Formaldehyde-Induced Alkylation of a 2′-Aminoglucose Rebeccamycin Derivative to Both A·T and G·C Base Pairs in DNA

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Rebeccamycin derivatives represent a promising class of antitumor agents. In this series, two glycosylated indolocarbazoles, NB-506 and NSC-655649, are currently undergoing clinical trials. Their anticancer activities are associated with their capacities to interact with DNA and to inhibit DNA topoisomerases. Previous studies revealed that the planar indolocarbazole chromophore can intercalate into DNA, locating the appended carbohydrate residue in one of the two helical grooves, probably the minor groove as is the case with the anthracyclines and other DNA-binding antibiotics. The sugar residue contributes significantly to the DNA binding free energy of NB-506. However, the exact positioning of the glycosyl residue of rebeccamycin derivatives in the drug−DNA complex remains poorly understood. To better understand how glycosylated indolocarbazoles interact with DNA, we investigated the interaction of a rebeccamycin derivative (**85**) bearing a 2′-amino group on the sugar residue. We show that the presence of the 2′-amino function permits the formation of covalent drug−DNA complexes in the presence of formaldehyde. Complementary biochemical and spectroscopic measurements attest that **85** reacts covalently with the 2-amino group of guanines exposed in the minor groove of the double helix, as is the case with daunomycin. In contrast to daunomycin, **85** also forms cross-links with an oligonucleotide containing only A·T base pairs. The covalent binding to A·T base pairs was detected using a gel mobility shift assay and was independently confirmed by thermal denaturation studies and by fluorescence measurements using a series of synthetic polynucleotides. The HCHO-mediated alkylation reaction of the drug with A·T base pairs apparently involves the 6-amino group of adenines exposed in the major groove whereas the covalent attachment to G·C base pairs implicates the 2-amino group of guanines situated in the opposite minor groove. Therefore, the results suggest that either the drug is able to switch grooves in response to sequence or it can simultaneously bind to both the minor and major grooves of the double helix. This study will help to guide the rational design of new DNA-binding antitumor indolocarbazole drugs and also provides a general experimental approach for probing minor versus major groove interactions between small molecules and DNA.

Introduction

Indolocarbazoles represent a structurally diverse and therapeutically important class of antitumor antibiotics.[1,2] The synthetic compound NB-506 and the rebeccamycin derivative NSC655649, which are both undergoing phase I/II clinical trials as anticancer agents,[3,4] bind to DNA and inhibit human topoisomerases.[5] NB-506 is a potent topoisomerase I poison, whereas the rebeccamycin derivative NSC655649 is a selective inhibitor of topoisomerase II. These indolocarbazole derivatives, which possess a hexacyclic planar ring system with associated carbohydrate moieties, can intercalate between the base pairs of DNA. Recent molecular modeling studies suggest that the chromophore penetrates the double helix with the imide F-ring approaching the major groove and the sugar lying in the minor groove.[6] This binding motif is common to the majority of DNA-interacting antibiotics, in particular the anthracyclines.

Daunomycin, the prototype anthracycline antibiotic, contains a DNA-intercalating anthraquinone ring system linked to the daunosamine moiety which acts as a sequence-selective minor groove binder.[7] Over the past decade, the atomic structure of several daunomycin−oligonucleotide complexes has been solved by X-ray crystallography or NMR.[8] In contrast, there is no structure yet available for an indolocarbazole complexed with DNA. For this reason, the exact positioning of the glycosyl residue of drugs such as NB-506 in the drug−DNA complex remains poorly understood. At first sight, it seems likely that the sugar locates into the minor groove, as is the case with the anthracyclines, but there are some biochemical data which are not fully compatible with this binding motif.

Although its sequence selectivity is rather modest, footprinting studies have revealed that rebeccamycin derivatives bind preferentially to sites containing GpC, GpT, or TpG sequences. Interestingly, we found that the

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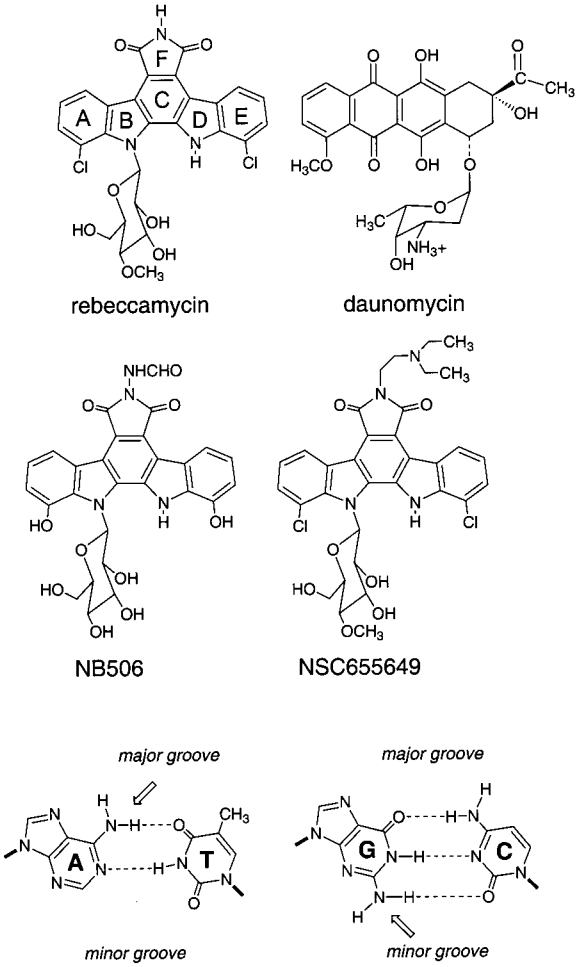
addition of a methyl group to pyrimidine residues in DNA creates new drug-binding sites.[9] In contrast to most DNA-binding small molecules, rebeccamycin analogues were found to be highly sensitive to modification of the exocyclic methyl substituent on the pyrimidine bases in the major groove of the double helix. These footprinting experiments raised the possibility that upon intercalation into DNA, the drug must somehow establish contacts with the DNA via the major groove of the double helix. While intercalation of the planar indolocarbazole chromophore is strongly supported by existing experimental evidence,[10] the location of the carbohydrate moiety when the drug is bound to DNA remains unclear. Does the glucose residue of NB-506 and related drugs locate in the minor or major groove of the double helix, or does it simply extend out from the helix? Existing experimental evidence cannot establish the exact location of the sugar moiety unambiguously, although it has been demonstrated that the sugar contributes significantly to the DNA binding free energy.[6,10] Clarification of exactly how glycosylated indolocarbazoles interact with DNA must await more precise structural investigations, but so far the NMR and X-ray studies have not been successful.

We have devised a chemical approach to investigate the positioning of the carbohydrate moiety of the indolocarbazole antibiotics in the drug−DNA complex. The strategy is based on the formation of stable drug−DNA cross-links in the presence of a highly reactive chemical, such as formaldehyde or glyoxal. This method has been previously used to covalently attach daunomycin[11] or *n*-butylamine[12−16] to DNA. Formaldehyde efficiently promotes base-specific and regioselective alkylation of daunomycin to DNA.[17] It can condense with the 3′-amino group of the daunosamine moiety to form an aminal linkage with the 2-amino group of the proximal deoxyguanosine in the drug−DNA complex. With these data in mind, we postulated that a similar approach can be used to induce cross-links between indolocarbazoles and DNA. Depending on the HCHO-activated reaction of the drug with either the 2-amino group of guanines in the minor groove or the 6-amino group of adenines in the opposite major groove (Chart 1), we may answer the question as to the location of the glycosyl residue of indolocarbazoles in the intercalated drug−DNA complex.

Chart 1. Structures of Daunomycin and the Glycosylated Indolocarbazoles Rebeccamycin, NB-506, and NSC655649[a]



[a]Also structures of hydrogen-bonded purine−pyrimidine base pairs. Broken lines represent hydrogen bonds.

Results

Drug Selection. The first set of experiments we performed was aimed at selecting the best indolocarbazole drug candidate for the alkylation experiments. By analogy with daunomycin, we reasoned that a rebeccamycin derivative bearing an amino group on the sugar moiety may react specifically with formadehyde, and if it is proximal to an amino group of a base, it may form DNA cross-links. Such a compound has recently been designed.[18] We have shown that **85** (shown in Figure 1) with a 2′-aminoglucose residue binds significantly more tightly to DNA than the corresponding analogue **7** with a 2′-hydroxy group on the sugar. At low pH, the association constant for compound **85** is more than 10 times higher than that of compound **7**. The replacement of the glucose residue with a 2′-aminoglucose significantly reinforces the interaction of the drug with DNA, without interfering with the drug effect on topoisomerase I.[18] But **85** has two NH_2 groups. It bears an aminoglucose in addition to an amino function on the imide nitrogen. Both amino groups may react covalently with DNA in the presence of formaldehyde. Therefore, to identify the reactive center of the molecule, we compared the cross-linking capacities of compounds **85** and **7** as well as the corresponding aglycone **14**. Each drug was incubated with a radiolabeled 117-base pair DNA fragment in borate buffer at pH 8.2 in the presence of HCHO. After the reaction (for 1 h at 37 °C), unbound drug molecules were extracted with phenol/chloroform and the DNA was precipitated and analyzed by electrophoresis. The results shown in Figure 1 indicate that only compound **85** bearing the 2′-aminoglucose moiety has reacted covalently with DNA, as judged from the appearance of a retarded band of DNA in the gel. No such retarded species can be detected with compounds **7** and **14** as well as in the control lanes without formaldehyde indicating that this chemical is absolutely required for the alkylation reaction to occur. The amino group on the imide nitrogen is apparently not reactive toward HCHO or it is not in a suitable position for covalent attachment to DNA. The lack of reactivity of the aglycone **14** indicates that the

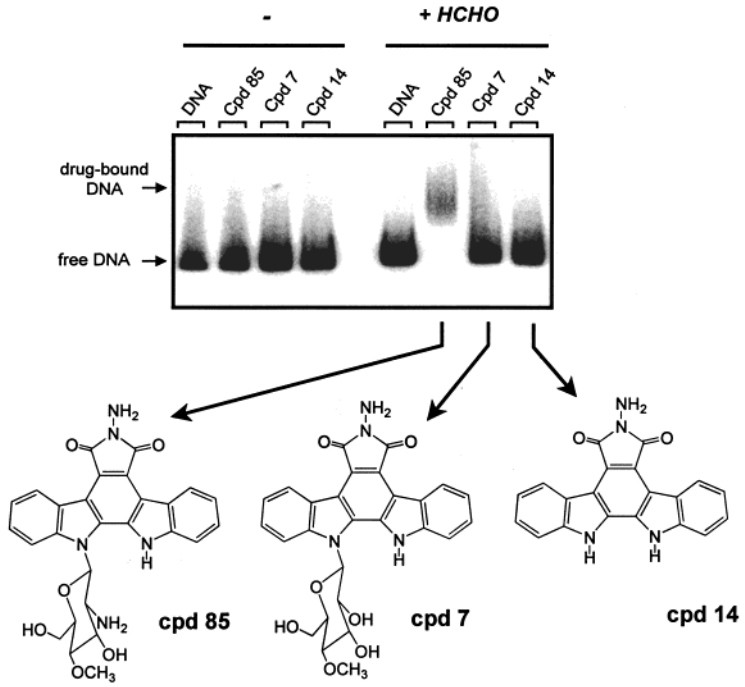


Figure 1. Drug selection. The 117-base pair DNA fragment was reacted with the test drug **7**, **14**, or **85** (50 μM each) in the presence of 1% HCHO in 50 mM borate buffer at pH 8.2. After 1 h incubation at 37 °C, the samples were extracted with phenol/chloroform and the DNA was precipitated with ethanol. The cross-linked DNA samples were then subjected to electrophoresis on a 6% polyacrylamide. Lane marked DNA contained no drug. **85** alkylates the DNA in the presence of HCHO only.

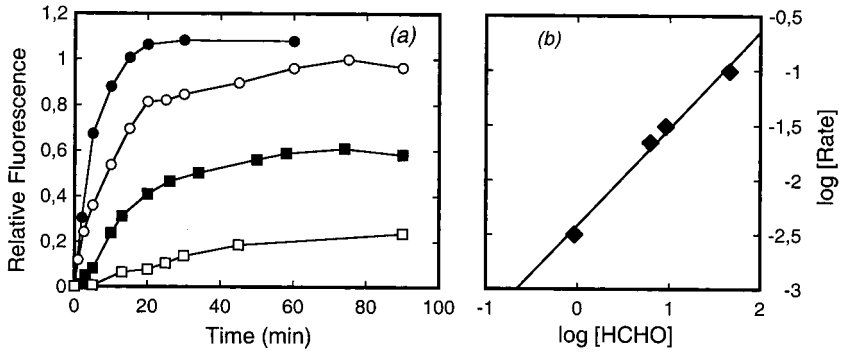


Figure 2. (a) Time course for the covalent reaction of **85** with DNA in the presence of different amounts of HCHO: □, 0.0074%; ■, 0.05%; ○, 0.074%; and ●, 0.37%. The fluorescence of the DNA-bound drug molecules was determined after phenol extraction. (b) Plot of log rate vs log [HCHO] for cross-linking of **85** to DNA. The slope of this plot was used to determine the apparent reaction order with respect to HCHO.

indole NH group is not the reactive site of the molecule. It must be admited, however, that the molecule lacking the sugar may bind in a different mode or position not conducive to cross-linking with formaldehyde. Compound **85** was selected for the subsequent experiments with HCHO.

Dependence of the Formaldehyde Concentration. Kinetic experiments using fluorescence to monitor adduct formation revealed that the formaldehyde concentration is an important parameter for optimal cross-linking of the drug to DNA. Like most indolocarbazole derivatives, **85** is weakly fluorescent in aqueous solution, but when bound to DNA its fluorescence emission increases considerably. Figure 2a shows the time course for the covalent binding of **85** to DNA in the presence of HCHO. The rate of the alkylation reaction increases with the HCHO level in the reaction. At 0.37% HCHO, the DNA was totally alkylated after 20 min of incubation. The extent of cross-linking is much reduced with using a concentration as low as 0.0074%, although the covalently bound drug molecules can still be easily detected. A double-logarithmic plot of the initial rate of the alkylation reaction versus the concentration of HCHO is shown in Figure 2b. The plot yields a straight line with a slope of 0.88 ($\pm$0.11), indicating that the covalent reaction is essentially first order with respect to the HCHO concentration. A similar stoichiometry of 1 for HCHO in the chemical reaction was measured previously with daunomycin.[17] We can conclude that one HCHO molecule must be involved in the formation of each cross-linking event, with both daunomycin and **85**. For ethidium bromide, which can react covalently with DNA in the presence of glyoxal, the cross-linking situation is different. In this case, the double-logarithmic plot yielded a slope of 3.8 suggesting that the stoichiometry of glyoxal in the reaction is 4.[19] The structure of the drug and its DNA-binding configuration, as well as the intrinsic chemical reactivity of the cross-linking agent, must be important for the covalent reaction with DNA.

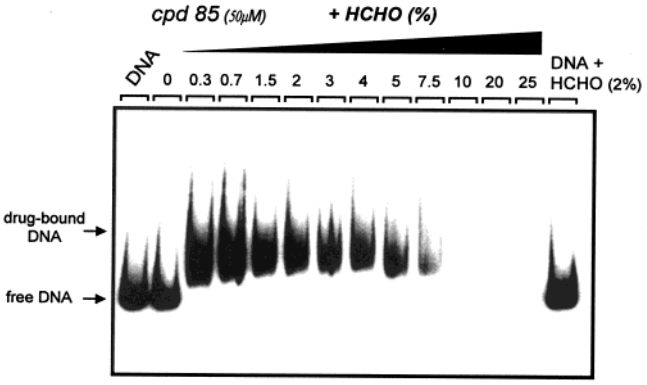


Figure 3. Mobility shifts of the radiolabeled 117-base pair DNA fragment cross-linked to **85** in the presence of increasing amounts of HCHO. The alkylation reactions were performed in 50 mM borate buffer at pH 8.2. After 1 h of incubation at 37 °C, the samples were extracted with phenol/chloroform and the DNA was precipitated with ethanol. The alkylated DNA samples were then subjected to electrophoresis on a 6% polyacrylamide. Lane marked DNA contained no drug and no HCHO, whereas the lane marked 0 contained the drug at 50 μM but no HCHO. 2% Formaldehyde has no effect on the mobility of the DNA in the absence of the drug (lane DNA + HCHO).

Gel shift experiments also indicated that the covalent reaction between DNA and **85** can be induced with relatively small amounts of HCHO. As shown in Figure 3, the use of 0.3% HCHO is sufficient to shift completely the electrophoretic mobility of the 117-base pair DNA fragment in the presence of **85**. Concentrations between 0.5% and 3% work as well, but for higher HCHO concentrations, the amount of cross-linked DNA material was significantly reduced due to nonspecific reaction between DNA molecules and/or because the DNA became denatured, at least partially. Formaldehyde, like other organic solvents, is known to weaken the double-helical structure of DNA. Therefore, the HCHO concentration was maintained at 1% in the subsequent experiments.

HCHO-Mediated DNA Alkylation by the Indolocarbazole Drug and Daunomycin. The radiolabeled DNA fragment was incubated with increasing concentrations of **85** or daunomycin and a fixed amount of DNA, in the absence and presence of 1% formaldehyde. After phenol extraction of the noncovalently bound drug molecules, the DNA was analyzed by electrophoresis. Figure 4 shows an autoradiogram from a typical nondenaturing gel electrophoresis experiment with the two drugs. The addition of formaldehyde to the drug−DNA complexes exerts a significant effect on the electrophoretic mobility of the DNA molecules, attesting that the covalent reaction has been induced. Cross-linked DNA migrates more slowly than free DNA with both daunomycin and **85**. There was absolutely no reaction in the absence of formaldehyde. The reactivity of daunomycin is apparently much higher than that of **85**, an observation that is consistent with the much higher DNA binding affinity of the anthracyline. With the indolocarbazole drug, the retarded DNA species can be detected using drug concentrations of 20 μM, whereas a 20-fold lower concentration of daunomycin is sufficient to reduce the electrophoretic mobility of the DNA. However, there is no doubt that the alkylation reaction between DNA and the indolocarbazole drug can be initiated by the addition of formaldehyde.

Cross-Linking to Supercoiled DNA. In the preceding experiments we showed that the indolocarbazole drug can form cross-links with a short restriction fragment 117 base pairs in length. The same reaction can occur using a full-length plasmid. Supercoiled DNA was mixed with the test drug in borate buffer pH 8.2 with increasing concentrations of **85** and 1% HCHO, and the DNA samples were analyzed by electrophoresis on agarose gels. Compound **85** reacts efficiently with supercoiled DNA in the presence of HCHO (Figure 5). Here again, the electrophoretic mobility of the DNA is significantly reduced when the alkylation reaction has been initiated with HCHO. Practically no reaction can be detected without HCHO. Note that the electrophoretic mobility of both the supercoiled DNA species (form I) and the nicked DNA species (form II) is profoundly affected by the HCHO-mediated drug attachment. At 5 μM, the electrophoretic mobility of the negatively supercoiled DNA form is markedly reduced. At 10 μM, the plasmid is totally unwound by the drug to produce relaxed DNA species which migrate much more slowly in the agarose gel than the supercoiled form. At higher concentrations, the unwinding effect of the drug generates positively supercoiled DNA species which again migrate more rapidly that the relaxed DNA species. Supercoiled DNA (~3000 base pairs in length) represents a very reactive substrate toward HCHO cross-linking with **85**.

Base-Dependent Cross-Linking of the Indolocarbazole Drug to DNA. To determine whether the addition of formaldehyde induces alkylation of **85** to A·T or G·C base pairs, we prepared two radiolabeled oligonucleotide duplexes containing exclusively one or the other base pairs. The AT and GC duplexes, both 30 base pairs in length, were labeled with ^{32}P at the 3′-end and reacted with the drug in borate buffer pH 8.2 in the presence of 1% HCHO. The experiments were performed in parallel with daunomycin and **85** for comparison. The results are shown in Figure 6. As expected, daunomycin was cross-linked exclusively to the GC oligonucleotide. There was no reaction at all with the AT duplex even using high concentrations of the anthracycline antibiotic. In sharp contrast, the indolocarbazole drug has reacted with both the AT and GC duplexes. However, the reaction with the GC duplex is slightly more efficient than with the AT duplex. Here again, formaldehyde is absolutely essential for the cross-links to occur. No retarded band of DNA can be detected without HCHO, be it with **85** or with daunomycin. These gel experiments with oligonucleotides containing either A·T or G·C base pairs strongly suggest that, unlike daunomycin, the indolocarbazole can be induced to react covalently with both types of purine bases in DNA, A and G. The alkylation reaction with the AT oligonucleotide was detected only with **85**. No covalent reaction was observed with **7** bearing a 2′-hydroxy group on the sugar or the aglycone **14** (data not shown). Thus the covalent bond to A·T base pairs also directly implicates the 2′-amino group on the sugar moiety of **85**. These striking results prompted us to investigate further the HCHO-mediated cross-linking properties of **85** using spectroscopic methods.

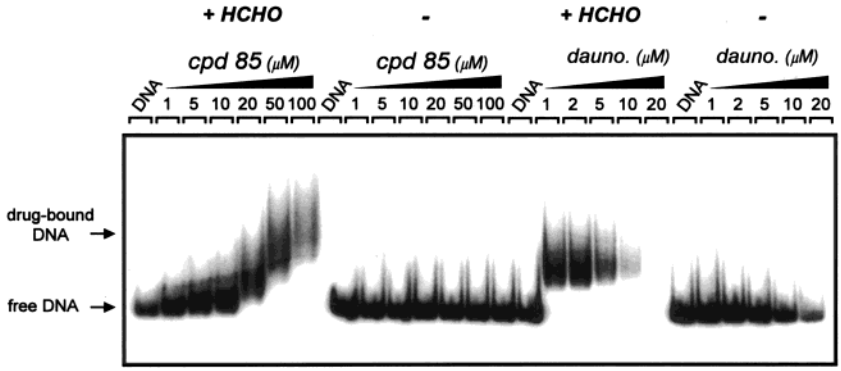


Figure 4. Comparison with daunomycin. The 117-base pair DNA fragment was reacted with daunomycin or **85** at the indicated concentration (μM), in the absence (−) or presence of 1% HCHO, in 50 mM borate buffer at pH 8.2. After 1 h of incubation at 37 °C, the samples were extracted with phenol/chloroform and the DNA was precipitated with ethanol. Cross-linked DNA samples were then subjected to electrophoresis on a 6% polyacrylamide gel under nondenaturing conditions. Lane marked DNA contained no drug.

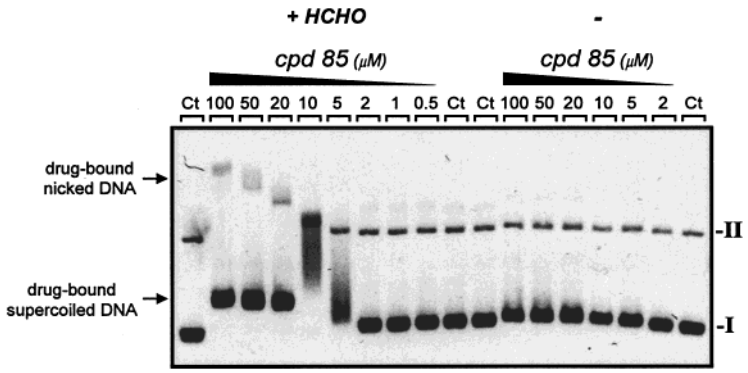


Figure 5. Cross-linking of the drug to plasmid DNA. Supercoiled plasmid DNA (0.5 μg) was reacted with **85** at the indicated concentration (μM), in the absence (−) or presence of 1% HCHO, in 50 mM borate buffer at pH 8.2. After 1 h of incubation at 37 °C, the samples were extracted with phenol/chloroform and the DNA was precipitated with ethanol. Cross-linked DNA samples were then subjected to electrophoresis on a 1% agarose gel. Lanes marked Ct contained no drug. I and II refer to the supercoiled and nicked DNA forms, respectively.

Thermal Denaturation Studies. Thermal denaturation experiments were performed in BPE buffer (16 mM Na^+) at pH 7.0. A recent study showed that **85** is only partially charged at neutral pH.[18] At physiological pH, a large fraction of the drug molecules must bear a nonprotonated 2′-amino group available to react freely with HCHO. Calf thymus DNA and the double-stranded synthetic polynucleotide poly(dA-dC)·poly(dG-dT) both have T_m values of 65 °C under the conditions used. With the polynucleotide, but not with the natural DNA, the addition of 1% HCHO decreased the T_m value by 2 °C (Table 1). A weak destabilizing effect was also observed with $[poly(dI\text{-}dC)]_2$. The noncovalently bound and covalently cross-linked drug molecules stabilize DNA to different extents. The ΔT_m values ($T_m^{drug-DNA} - T_m^{DNA}$) are considerably higher for HCHO cross-linked drug relative to the noncovalently bound drug (−HCHO) (Table 1). For example, for calf thymus DNA at a drug/DNA ratio of 0.25, the ΔT_m is 3 °C with **85** alone whereas it exceeds 20 °C in the presence of HCHO. This profound difference no doubt results from the dissociation or redistribution of the noncovalently bound drug over the course of the melting transition. For the covalently attached drug, dissociation or redistribution would not be possible.

Experiments were also performed with the alternating polynucleotide $[poly(dA\text{-}dT)]_2$ containing only A·T base pairs. In this case, the T_m of the polymer is not affected by the HCHO and is only weakly increased in the presence of the drug (Table 1). But the T_m is considerably increased when the preformed drug−$[poly(dA\text{-}dT)]_2$ complex has been treated with HCHO. At a drug/DNA ratio of 0.1, the T_m was raised by 12 °C in the presence of HCHO. The ΔT_m values increases proportionally to the binding ratio (Table 1). In sharp contrast, little difference in T_m was found in experiments performed with $[poly(dI\text{-}dC)]_2$ and **85** in the absence and presence of HCHO (Table 1). In BPE buffer, the T_m of $[poly(dI\text{-}dC)]_2$ is 41 °C, and this value is only weakly increased in the presence of **85** in both the presence and absence of HCOH (Table 1). In the presence of HCHO, **85** reacts poorly $[poly(dI\text{-}dC)]_2$ whereas it can be efficiently cross-linked to both $[poly(dA\text{-}dT)]_2$ and $[poly(dG\text{-}dC)]_2$. It is thus most likely that the covalent attachment of the drug to GC sequences required the exocyclic 2-amino group of guanine, as is the case with daunomycin. It must be noted that $[poly(dG\text{-}dC)]_2$ cannot be used in the T_m experiments because the polynucleotide only melts at a very high temperature (>90 °C) even in the absence of a ligand. But cross-linking of the drug to $[poly(dG\text{-}dC)]_2$ was evidenced by fluorescence.

Fluorescence Measurements. The fluorescence emission of **85** increases considerably when bound to DNA. This property provides a useful mean for accurately determining its DNA binding affinities. Fluo-

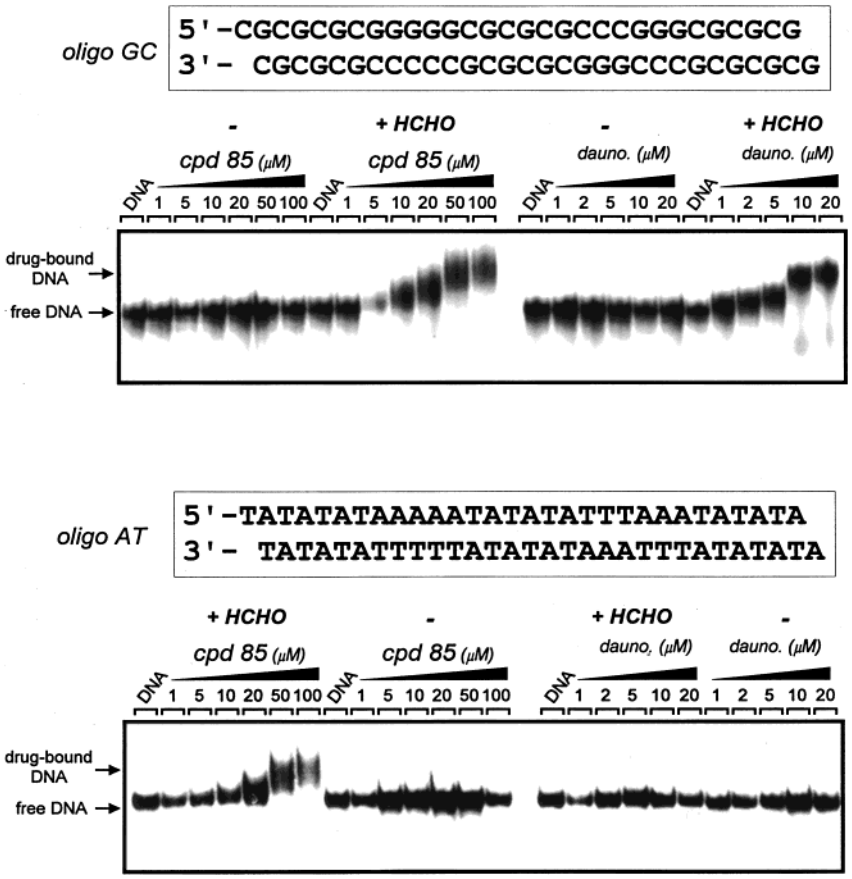


Figure 6. Cross-linking to the AT and GC oligonucleotides. In both cases, the 30-base pair oligonucleotide (containing exclusively A·T or G·C pairs) was reacted with either daunomycin or **85** at the indicated concentration (μM), in the absence (−) or presence of 1% HCHO, in 50 mM borate buffer at pH 8.2. After 1 h of incubation at 37 °C, samples were extracted with phenol/chloroform and the DNA was precipitated with ethanol. Cross-linked DNA samples were then subjected to electrophoresis on a 10% polyacrylamide gel. Lane marked DNA contained no drug.

rescence titration experiments were performed with **85** bound to $[poly(dA\text{-}dT)]_2$ or $[poly(dG\text{-}dC)]_2$. The fluorescence intensity was found to be much higher with the drug bound to the AT polymer than with the GC polymer (data not shown). Specifically, the ratio of the fluorescence of the fully bound drug to the free form was found to be 49 for the AT polymer but only 5 for the GC polymer. Nonlinear least-squares analysis of the titration curves yielded binding constants of 6.2×10^4 and 1.9×10^4 $(M\ bp)^{-1}$ for the AT and GC polymers, respectively. A binding constant of 10.6×10^4 $(M\ bp)^{-1}$ was measured with calf thymus DNA under the same experimental conditions (BPE buffer, pH 7.0).

The fluorescence emission spectrum of the drug cross-linked to $[poly(dA\text{-}dT)]_2$, $[poly(dG\text{-}dC)]_2$, or calf thymus DNA was recorded after phenol extraction of the noncovalently bound molecules. As shown in Figure 7A, there was no fluorescence at all in the control sample without HCHO (spectrum A) whereas a high fluorescence peak centered at 560 was measured with both calf thymus DNA and $[poly(dA\text{-}dT)]_2$ (spectra C and D). The fluorescence intensity for the drug cross-linked to $[poly(dG\text{-}dC)]_2$ (spectrum B) is much weaker than that observed with the AT polynucleotide, but this is most probably a consequence of the lesser fluorescence enhancement for binding to the GC polymer rather that a reflection of alkylation efficiency. Similar behavior was observed when the alkylation experiments were performed in borate buffer at pH 8.2 (Figure 7B).

Natural DNA samples were also used for fluorescence measurements. **85** binds well to the DNA from calf thymus (42% GC) and *Clostridium perfringens* (26% GC). The nonlinear least-squares analysis of the titration curves yielded binding constants of 10.6×10^4 and 3.0×10^4 $(M\ bp)^{-1}$ for the calf thymus DNA and *C. perfringens* DNA, respectively. In the presence of HCHO, the drug was easily cross-linked to these two DNA species, as judged from the fluorescence emission spectra presented in Figure 8. More interesting was the observation that the drug could also be reacted with the DNA from *Escherichia coli* phage T4 (35% GC) which major groove is partially occluded by glucose residues attached to 5-(hydroxymethyl)cytosine and 5-(hydroxy)-cytosine residues. In this case, the fluorescence analysis yielded a binding constant of 7.0×10^4 $(M\ bp)^{-1}$ and the fluorescence emission spectrum for the drug cross-linked to T4 DNA almost superimposed with that obtained with $[poly(dA\text{-}dT)]_2$ (Figure 8). The blockage of the major groove is apparently not an obstacle for the drug to interact with the DNA double helix. These observations are reminiscent to those reported with daunomycin which can also interact with a natural DNA (from *Trypanosoma brucei*) containing β-D-glucosyl(hydroxymethyl)uracil residues obstructing the major groove.[20]

Discussion

Results from gel mobility shift experiments, thermal denaturation studies, and fluorescence measurements

Table 1. Results from Thermal Denaturation Experiments[a]

[drug]/[DNA bp]	ΔT_m (°C) (−HCOH)	ΔT_m (°C) (+HCOH)
Calf Thymus DNA		
0	0	0
0.05	2	6
0.1	4	11.9
0.25	6.7	22.3
0.5	6.5	26.2
1.0	6.7	28.7
Poly(dA-dC)•Poly(dG-dT)		
0	0	−2
0.05	1	6.7
0.1	1.8	12.4
0.25	2.9	21.2
$[Poly(dA-dT)]_2$		
0	0	0
0.05	2	5.9
0.1	3	11.9
0.25	4.8	31
0.5	6.6	50.8
1.0	8.9	52.6
$[Poly(dI-dC)]_2$		
0	0	−1
0.05	2	3.1
0.1	3.9	6.1
0.25	8.9	10

[a] T_m measurements were performed in BPE buffer pH 7.0 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA) using 20 μM DNA or polynucleotide (nucleotide concentration) in 1-mL quartz cuvettes at 260 nm with a heating rate of 1 °C/min. Each drug concentration was tested in duplicate. The T_m values for the drug-free nucleic acids were 65 °C for calf thymus DNA and poly(dA-dC)•poly(dG-dT), 44 °C for $[poly(dA-dT)]_2$, and 41 °C for $[poly(dI-dC)]_2$.

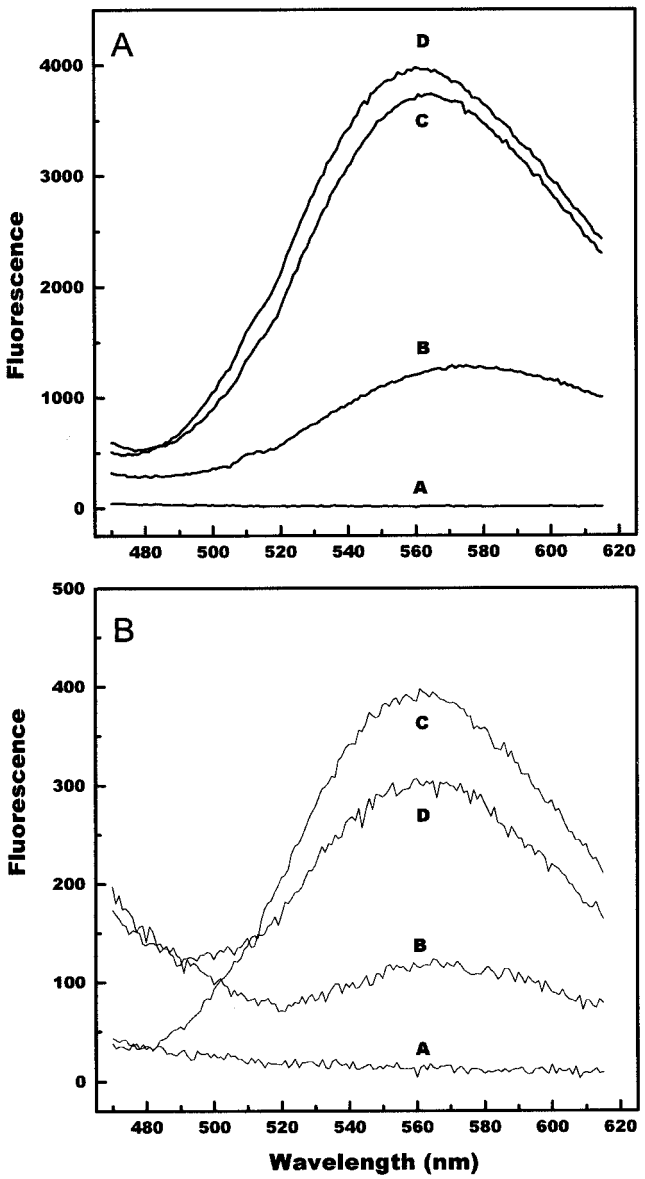


Figure 7. Fluorescence emission spectra of **85** obtained in the absence and presence of HCHO (2%, w/w) in (A) BPE at pH 7.0 or (B) borate buffer at pH 8.2: (spectrum A) no HCHO, (spectrum B) cross-linked to $[poly(dG-dC)]_2$, (spectrum C) cross-linked to calf thymus DNA, and (spectrum D) cross-linked to $[poly(dA-dT)]_2$. All spectra were recorded after phenol extraction of the noncovalently bound molecules and further dialysis of the HCHO-assembled drug−DNA samples against the same buffer, BPE or borate. The excitation wavelength was 320 nm. The drug concentration was fixed at 5 μM, and the DNA and polynucleotides were used at 75 μM.

all show that the indolocarbazole **85** containing a primary amino group at position 2′ on the carbohydrate residue can form covalent adduct with DNA in the presence of formaldehyde. The chemical reagent, HCHO, is absolutely essential for the formation of covalent drug−DNA adducts. In this respect, the situation reported here with the indolocarbazole drug is analogous to that previously described with daunomycin which naturally contains an amino sugar residue (the daunosamine) that can react with DNA in the presence of HCHO. The covalent reaction of daunomycin with DNA in the presence of HCHO was first characterized in 1991 by Wang after the observation that the disaccharide daunomycin analogue MAR70 was covalently attached to the hexamer $d(CGTDCG)_2$ due to the presence of trace amount of HCHO in the crystallization solvent.[11] Their serendipitous discovery opened up a new experimental route for the structural evaluation of the interaction between the anticancer drug daunomycin and different DNA sequences.[21] The 3′-amino group on the daunosamine moiety was shown to be essential for HCHO-mediated covalent binding of daunomycin to DNA. Removal of the 3′-amine or moving the amino group from the 3′ to the 4′ position resulted in a loss of cross-linking reactivity.[17] On the DNA, the reaction involved the amino group of guanine, accessible via the minor groove of double helix.[17] The same G-specific covalent reaction can also occur with *n*-butylamine[22] and adriamycin which is structurally close to daunomycin.

Whereas the HCHO-mediated covalent reaction of daunomycin with DNA is clearly restricted to guanine residues, the indolocarbazole drug studied here can react with both A•T and G•C base pairs in the presence of HCHO. The gel in Figure 6 indicates that in the presence of HCHO, **85** strongly reduces the electrophoretic mobility of a short oligomer duplex containing exclusively A•T base pairs. Under the same experimental conditions, daunomycin has no effect. We have not yet characterized the adducts formed between **85** and the DNA bases. By analogy with daunomycin, it is likely that the covalent reaction with the GC oligomer involves the 2-amino group of guanine pointing out into the minor groove. We have previously shown that this exocyclic substituent plays an important role in deter-

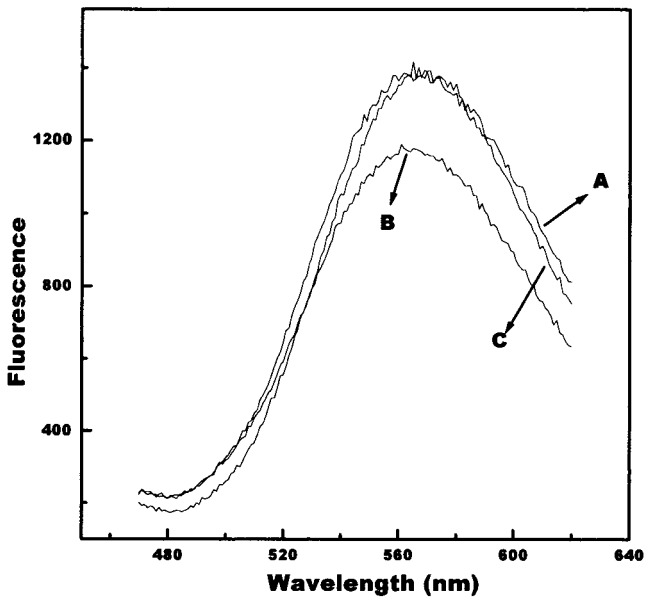


Figure 8. Fluorescence emission spectra of **85** cross-linked to (spectrum A) [poly(dA-dT)]$_2$, (spectrum B) DNA from *C. perfringens*, or (spectrum C) DNA from coliphage T4. All spectra were recorded in BPE buffer at pH 7.0, after phenol extraction of the noncovalently bound molecules and further dialysis of the HCHO-assembled drug−DNA samples against BPE buffer. The excitation wavelength was 320 nm. The drug concentration was fixed at 30 μM, and the DNA and polynucleotides were used at 30 μM.

mining how rebeccamycin derivatives recognize their binding sites in DNA.[7] The amino group of cytosine residues exposed in the major groove of DNA could in theory represent a target site for **85**, but this hypothesis is considered unlikely. The alkylation experiments with the DNA from coliphage T4 attest that the drug still reacts with this DNA for which the major groove is obstructed with glucose residues attached to the cytosines. For this reason, the minor groove hypothesis involving the 2-amino group of guanines is preferred. At AT sequences, we believe that the only possibility would be that the drug has reacted with the 6-amino group of adenines. This substituent exposed into the major groove is the only amino group available on A·T base pairs, and it is highly reactive toward HCHO. The 6-amino functions of two adenosine residues can be linked by a methylene bridge in the presence of HCHO.[23] To react with the 6-amino group of adenines the 2′-aminoglucose residue of **85** would have to enter the DNA from the major groove. At first sight this binding configuration may seem unlikely because the vast majority of small molecules fit into the minor groove of DNA. However, there are a few exceptions. For instance the antitumor drug menogaril contains a bicyclic aminoglucose sugar which locates into the major groove of DNA when the aglycone chromophore is intercalated.[24] The NMR structure of menogaril bound to the octanucleotide d(GACATGTC)$_2$ showed that the planar chromophore intercalates at the CA·TG step placing the aminoglucose residue into the major groove at the ACA·TGT segment. It is possible that a similar binding situation occurs with **85** which intercalates preferentially at CA·TG step and contains an aminoglucose residue. The binding configuration at AT sites would thus be directly opposite to that occurring at GC sequences. Further structural studies will be needed to determine the orientation of the drug-bound GC and AT sites, but the data reported here suggest that the drug can adopt two distinct orientations depending on the target sequence. The indolocarbazole chromophore may intercalate into the double helix placing the appended sugar residue in the minor groove at GC sites (to permit alkylation of 2-amino-G) and/or in the opposite major groove at AT sites (to permit alkylation of 6-amino-A). In other words, the drug may be able to switch grooves in response to sequence. Alternatively, the drug may be able to bind to both grooves, occupying simultaneously the minor and major grooves of the double helix. Both hypotheses would account satisfactorily for the footprinting data reported previously showing that rebeccamycin analogues are highly sensitive to any modification of the exocyclic substituents on the bases in both the major and minor grooves of the double helix.[7] The unique DNA binding characteristics of **85** warrant further structural investigations.

Our approach to investigate further the molecular interactions between indolocarbazoles is based on the design of a rebeccamycin analogue bearing an aminoglucose residue which can be efficiently cross-linked to DNA via the reaction with formaldehyde. This cross-linking methodology has proven useful to comprehend further the recognition of DNA by daunomycin and adriamycin.[17] Over the past few years, the same approach has been employed with marked success to study the recognition of DNA by other anthracyclines containing the daunosamine moiety or a related aminosugar residue, such as 2′-bromo-4′-*epi*-daunorubicin, named WP401,[25,26] or the daunorubicin derivative MAR70.[27] More recently, the aldehyde-mediated alkylation strategy was used to study the DNA interactions of ethidium and related phenanthridines[19] and the anticancer drug mitoxantrone.[28] The method is generally applicable to a variety of DNA-interacting agents containing reactive amino groups. HCHO-mediated alkylation experiments provides a general experimental approach for probing minor versus major groove interactions between small molecules and DNA.

Experimental Section

Drugs and Chemicals. Daunomycin was purchased from Sigma Chemical Co. (La Verpillière, France). The synthesis of the 2′-amino rebeccamycin derivative used in this study has been described recently.[18] Drugs were dissolved in dimethyl sulfoxide (DMSO) at 5 mM and then further diluted with water. The stock solutions of drugs were kept at −20 °C and freshly diluted to the desired concentration immediately prior to use. All other chemicals were analytical grade reagents.

Biochemicals. DNA from calf thymus and the double-stranded polynucleotides [poly(dA-dT)]$_2$, [poly(dI-dC)]$_2$ and poly(dA-dC)·poly(dG-dT) were from Sigma Chemical Co. Their concentrations were determined applying the molar extinction coefficients given in the literature.[29] Calf thymus DNA was deproteinized with sodium dodecyl sulfate (protein content < 0.2%) and all nucleic acids were dialyzed against BPE buffer pH 7.0 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA). The synthetic oligonucleotides were purchased from Eurogentec (Belgium) and purified. To prepare the AT and GC targets, a synthetic 30-mer oligonucleotide and its complement were mixed at a 1:1 ratio, heated to 70 °C and slowly cooled to form the desired duplex which was then labeled at the 3′-end using [α-^{32}P]dATP or [α-^{32}P]dCTP (6000 Ci/mmol; Amersham) and the Klenow fragment of DNA polymerase I (Boehringer).

DNA Restriction Fragment. The pBS plasmid (Stratagene, La Jolla, CA) was isolated from *E. coli* by a standard sodium dodecyl sulfate−sodium hydroxide lysis procedure and purified by banding in CsCl−ethidium bromide gradients. The plasmid was digested with *Pvu*II and *Eco*RI and the resulting 117-base pair fragment was labeled at the *Eco*RI site with [α-^{32}P]dATP and AMV reverse transcriptase (Boehringer, Mannheim, Germany). Electrophoresis on a nondenaturing 6% (w/v) polyacrylamide gel served to remove excess radioactive nucleotide, with the desired 3′-end-labeled product being cut out of the gel and eluted overnight in 500 mM ammonium acetate, 10 mM magnesium acetate. The purifed DNA was then precipitated twice with 70% ethanol prior to being resuspended in the reaction buffer.

Gel Shift Studies. A typical alkylation reaction consisted of 5 μL of radiolabeled DNA, 10 μL of 10X buffer (500 mM Na Borate, pH 8.2), 75 μL of water and 10 μL of the test drug at the desired concentration. After 20 min of incubation at 37 °C to ensure equilibration of the binding reaction, alkylation was initiated by the addition of HCHO. The reaction was left to proceed for 1 h (unless otherwise stated) at 37 °C prior to the extraction with 100 μL of phenol/chloroform/isoamyl alcohol (25:24:1). After a brief centrifugation, the aqueous layer (80 μL) was removed from the tube and the DNA was precipitated with 400 μL of cold ethanol. The DNA was finally resuspended in 10 μL of water and 5 μL of a 50% glycerol solution containing tracking dyes. DNA samples were resolved by electrophoresis under nondenaturing conditions in 6% or 10% acrylamide gels, depending on the length of the DNA substrate. Electrophoresis was performed for about 1.5 h at 120 V in TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM Na_2 EDTA, pH 8.3). Gels were transferred to Whatman 3MM paper, dried under vacuum at 80 °C, and then analyzed on the phosphorimager (Molecular Dynamics 445SI).

Thermal Denaturation Studies. Melting curves were measured using an Uvikon 943 spectrophotometer coupled to a Neslab RTE111 cryostat. For each series of measurements, 12 samples were placed in a thermostatically controlled cellholder, and the quartz cuvettes (10-mm path length) were heated by circulating water. The measurements were performed in BPE buffer pH 7.0 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM EDTA). The temperature inside the cuvette was measured with a platinum probe; it was increased over the range 20−100 °C with a heating rate of 1 °C/min. The "melting" temperature T_m was taken as the midpoint of the hyperchromic transition.

Fluorescence Titration Experiments. The stock solution of **85** was freshly prepared at a concentration of 2 mM in DMSO and diluted into buffer solution at the desired concentration. Calf thymus DNA was purchased from Pharmacia (lot no. 27-4562-02) and was sonicated and purified as described earlier.[30] Before further use, the DNA was dialyzed in the appropriate buffer for 24 h, and its concentration was determined by UV absorption at 260 nm by using a molar extinction coefficient, $\epsilon_{260} = 12\ 824\ cm^{-1}\ M^{-1}$. Titration experiments were carried out in a buffer (BPE) consisting of 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.0, unless noted otherwise. Fluorescence titration data were recorded at room temperature using an ISS Greg 200 fluorometer. Excitation was at 320 nm and fluorescence emission was monitored over the range 340−620 nm. Samples used for titration experiments were prepared separately at a constant drug concentration of 5 μM, and DNA concentrations ranging from 0.1 μM bp to 1 mM bp.

Fluorescence titration data were fit directly to get binding constants, using a fitting function incorporated into FitAll (MTR Software, Toronto, Canada). Simply, the observed fluorescence is assumed to be a sum of the weighted concentrations of free and bound ligand:

$$F = F^0(C_t - C_b) + F^bC_b \qquad (1)$$

where F is the apparent fluorescence at each DNA concentration, F^0 is the fluorescence intensity of free ligand, and F^b is the fluorescence intensity of the bound species. For the interaction of a ligand D with a DNA site S, it may be easily shown that:

$$Kx^2 - x(KS_0 + KD_0 + 1) + KS_0D_0 = 0 \qquad (2)$$

where $x = C_b$, K is the association constant, S_0 is the total concentration, and D_0 is the total ligand concentration. Equation 2 is readily solved using the quadratic formula. Data in the form of fluorescence response F as a function of total DNA site concentration at fixed concentration of ligand may then be fit by nonlinear least-squares methods to obtain K, F^0, and F^b.

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