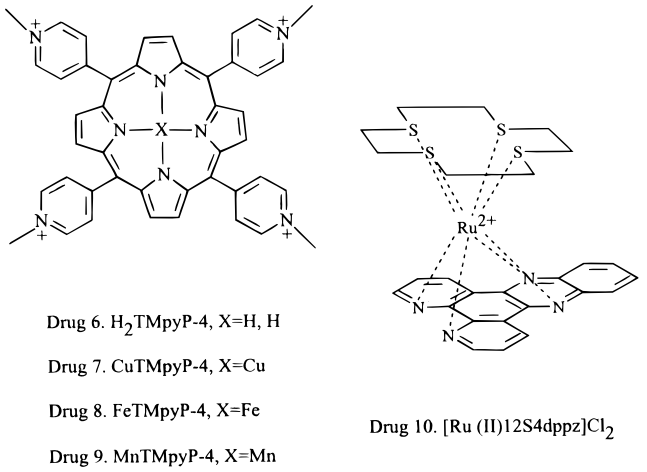

Drug 6. H_2TMpyP-4, X=H, H

Drug 7. CuTMpyP-4, X=Cu

Drug 8. FeTMpyP-4, X=Fe

Drug 9. MnTMpyP-4, X=Mn

Drug 10. [Ru (II)12S4dppz]Cl_2

Figure 2. Structures of group-II drugs.

Experimental Section

Materials. All oligodeoxynucleotides were synthesized (on the 0.2-μmol scale) by the Nucleic Acid Chemistry Laboratory at Washington University and were used without further purification.

Group-I drugs (distamycin, Hoechst 33258, Hoechst 33342, berenil, and actinomycin D) were purchased from Sigma Chemical (St. Louis, MO), and group-II drugs (porphyrin H_2-TMpyP-4 and metalloporphyrins CuTMpyP-4, FeTMpyP-4 and MnTMpyP-4) were obtained from Porphyrin Products Inc. (Logan, UT). Drug 10 ([Ru(II)12S4dppz]Cl_2) was donated by Professor Graca Santana Marques (Department of Chemistry, University of Aveiro, Portugal).

Sample Preparation. Solutions of 10 μL of 5 mM, self-complementary oligodeoxynucleotide stock solutions were annealed in 50 μL of 1 M ammonium acetate by heating to 85 °C for 10 min and cooling to room temperature slowly (over 2−3 h). Then 10 μL of the solution containing the annealed duplex oligonucleotides was interacted with 10 μL of 0.8 mM drugs (in water) to make the complexes. Each 20-μL solution containing the complex was diluted with spray solvent (50/50 V/V MeOH/100 mM aqueous ammonium acetate) to 100 μL for mass spectrometry analysis.

ESI-MS with an Ion-Trap Mass Spectrometer. Negative-ion ESI-MS spectra were obtained with the Finnigan LCQ mass spectrometer (San Jose, CA). The solutions containing noncovalent complexes were infused at 3 μL/min directly into the mass spectrometer. The spray voltage was 4.0 kV. The capillary temperature was 100 °C for the noncovalent complexes of duplex oligonucleotides of 8 bases or less; otherwise the temperature was 150 °C. The N_2 bath gas flow was increased by approximately 1.5 times over that normally used for electrospray at 200 °C to ensure efficient desolvation. The analyzer was operated at a background pressure of 2×10^{-5} Torr, as measured by a remote ion gauge. In all experiments, helium was introduced to an estimated pressure of 1 mTorr for improving the trapping efficiency. Data were collected for approximately 10 scans and analyzed with both the instrument software and the ICIS software developed by the manufacturer.

Results and Discussion

Electrospray of Duplex Oligodeoxynucleotides. We employed self-complementary oligonucleotides throughout the study because less sample preparation is involved and their mass spectra are simple and easy to interpret. Peak assignment, however, is sometimes problematic because single-stranded and double-stranded oligonucleotides with even number of charges may have the same mass-to-charge ratio. Although one could assign those peaks based on the incremental mass-to-charge ratio difference of Na-adduct ions,[41] it was not possible to make an unambiguous assignment when both [single-stranded]$^{n-}$ and [double-stranded]$^{2n-}$ ions are present. To avoid any uncertainty about ion identity, we chose to study exclusively the ions with odd-numbered charge states. Figure 3 shows the ESI spectra of 0.08 mM solution of annealed 6-mer and 12-mer oligonucleotides.

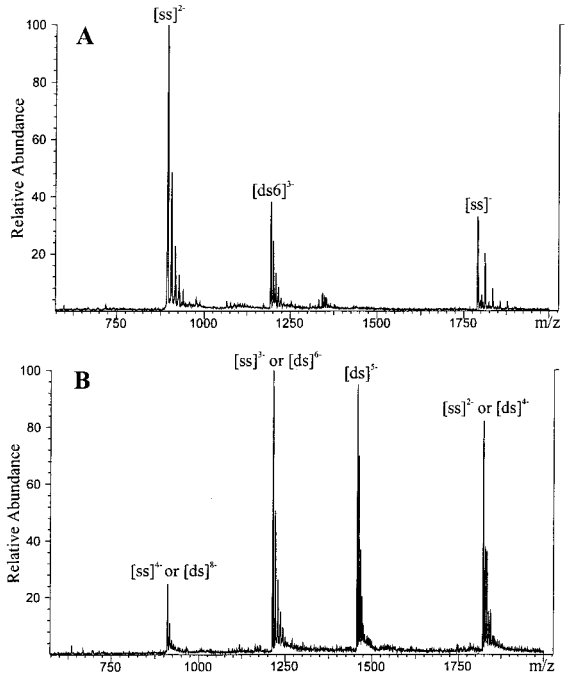

Figure 3. ESI/MS spectra of duplex oligodeoxynucleotides: (A) d(GCATGC)$_2$, (B) d(CGCGAATTCGCG)$_2$.

To detect the noncovalent duplex in the gas phase, we annealed the self-complementary strands in 1 M ammonium acetate and sprayed the annealed samples in 100 mM ammonium acetate. Lower capillary temperature and less organic solvent favor the preservation of duplexes.

Nonspecific aggregation is always a concern in the study of noncovalent associations. Therefore, we evaluated the effects of concentration and of annealing for two self-complementary oligonucleotides and for one random-sequenced oligonucleotide (Table 1). Clearly, the annealing process promotes duplex formation for self-complementary oligonucleotides. This observation contrasts with that of Schnier et al.[46] For the self-complementary oligonucleotides without annealing, the [duplex 6-mer]$^{3-}$ could also be observed when the concentration was 40 μM, whereas the [duplex 12-mer]$^{5-}$ was detected at half that concentration. The random-sequence 12-mer did not show any duplex signal in the concentration range studied with or without annealing. Although the duplex signals were of lower intensity compared to those for the annealed samples, the results suggest that duplexes do form in the presence of counterions (100 mM ammonium acetate) without annealing.

We collisionally activated the [duplex 6-mer]$^{3-}$ and [duplex 12-mer]$^{5-}$ ions that were obtained with and without annealing.

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Table 1. Effect of Annealing on the Abundance of Duplex Oligonucleotides[a] in ESI-MS

	8 μM		20 μM		40 μM		80 μM	
intensity	annealed	nonannealed	annealed	nonannealed	annealed	nonannealed	annealed	nonannealed
$[d(GCATGC)_2]^{3-}$	0.7E3	N. D[b].	6.1E3	N. D.	8.2E3	7.3E3	17.3E3	8.0E3
$[d(CGCGAATTCGCG)_2]^{5-}$	3.0E3	N. D.	7.6E3	2.3E3	17.4E3	6.0E3	17.5E3	7.6E3
$[d(GAGTATTATGAG)_2]^{5-}$	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.

[a] Oligonucleotides in 50/50 MeOH/100 mM ammonium acetate, capillary temperature = 150 °C. [b] N. D. = Not Detected

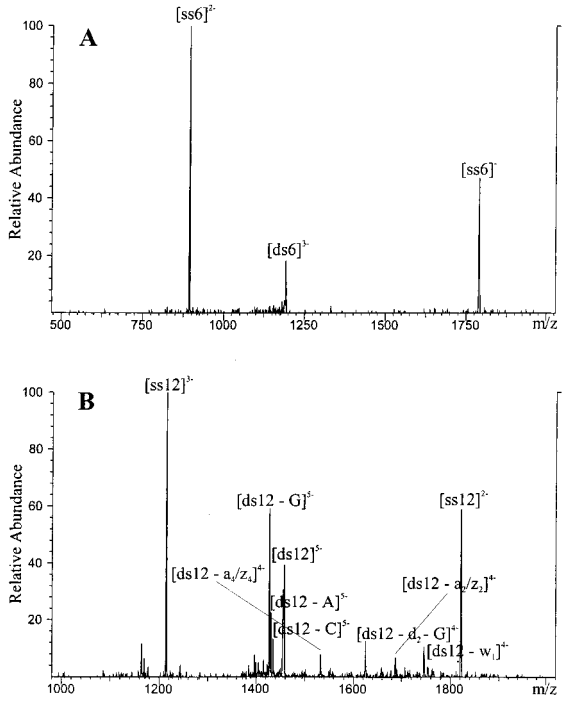

Figure 4. Product-ion mass spectra (MS/MS) of (A) $[d(ATATAT)_2]^{3-}$ and (B) $[d(CGCGAATTCGCG)_2]^{5-}$.

The product-ion mass spectra (Figure 4) of the annealed duplexes are identical to those of the nonannealed ones (data not given), suggesting that the gas-phase structures are the same, irrespective of whether they are formed in solution with or without annealing.

Choice of Group-I Model Drugs. Distamycin (drug 1) is an oligopeptide antibiotic that inhibits binding of RNA polymerase and hence transcription in vitro.[47] It acts by binding to the minor groove of AT-rich regions[48] of DNA. NMR[49−51] and X-ray studies[2] reveal that the crescent-shaped distamycin fits into the 5′-AATT-3′ minor-groove binding site with a binding constant in the range of 10^7-10^8 M^{-1}. Two distamycin ligands can bind simultaneously, overlapping in the minor groove, with each drug sliding between 5′-AATT-3′ and 5′-ATTT-3′ binding sites.

Hoechst 33258 (drug 2) is a fluorochrome widely used in chromosome staining.[52] It also possesses antihelminthic properties.[53] X-ray[54−56] and electric linear dichroism[57] studies show two binding modes for this drug. The primary mode is in the minor groove particularly at sites with consecutive AT base pairs with a binding constant of 10^7 to 10^8 M^{-1}. The drug is also able to interact with repeating GC base pairs by intercalation, but the affinity constant is 50-fold times lower. Hoechst 33342 (drug 3) is a derivative of Hoechst 33258, and it should have similar binding properties.

Berenil (drug 4) is applied in veterinary medicine as an antitrypanosomal agent and has cytotoxic and anti-viral properties.[58] NMR[59−61] and X-ray[62] studies show that the drug binds in the minor groove of DNA in regions rich in AT base pairs. The binding is asymmetric with the 5′-AAT-3′ sequence (binding constant $\approx 10^6$ M^{-1}). Besides binding in the minor groove, berenil also intercalates in regions rich in GC sequences; the binding is via a nonclassical intercalation process as was shown by electric linear dichroism.[23] Unlike drugs 1, 2, and 3, berenil has two positively charged sites.

Actinomycin D (drug 5) has very potent antitumor activity and has been used clinically as a chemotherapeutic agent. Its mechanism of action at the molecular level has been attributed to its inhibition of DNA-directed RNA synthesis.[63] Its phenoxazone chromophore intercalates at 5′-CG-3′ sites, and the two cyclic pentapeptide lactone moieties bind in the nearby minor groove on either side of the chromophore.[64,65]

Observation of Noncovalent Complexes Between Group-I Drugs and Duplex Oligonucleotides. The binding stoichiometry was measured by mixing $d(CGCGAATTCGCG)_2$ with the various substrates (drugs 1−5) in a 1:2 molar ratio and submitting the solution to ESI-MS (relative abundances of the complexes are tabulated in Table 2). When the duplex dodecamer-to-drug ratio is 1:2, no complexes with more than two drug ligands were detected. Among the five drugs that were studied, distamycin (drug 1) showed the highest relative abundance for forming 1:2 oligonucleotide duplex/drug complexes. It is well-known that two drug 1 molecules can bind in the minor groove of duplex DNA in a side-by-side antiparallel fashion. Hoechst 33258 (drug 2) and Hoechst 33342 (drug 3) have a dominant 1:1 binding stoichiometry; less than 7% of a 1:2 oligonucleotide duplex/drug complex formed even when the duplex dodecamer-to-drug ratio was 1:2. The 1:2 complexes may result from nonspecific binding when the ligand concentration is high.

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Table 2. Stoichiometry of Various Complexes of Duplex Oligodeoxynucleotides and Drugs[a]

$d(CGCGAATTCGCG)_2$:drug = 1:2						$d(ATATAT)_2$:drug = 1:2					
RA%	D1	D2	D3	D4	D5	RA%	D6	D7	D8	D9	D10
$[1:1]^{5-}$	100.00	100.00	100.00	100.00	77.00	$[1:1]^{3-}$	20.00	14.00	8.00	7.00	10.00
$[1:2]^{5-}$	32.00	5.00	7.00	15.00	<5	$[1:2]^{3-}$	15.00	17.00	N. D.	N. D.	7.00
[1:>2]	N. D.	N. D.	N. D.	N. D.	N. D.	[1:>2]	N. D.	N. D.	N. D.	N. D.	N. D.

[a] N. D. = Not Detectable

Table 3. Effect of the Size of Duplex Oligodeoxynucleotides on Binding for Group-I Drugs

	GCATGC $[ds+D]^{3-}/[ds]^{3-}$	CAAATTTG $[ds+D]^{3-}/[ds]^{3-}$	GCGAAATTTCGC $[ds+D]^{5-}/[ds]^{5-}$	CGCGAATTCGCG $[ds+D]^{5-}/[ds]^{5-}$
D1	0.19	1.4	17.5	50
D2	0.15	9	13.5	14
D3	0.12	12	14	25
D4	0.43	3.6	1.3	2.4
D5	0.33[a]	0.34[a]	0.45[b]	0.5[b]

[a] The ratio is $[ds + D]^{3-}/[D]^{-}$. [b] The ratio is $[ds + D]^{5-}/[D]^{-}$.

Berenil (drug 4) also showed a dominant 1:1 binding stoichiometry. The nonspecific 1:2 oligonucleotide duplex-drug complex, however, was approximately twice as abundant as those observed for drugs 2 and 3. The increased formation of the 1:2 complex may be due to the stronger electrostatic interactions between duplex DNA and berenil, which has two positive charges instead of one, than those between the duplex DNA and other group-I drugs.

We then investigated the effect of oligonucleotide size on binding. Our concern was whether small duplex oligonucleotides (6 base pairs per strand) are sufficiently large to be used to evaluate binding properties. The use of small duplex oligonucleotides has three advantages. First, the ion-trap instrument that we used has a upper mass limit of *m*/*z* 2000. Even for a duplex 6-mer, it is not possible to observe the whole charge distribution for either the duplex or the noncovalent complex. With self-complementary oligonucleotides, we are restricted to work with species having odd-numbered charge states, making it even more critical to have as much of the noncovalent species fall into the mass range of the ion trap. Second, small oligonucleotides are easy to synthesize and handle. Third, tandem mass spectra of small duplex oligonucleotide-drug complexes, if needed, are relatively easy to interpret.

Table 3 summarizes the effect of the size of duplex oligonucleotides on binding for drugs 1−5. Because we could not get the whole charge distribution for each species, the ratio of the abundance of the odd-charge complex to that of the odd-charge free duplex was used to evaluate the binding affinity. We found that the abundance ratio of complex to free duplex increased dramatically for drugs 1, 2, and 3 as the size of duplex increased. This is expected because the larger oligonucleotides have greater stabilization energy from π-stacking, assisting the larger duplexes to survive the ESI process. For small duplexes, on the other hand, dissociation of the two strands and local unwinding, especially terminal unzipping,[41] during the ESI process can destroy the proper conformation for tight binding and release the drug from the complex.

Berenil (drug 4) did not show a significant increase in binding when the size of duplex was increased presumably because the drug carries additional positive charge that provides extra stabilization for a small complex. Actinomycin D (drug 5) maintains nearly the same binding for $d(GCATGC)_2$ and $d(CAAATTTG)_2$ because there is a lack of specific intercalating sites (5′-CG-3′) in these two sequences. This drug also binds slightly more strongly with the 12-mer than with the 10-mer. This suggests that intercalation relies less on the right conformation of the duplex oligonucleotide than does minor-groove binding, and more on base sequence. Although larger duplexes give more abundant complexes, 6-mer duplexes do form sufficiently abundant noncovalent complexes for these binding studies.

The Assay: Group-I Drugs. The mode of binding is crucial for understanding the molecular basis for the drug action. For Group-I drugs, NMR, X-ray crystallography, gel footprinting, circular dichroism, and electric linear dichroism revealed there are two binding modes: (i) minor-groove binding at AT-rich regions and (ii) intercalation at GC-rich regions. On the basis of these now commonly accepted binding modes, we developed a fast assay to determine the type of binding to duplex oligodeoxynucleotides of various drugs and drug candidates. The assay in the present stage of development uses five different self-complementary duplex 6-mers. The premises are that duplex oligodeoxynucleotides with tailored sequences will bind selectively to various substrates and that the relative extent of binding can be used as an "indicator" to assess the mode of binding. We designed a set of self-complementary hexadeoxynucleotides so that the AT/GC content in those duplexes varied from GC-rich to AT-rich. Then group-I drugs were mixed in separate solutions with each duplex at 2:1 molar ratios, and the resulting mixtures were subjected to ESI/MS measurement.

The abundance ratio of $[duplex + drug]^{3-}$ to $[duplex]^{3-}$ (Figure 5) shows that distamycin (drug 1) and berenil (drug 4) have a clear preference for binding with $d(ATATAT)_2$, indicating they are minor-groove binders. Actinomycin D (drug 5), on the other hand, showed a clear propensity to bind with $d(GCGCGC)_2$, indicating that it is an intercalator. Hoechst 33258 (drug 2) and Hoechst 33342 (drug 3) preferentially bind to both $d(ATATAT)_2$ and $d(GCGCGC)_2$, indicating that mixed binding modes exist for these two drugs. These results are entirely consistent with findings by other physical or chemical methods that have been applied to determine the binding modes for these drugs.[2]

A competition study was carried out for drugs 1, 2, 3, and 4 with $d(GCGAAATTTCGC)_2$ to test whether the extent of drug binding as determined by relative ion abundances matches solution affinities. In each competition experiment, solutions of two test drugs were mixed with a solution of a duplex oligonucleotide to give final concentrations of [drug] = 0.04 mM, [duplex] = 0.08 mM. All six reactions were interrogated by ESI-MS by using the same spray solvent under identical ESI interface conditions. On the basis of relative abundances of the noncovalent complexes, we established the following ordering

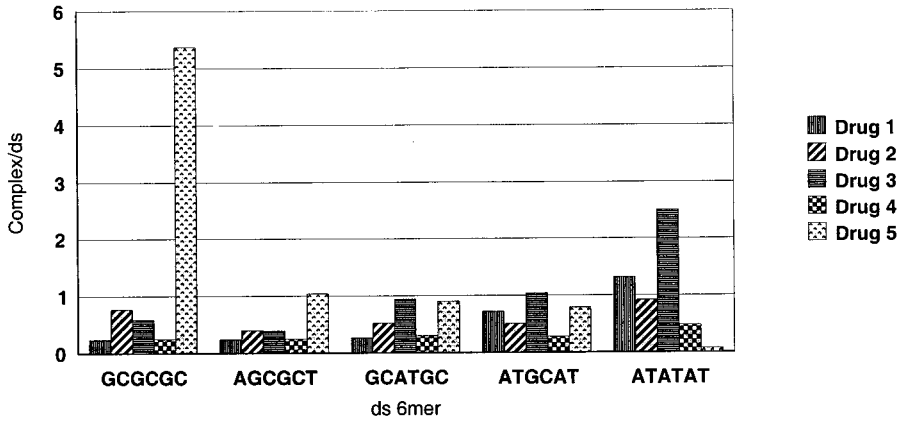

Figure 5. The relative binding of group-I drugs to various double-stranded oligomers ranging from GC- to AT-rich. The relative binding is the ratio of [complex]/[duplex].

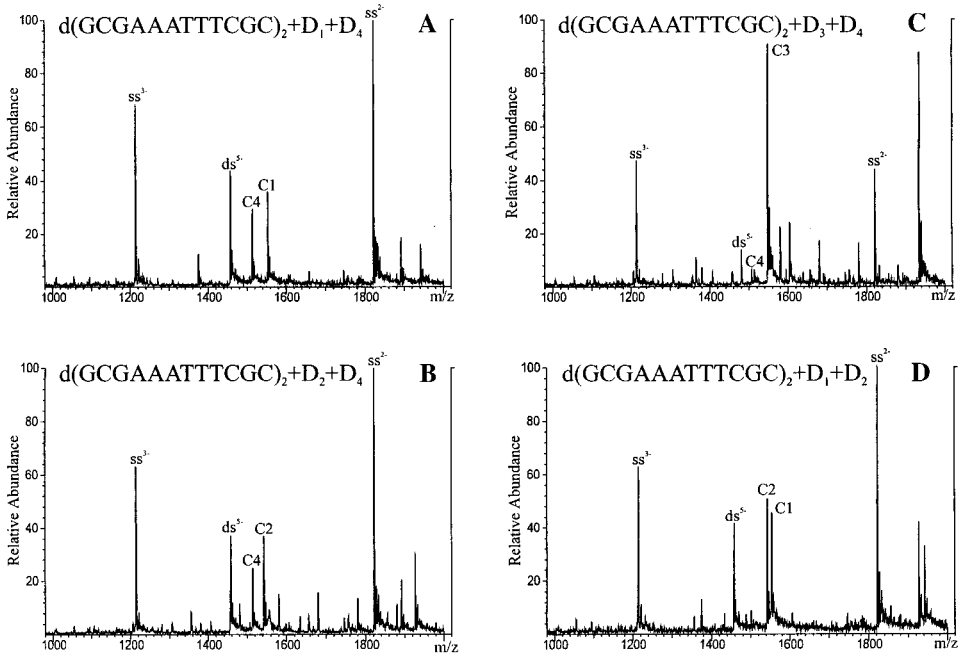

Figure 6. Competitive binding of group-I drugs 1−4. C1, C2, C3, and C4 are complexes of the specified duplex with drugs 1, 2, 3, and 4, respectively. The designation ss and ds are single-stranded and double-stranded.

of pairs of complexes: C1 > C4 (Figure 6, panel A), C2 > C4 (panel B), C3 > C4 (panel C), C2 > C1 (panel D), C3 > C1 (not shown), and C3 > C2 (not shown). From such a series of pairwise comparisons, we determined that the overall order of binding preferences for drugs is D3 > D2 > D1 > D4.

It is difficult to compare the relative binding affinities determined here with those obtained by using other techniques because those other measurements were made under different solution conditions and with oligonucleotides of different sequences. Nevertheless, the order we determined is consistent with the results of other measurements that show berenil (drug 4) has the lowest binding affinity, whereas drugs 1, 2, and 3 have at least 10 times greater binding affinity. In fact, if the mass spectrometric determinations do reflect solution stability even though the abundances of gas-phase ions are compared, the true solution-phase order is D3 > D2 > D1 > D4.

Observation of Noncovalent Complexes Between Group-II Drugs and Duplex Oligonucleotides. The interaction of cationic porphyrins with DNA has been a topic of considerable interest because the substrates have found use in photodynamic therapy, cancer detection, and virus inhibition.[66] The results from absorption, circular dichroism, electron paramagnetic resonance, resonance Raman, viscosity, NMR, and fluorescence spectroscopy suggest that porphyrin free bases, H_2TMpyP, and square-planar complexes such as those with Cu^{2+} and Ni^{2+}, intercalate between base pairs, whereas metalloporphyrins with axial ligands (e.g., FeTMpyP and MnTMpyP) bind to the minor groove of AT regions.[66] Results from circular dichroism spectroscopy also demonstrate the possibility that the major groove of a GC region is an acceptor for FeTMpyP and MnTMpyP.[67] Further, outside binding with self-stacking was reported for solution-phase in which the porphyrin-to-DNA molar ratio is high.[66] Porphyrins intercalate into DNA with binding constants of approximately 10^6 M^{-1}.[68−70]

The binding stoichiometry was measured for drugs 6, 7, 8, 9, and 10 by mixing, in separate experiments, $d(ATATAT)_2$

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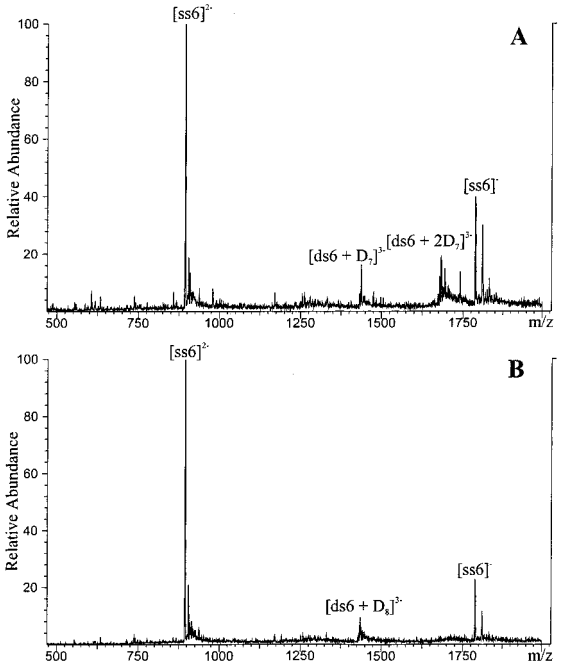

Figure 7. Product-ion mass spectra (MS/MS) of d(ATATAT)$_2$ with drug 7 (A) and drug 8 (B).

Table 4. Effect of the Size of Duplex Oligodeoxynucleotides on Binding for Group-II Drugs

	GCATGC $[ds+D]^{3-}/[ds]^{3-}$	TATGCATA $[ds+D]^{3-}/[ds]^{3-}$	ATATGCATAT $[ds+D]^{5-}/[ds]^{5-}$
D6	0.87	2.40	100.00
D7	0.61	2.70	32.00
D8	0.24	0.56	51.00
D9	0.11	0.34	19.00

with the drug to give a 1:2 molar ratio, respectively. The relative abundances of the complexes that formed upon ESI are tabulated in Table 2. At a 6-mer duplex-to-drug ratio of 1:2, no complexes with more than two drug ligands were detected. Drugs 6 and 7 formed comparably abundant 1:2 oligonucleotide duplex/drug complexes, whereas 1:2 duplex/drug complexes were not detected for drugs 8 and 9 (Figure 7 shows some resulting spectra). The absence of the latter complexes is probably because drugs 8 and 9 have axial ligands that prevent them from binding simultaneously in the narrow and short minor groove of the small duplex 6-mer.

We then investigated the effect of the size of the double-stranded oligonucleotides on binding stoichiometry. At a 1:2 duplex-to-drug molar ratio, both 1:1 and 1:2 noncovalent duplex/drug complexes were observed for drugs 6 and 7 with all the duplexes (from the duplex 6-mer to 10-mer). On the other hand, drugs 8 and 9, which have axial ligands, show only 1:1 complexes with the five different 6-mer duplexes, but 1:2 complexes are detectable for longer duplex sequences (8- and 10-mers). The larger duplexes may be able to provide enough space for simultaneous binding of two drugs that have axial ligands because they possess an elongated minor groove.

Table 4 shows the effect of the size of duplex oligonucleotides on the binding affinity for drugs 6, 7, 8 and 9. Larger duplexes show strong drug binding (higher relative abundance of the complex), as was observed for Group-I drugs. Group-II drugs were also submitted to the assay to reveal their binding modes. After mixing each duplex oligonucleotide with a drug at 1:2 molar ratio, we measured the resulting abundance ratio of $[\text{duplex} + \text{drug}]^{3-}$ to $[\text{duplex}]^{3-}$ (Figure 8). Drugs 6 and 7, which do not have axial ligands, show preferred binding to two

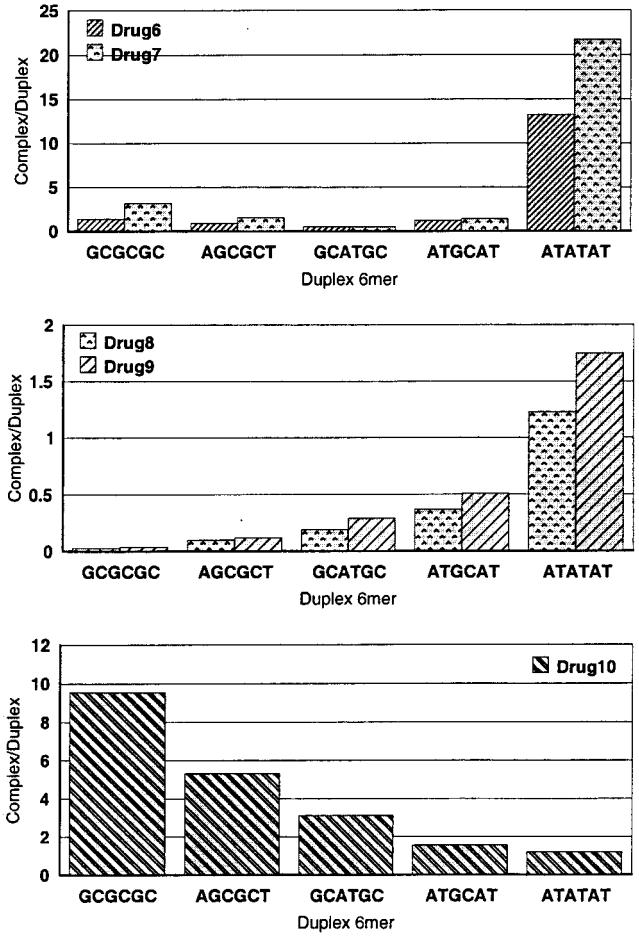

Figure 8. The relative binding of group-II drugs with double stranded oligomers ranging from GC- to AT-rich.

sequences. A preferred sequence is d(ATATAT)$_2$, indicating that there is significant minor-groove binding to duplexes containing repetitive AT sequences. The other sequence is d(GCGCGC)$_2$, suggesting intercalation or major-groove binding for these drugs. Tetrapyridylporphyrins are much larger than conventional intercalators, and one might expect kinetic and thermodynamic barriers to their intercalation. An X-ray crystallography study confirmed the severe conformational distortion of d(CGATCG)$_2$ upon binding to CuTMpyP-4.[71] As a result, the authors designated the binding mode as "hemi-intercalation" to indicate intercalation with one strand of duplex DNA but not the other.[71] The lower binding affinities of drugs 6 and 7 to a GC-rich compared to an AT-rich duplex are consistent with results in the literature.[66]

Another possible mode of binding for GC-rich duplexes involves the major groove. This groove is wider than the minor groove in B-DNA, thus providing fewer van der Waals contacts with drug molecules. Noncovalent complexes that involve the major groove of a GC region, however, are still possible because sufficient electrostatic interactions with cations can be provided. Theoretical calculations show that major grooves rich in GC have more negative electrostatic potential than minor grooves rich in AT when the duplex is in water but have slightly less negative electrostatic potential when the duplex is in the gas phase.[4,3] Drugs 8 and 9, which have axial ligands, show one preferred binding sequence (i.e., to the AT-rich region of a duplex), indicating that they are minor-groove binders. Their

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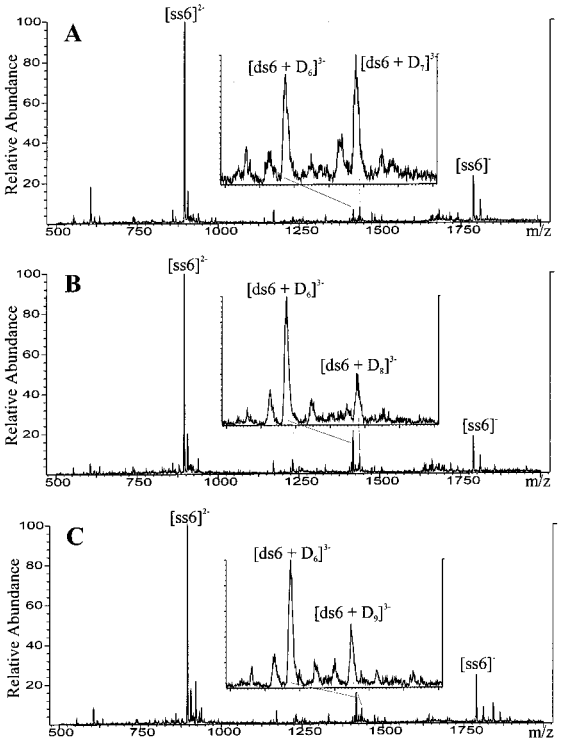

Figure 9. Competition study of group-II drugs. (A) $d(ATATAT)_2$ + D6 + D7, (B) $d(ATATAT)_2$ + D6 + D8, and (C) $d(ATATAT)_2$ + D6 +D9.

axial ligands prevent them from intercalating into the GC-rich regions in solution. Drug 10 prefers sequences rich in GC over those rich in AT, suggesting that this drug is either an intercalator or binds to the major groove of GC-rich region.

We then carried out a competitive binding study for drugs 6, 7, 8, and 9 to compare their binding affinities toward $d(ATATAT)_2$. In each competition experiment, a solution of the duplex oligonucleotide was mixed with two different drugs such that their concentrations were [duplex] = 0.08 M and [drug] = 0.04 M. The relative binding affinities are in the order: D6 ≈ D7 > D8 ≈ D9 (Figure 9), as was determined from the relative abundances of the complexes. The low binding affinities of D8 and D9 may be due to the axial ligands of metalloporphyrins; these ligands cause steric hindrance for binding in the narrow minor groove of an AT region.

Conclusions

The analysis of noncovalent associations between double-stranded oligonucleotides and small organic molecules (e.g., drugs) can be accomplished by using ESI mass spectrometry under conditions of appropriate annealing, ionic strength, and sufficiently gentle ESI-interface conditions. The binding stoichiometry can be established, and the relative binding affinities can be determined quickly and easily.

Strong binding pertains to larger duplexes, but 6-mers do bind noncovalently with the various drugs at sufficient abundance to provide a basis for a binding assay. Sequence selectivity can be obtained directly, and the binding mode can be ascertained by examining the selectivities of test duplexes to the candidate drug. The sample consumption is less than 1 nmol per analysis, which makes the method useful when only small amounts of compounds are available (e.g., from the beads of a combinatorial chemistry library).

In a sequel article, we will examine the gas-phase properties of the duplexes to various DNA-binding drugs by using tandem mass spectrometry.

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