How and How Much Can Hoechst 33258 Cause Unwinding in a DNA Duplex?

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The effect of Hoechst 33258 binding on the geometry of a DNA duplex (plasmid pBR322) has been examined using topoisomerase II relaxation followed by gel electrophoresis. Of this drug–DNA system, fluorescence, optical absorption, and calorimetric measurements were also made at various drug and DNA concentrations and in the same buffer as that for the topoisomerase reaction. It has been confirmed that there are two modes of drug–DNA interaction. When the drug concentration is much lower than the DNA base pair concentration, the Hoechst 33258 molecule binds in the minor groove of the DNA duplex and occupies a site formed of five continuous base pair sequences that contain no G·C pair. Here, the equilibrium constant $K_2$ is $1.8 \times 10^3$ M$^{-1}$ (at 37°C), and the enthalpy of binding $\Delta H_2$ is $-865$ cal/mol. When the drug concentration is much higher, on the other hand, it shows another binding mode which is much weaker, so that $K_2=2.25 \times 10^4$ M$^{-1}$ and $\Delta H_2$ is $-464$ cal/mol, which gives fluorescence quenching, which has no base pair preference, and which causes an unwinding of the duplex by 1 degree.

Key words unwinding of DNA duplex; Hoechst 33258; plasmid; topoisomerase I; topoisomerase II; topoisomer; calorimetry

Hoechst 33258 (2'-(4-hydroxyphenyl)-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole) (Fig. 1) is a well known DNA binding agent and a therapeutic drug. By X-ray crystallographic studies of the complexes formed between Hoechst 33258 and various oligonucleotides, it has been established that this drug binds preferentially in the minor groove of AT rich sequences of a DNA duplex. This binding mode has also been found to be the case in solution according to a high resolution NMR study. Would such non-intercalative binding cause any unwinding of the DNA duplex? This question motivated our present study, because we have been engaged in the precise determination of the unwinding angles that are caused by intercalative drugs such as aclacinomycin A, daunomycin, ethidium bromide, and chromomycin A$_3$.

Some of the previous investigations, however, suggested that the minor groove binding mechanism is not the sole means whereby Hoechst 33258 can interact non-covalently with DNA. Equilibrium binding experiments using fluorescence and absorption techniques indicated some other modes of binding for this drug when it is placed in solutions of various concentrations of calf thymus DNA, poly (dA-dT)-poly (dA-dT), poly (dG-dC)-poly (dG-dC), and some sequence controlled oligo-DNAs.

In view of such a situation, the aim of our present study became twofold. First, we attempted to determine a precise set of parameter values for this drug–DNA system, namely, the equilibrium constant ($K$), size of site ($n^*$), and enthalpy of binding ($\Delta H$), under a specially selected unified common condition: at 37°C, in buffer-T2, and with pBR322 DNA as the substrate. Secondly, we attempted to establish whether the binding of this drug produced any unwinding of the DNA duplex, and if so, under what condition, and how much. In the course of this study, we found that this drug acts as an inhibitor of topoisomerase I and topoisomerase II. Because this may be another important aspect of the biological function of this drug, we shall also report below some details of this finding.

Experimental

Materials The sample of Hoechst 33258 was purchased from Sigma Chemical Co. The adsorption of this drug from neutral aqueous solution onto glass, quartz, and some plastic (Parafilm) surfaces was avoided by use of a polystyrene tube. The quartz cuvettes for spectroscopic measurements were pre-coated with Sigmacote (chlorinated organopolysiloxane in heptane) (Sigma Chemical Co.).

Some of the samples of plasmid pBR322 DNA were prepared using a Qiagen plasmid kit (Qiagen GmbH, Max-Volmer-Strasse 4 40724 Hilden, Germany). This kit was purchased through Funakoshi Co. Further purification was done through treatment with sodium dodecyl sulfate (SDS)-proteinase K, followed by treatment with phenol and then by dialysis. Some of the samples of plasmid pBR322 DNA were purchased from Takara Shuzo Co. These two kinds of plasmid samples did not show any difference as far as our experiments were concerned.

Topoisomerase I from calf thymus was purchased from Takara Shuzo Co. Topoisomerase II was purchased from Topo Gen, Inc., through Cosmo Bio Co. Both of these topoisomerases were used without further purification.

The drug plus plasmid plus topoisomerase I reactions were produced in a buffer, which was proper for topoisomerase I activity measurement. This buffer contained 35 mM Tris–HCl (pH = 8.0), 72 mM KCl, 5 mM MgCl$_2$, 5 mM dithiothreitol, 5 mM spermidine, and 0.01% bovine serum albumin. Hereafter, this is called buffer-T1 in this paper.

Most of the other drug plus pBR322 DNA reactions were produced in a buffer, which was proper for topoisomerase II activity measurement. This buffer contained 50 mM Tris–HCl (pH = 8.0), 120 mM KCl, 10 mM MgCl$_2$, 0.5 mM ATP, and 0.5 mM dithiothreitol. Let us call this buffer-T2.

The sample of relaxed pBR322 DNA was prepared using topoisomerase I in buffer-T1 at 37°C. After the enzymatic reaction, the solution was treated with SDS-proteinase K, then DNA was purified by phenol treatment and dialysis.

Absorption Spectroscopic Measurements A Shimadzu automatic recording spectrometer UV-2200A was used. The temperature of the sample solution was kept at 37°C using a Taieto EL-8F Coolbat Bath.

Fluorescence Measurements A Jasco FP-770 spectrometer was used. The temperature of the sample solution was kept at 37°C using a SCINICS

Fig. 1. Structure of Hoechst 33258

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Electrophoresis Analysis A proper amount of plasmid pBR322 DNA was dissolved in buffer-T1 (or buffer-T2), and a proper amount of drug was added. The mixture solution was incubated at 37 °C for 10 min to complete the binding reaction. Next, the proper amount (6 units for 50 μl of the mixture solution) of topoisomerase I (or topoisomerase II) was added, and the solution was incubated at 37 °C for 2 h to cause a complete relaxation of the supercoiled plasmid (with bound drug). Then, the drug and enzyme were removed through phenol extraction, and DNA was isolated by ethanol precipitation. The isolated DNA was then subjected to 1% agarose gel electrophoresis. The buffer used here was TBE (90 mm Tris-borate and 2 mm EDTA). The experiment was done by imposing 40 V at room temperature for 20 h. After that, the gel was stained with ethidium bromide and subjected to photography.

The mobility of pBR322 DNA is related to its writhing number τ. The relation was examined in detail using agarose gel containing various amounts of chloroquine. When 0.5 μg/ml of chloroquine, for example, was added in the agarose gel as well as in the TBE buffer, the τ value of pBR322 DNA was elevated by 10. It was found that pBR322 DNA with $\tau = -1, -2, \ldots$ had a slightly greater mobility than pBR322 DNA with $\tau = +1, +2, \ldots$. 

Isothermal Titration Calorimetry (ITC) An MCS isothermal titration calorimeter from MicroCal, Inc. was used. A 1.5 mm solution of pBR322 DNA in buffer-T2 was placed in the cell. To this, a 1.0 mm solution of Hoechst 33528 was injected 12 times at 37 °C, with 20 μl each time; the time between injections was 400 s. Data collection and analysis were made using the software “Origin,” which was provided by MicroCal, Inc.

Results and Interpretations

Inhibitory Actions of Hoechst 33528 against Topoisomerases Figure 2 shows the result of our electrophoresis analysis of topoisomerases of pBR322 DNA caused by topoisomerase I action. As seen here, the superhelical plasmid (lane 1) was relaxed by topoisomerase I (lane 2). The coexistence of Hoechst 33528 caused some changes in topoisomer distribution. Particularly, the addition of Hoechst 33528 in an amount as much as $9.37 \times 10^{-8}$ M caused superhelices that looked similar to the intact plasmid (see lane 6 as well as lane 1). We suspected that this might be due to the inhibitory action of Hoechst 33528. To confirm whether this was the case, we next examined the actions of the drug and enzyme on an already relaxed plasmid. The results are shown in Fig. 3. Here, the DNA in lane 1 showed the same topoisomer distribution as that of the relaxed pBR322 DNA, and was different from lane 5 of Fig. 2. Even when the amount of the added Hoechst 33528 was as great as $9.37 \times 10^{-8}$ M the relaxed plasmid remained as it was, and never went into the superhelices shown in lane 6 of Fig. 2. Thus, it is clear that the topoisomer distributions found in lanes 5 and 6 of Fig. 2 are not what are produced through the unwinding action of Hoechst 33528, but must be due to the inhibitory action of this drug.

Lanes 3 and 4 of Fig. 3 are the result of similar examinations of the action of Hoechst 33528 and topoisomerase II. As seen here, the topoisomer distribution in lane 3 was appreciably different from that of the completely relaxed plasmid. This shows that topoisomerase II was active here. If the concentration of Hoechst 33528 was as low as $9.37 \times 10^{-8}$ M, it did not inhibit topoisomerase II, although it was definitely inhibitory when the concentration was as high as $9.37 \times 10^{-7}$ M (see lane 4, Fig. 3).

Display of the Unwinding Ability Since we knew that topoisomerase II retained its ability to relax pBR322 DNA in a solution with a lower concentration of Hoechst 33528, we next proceeded with the use of this enzyme in an examination of how this drug unwinds the DNA duplex. We started from a completely relaxed pBR322 DNA ($\tau = 0 \pm 2$, see lane 2 of Fig. 4). Next, a reaction was examined in buffer-T2 by adding topoisomerase II (lane 3). Here the topoisomer distribution was slightly different from that shown in lane 2, which was obtained by the relaxation reaction in buffer-T1 by the use of topoisomerase I. In the same system as that of lane 3, a proper amount of Hoechst 33528 was added, then relaxation of the plasmid (with the bound drug) was achieved by topoisomerase II in buffer-T2. If the drug binding causes an unwinding of the DNA duplex, a superhelix is expected to appear after the drug molecules are stripped off the plasmid molecule. Thus, the change in θ (the angle of clockwise rotation viewed along the local duplex axis in order to reach a
**Fig. 4** Electrophoresis Analysis of Topoisomers of pBR322 DNA, Produced by Hoechst 33258 Binding and Topoisomerase II Relaxation. Followed by the Removal of the Drug and Enzyme.

Lane 1, purified intact pBR322 DNA. Lane 2, completely relaxed pBR322 DNA. Lanes 3—9, Hoechst 33258 was added before topoisomerase II was introduced. DNA concentration=1.72×10⁻¹⁵ M. Drug concentration: lane 3, 0 μM; lane 4, 9.37×10⁻⁴ μM; lane 5, 1.87×10⁻⁴ μM; lane 6, 3.75×10⁻⁴ μM; lane 7, 5.65×10⁻⁴ μM; lane 8, 7.50×10⁻⁴ μM; lane 9, 9.37×10⁻⁴ μM.

**Fig. 5** Electrophoresis Analysis of Topoisomers of pBR322 DNA in a 1% Agarose Gel Containing 0.5 μg/mL of Chloroquine

Samples 1—9, here, are the same respectively as samples 1—9 described in the caption of Fig. 4.

base pair from an adjacent base pair located closer to the viewer), Δθ=θ−θ₀, should cause a change in h (the number of base pairs involved in one pitch of the DNA duplex), and should cause a change in β (twisting number) by

$$β−β₀=NΔθ/360$$

Here, $θ₀$ and $θ$ are the angles $θ$ (degrees) before and after the drug binding, $β₀$ and $β$ are the twisting numbers before and after the drug binding, and $N$ is the total number of base pairs involved in the closed circular duplex pBR322, that is 4362. An unwinding means a negative value of $Δθ$ (because $β<β₀$), and it should cause an increase in h value, as well as a decrease in the $β$ value. When $β$ is reduced by drug binding, the topoisomerase should reduce $α$ (linking number) to keep $τ$ (writhing number) at 0±2, because

$$α=β+τ$$

What was expected actually proved to be the case, as seen in lanes 4—7 of Fig. 4. As the concentration of added Hoechst 33258 increased, the amount of lowering of the linking number of the topoisomer produced increased. This was more clearly shown by the electrophoresis experiment in an agarose gel containing 0.5 μg/mL of chloroquine (Fig. 5). Here, the writhing number of every topoisomer was reduced by 10, and the change caused by the drug binding can be easily read; without chloroquine, the overlapping of bands for $τ=+1$ and −1, and those for $τ=+2$ and −2 were somewhat disturbing in analysis. When the drug concentration was as high as 7.50×10⁻⁵ M (or 9.37×10⁻⁵ M), the topoisomer distribution was the same as that of completely relaxed plasmid (see lane 8 and lane 9 of Fig. 4 or of Fig. 5). This indicates that Hoechst 33258 at concentrations higher than 7.50×10⁻⁵ M is a perfect inhibitor of topoisomerase II.

**Binding Characteristics in Buffer-T2 in the Low Ratio of [Drug]/[Base pair] Concentrations** Figure 6 shows a result of the fluorescence spectral measurements. As seen here, 4.24×10⁻⁷ M Hoechst 33258 gave only weak fluorescence in buffer-T2 (curve “a” in the left panel of Fig. 6), but on adding pBR322 DNA its fluorescence became stronger. The fluorescence intensity at 450 nm was plotted against the amount of DNA added in the right portion of Fig. 6. After the DNA/drug concentration ratio reached 150, the fluorescence intensity showed almost no additional increase. In the concentration ratio range of 0—60, more detailed fluorescence spectroscopic measurements were made, and the result is shown in Fig. 7. From the intensity measurements at 450 nm, the concentration of the drug bound to DNA ($L_0$) and concentration of free drug ($L_1$) were determined for each solution. Then, the binding ratio $γ=L_0/D$ (where $D$ is the concentration of plasmid base pair) was calculated and $γ/L_1$ was plotted against $γ$ (Scatchard plot). The result is shown in Fig. 8. On an assumption that this plot corresponds to the

[Figure 4, Figure 5, Figure 6, Figure 7, Figure 8]
Fig. 7. Fluorescence Spectra of Hoechst 33258 in Buffer-T2 at 37°C, Excited at 360 nm

(a) Hoechst 33258 1.27×10^{-7} M only. (Curves with no labels). Solutions consist of 3000 μl of 1.27×10^{-7} M of Hoechst 33258 + 10 μl × J of 2.11×10^{-3} M (in base pair) of pBR322 DNA, where J=1—10. (b) Solution consists of 3000 μl of 1.27×10^{-7} M of Hoechst 33258 + 100 μl of 2.11×10^{-5} M pBR322 DNA + 4 μl of 9.35×10^{-1} M pBR322 DNA.

Fig. 8. Scatchard Plot of Hoechst 33258 Bound to Plasmid pBR322 DNA

- Concentrations of the bound and free drugs (L_b and L_f, respectively) were determined from fluorescence intensity measurements at 450 nm. The solid line shows a theoretical curve on the assumption that equilibrium constant \( K = 1.85 \times 10^7 \text{ M}^{-1} \) and the number of the drug molecules bound to one base pair is \( n^* = 2.8 \times 10^{-2} \).

Scatchard relation,

\[
\frac{\gamma}{L_f} = K(n^* - \gamma)
\]

the equilibrium constant,

\[
K = \frac{\text{[bound drug]}}{\text{[free drug] [free site]}}
\]

was determined to be \( 1.85 \times 10^7 \text{ M}^{-1} \), and \( n^* \) (the number of drug sites per base pair) to be \( 2.8 \times 10^{-2} \).

The \( n^* \) value, determined here, must involve a piece of information of the nucleotide sequence specificity of Hoechst 33258.

Fig. 9. Absorption Spectra of Hoechst 33258 in Buffer-T2 at 37°C

(a) Hoechst 33258 9.37×10^{-8} M only. (Curves with no labels). Solutions consist of 3000 μl of 9.37×10^{-8} M Hoechst 33258 + 5.5 μl × J of 9.35×10^{-7} M (in base pairs) pBR322 DNA, where J=1—6. (b) 3000 μl of 9.37×10^{-8} M Hoechst 33258 + 453 μl of 9.35×10^{-7} M pBR322 DNA, corrected for dilution effect.

Table 1. The Number Indicating How Often a Given Particular Base Sequence Appears along the Whole Sequence of pBR322

<table>
<thead>
<tr>
<th>Base sequence (5'→3')</th>
<th>Frequency of appearance</th>
</tr>
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<tbody>
<tr>
<td>AATT</td>
<td>8</td>
</tr>
<tr>
<td>TATA</td>
<td>7</td>
</tr>
<tr>
<td>GTTTAT</td>
<td>1</td>
</tr>
<tr>
<td>ATTTT</td>
<td>3</td>
</tr>
<tr>
<td>TTAATG</td>
<td>1</td>
</tr>
<tr>
<td>AATAA</td>
<td>2</td>
</tr>
<tr>
<td>TTCT</td>
<td>6</td>
</tr>
</tbody>
</table>

From a footprinting study, Harshman and Dervan concluded that Hoechst 33258 binds on the AAATT, TATA, GTTTAT, ATTTT, TTAATG, AATAA, and TTCT sequences. However, since Teng et al. showed that this drug does bind on the AATT, let us replace AAATT in Harshman's list with AATT. On the basis of the known sequence of pBR322 DNA, the number of each possible site is given in Table 1. If the drug molecules bind to all of these available sequences, the total number of drug sites per base pair is 127/4362 = 2.9×10^{-2}, in agreement with the experimental value from the Scatchard plot.

**Binding Characteristics in Buffer-T2 in the High Ratio of [Drug]/[Base pair] Concentrations** Figure 9 shows the result of measurements of the absorption spectra. As seen here, 9.37×10^{-8} M of Hoechst 33258 in buffer-T2 showed an absorption maximum at 339 nm. On adding pBR322 DNA, the absorbance at 339 nm was lowered, and a new absorption band appeared at a longer wavelength. By measuring the peak intensity at 339 nm, and by assuming that it is proportional to the concentration of the free drug, a Scatchard plot has been made, and the result is shown in Fig. 10. In a range where the [drug]/[base pair] concentration ratio is as high as 0.09—0.55, the drug—drug interference on the DNA chain would be appreciable. Therefore, the Scatchard plot must be interpreted on the basis of the McGhee—von Hippel theory, rather than on the basis of the Scatchard relation itself. The experimental results shown in Fig. 10 are explained by assuming that the equilibrium constant \( K_{ij} = 22500 \text{ M}^{-1} \) and the number of drug sites per base pair is \( n^* = 0.88 \) (Fig. 10). Thus, it is concluded that there is a radically different mode of binding from the groove binding when the concentration of Hoechst 33258 increases. The fact that \( n^* = 0.88 \) indicates that the secondary binding mode has no base pair preference.

**Calorimetric Characterization** Such co-existence of two modes of binding of Hoechst 33258 has been supported...
by calorimetric measurement. Figure 11 shows the plot of our data for the ITC. Here, both the drug and DNA solutions were prepared in buffer-T2. At 37°C, drug was injected into the DNA solution, so that the [drug]/[base pair] concentration ratio was elevated stepwise. For each step, the "heat of dilution" was measured and plotted against the drug concentration added. The "pure" heat of dilution that had been measured without DNA was subtracted, and only the heat of drug–DNA interaction was plotted here. A theoretical curve was obtained by the use of the software "Origin" provided by MicroCal, Inc. In using the software, it was assumed that there were two modes of binding, and that four parameters $n_1^*, K_1, n_2^*$, and $K_2$, out of six were fixed at 0.028, 1.8×10^7 M^{-1}, 0.88, and 2.25×10^6 M^{-1}, respectively. Here, $n_1^*$ and $n_2^*$ are the number of the drug molecules bound to one DNA base pair, and $K_1$ and $K_2$ are the equilibrium constants for the binding reactions. Subscripts 1 and 2 indicate the two different modes of binding. The remaining two parameters (out of 6) are $\Delta H$ and $\Delta S$, the enthalpies of binding. In using the software, $\Delta H$ and $\Delta S$ came out as −865 and −464 cal/mol, respectively. The theoretical curve explains the experimental points (see Fig. 11), and the assumed $n_1^*, K_1, n_2^*$, and $K_2$ parameter values are considered to be supported by the calorimetric experiment, as well as by spectroscopic experiments.

The Angle of Unwinding We have already shown that 9.37×10^{-6} m of Hoechst 33258 causes a change in the unwinding number (\Delta \tau) by \pm 1 (Fig. 5); 1.87×10^{-6} m of it causes \Delta \tau=4±1, 3.75±10^{-5} M \Delta \tau=6±1, and 5.65×10^{-5} M \Delta \tau=6±1. The DNA base pair concentration was 1.72×10^{-5} M. On the basis of value $K_2=2.25×10^6$ M^{-1} (see above), therefore, the concentrations of the drug–DNA (base pair) complex must be $2.35×10^{-6}$ m, $4.23×10^{-6}$ m, $7.00×10^{-6}$ m, and $8.87×10^{-6}$ m, respectively. From these values, the numbers (m) of drug molecules bound to one plasmid molecule are calculated to be 596, 1073, 1775, and 2249, respectively. The number, m, and \Delta \tau are considered to be proportional to each other (Fig. 12). By assuming this relation, \Delta \tau per one drug molecule is found to be $3.0×10^{-6}$. In other words, one Hoechst 33258 molecule is now considered to unwind the DNA duplex by $3.0×10^{-6}×360°=1.08°$ (Eqs. 1 and 2).
taking into account the errors in estimating $\Delta r$ as well as $K_2$, the unwinding angle is estimated to be $1.1 \pm 0.1^\circ$.

**Discussion**

In our present study, it has been confirmed that there are two modes of Hoechst 33258–DNA interaction. When the [drug]/[base pair] concentration ratio is lower, Hoechst 33258 molecule binds in the minor groove of a DNA duplex and occupies a site formed of five continuous base pair sequences that contain no G·C pair. Here, the equilibrium constant ($K_2$) is $1.8 \times 10^4$ M$^{-1}$ at 37°C and enthalpy of binding ($\Delta H_2$) is $-865$ cal/mol. When the drug concentration is much higher, Hoechst 33258 shows another binding mode which is much weaker, so that $K_2 = 2.25 \times 10^4$ M$^{-1}$ and $\Delta H_2 = -464$ cal/mol. This binding causes fluorescence quenching, has no base pair preference, has a stoichiometry of (drug sites)/(DNA base pair) = 0.88, and causes an unwinding of the DNA duplex by 1.1 ± 0.1 degrees. Such two-mode binding was found also for another “classical groove binding molecule”, namely 4',6-diamino-2-phenylindole (DAPI).[13,14] The secondary mode (i.e., the mode other than the groove binding) was suggested to be an intercalation, especially for RNA. This mode, however, is not yet evident for the drug in question in DNA. In connection with such a secondary mode, it is interesting to note that two drug molecules may bind simultaneously to the same site.[15–19] When the drug/DNA ratio is higher, an anti-parallel chain dimer of distamycin (or netropsin) was found to sit on the minor groove of a dodocamer DNA duplex. Although such binding is not yet evident for the drug molecule now in question, this suggests another possible candidate for the secondary binding mode of Hoechst 33258.

As a by-product of this study, it has been found that Hoechst 33258 is a strong inhibitor of topoisomerase I. Even when the drug concentration is as low as $9.37 \times 10^{-6}$ M, it inhibits topoisomerase I activity. At this concentration, it does not inhibit topoisomerase II activity. It does inhibit topoisomerase II, however, when its concentration is as high as $7.50 \times 10^{-5}$ M. The inhibitory action of Hoechst 33258 was previously studied by Beerman et al.[20] They showed that $1.0 \times 10^{-5}$ M, for example, of Hoechst 33258 induced a strong inhibition of topoisomerase I, but practically no inhibition of topoisomerase II. As for the amount of drug required for the inhibition of enzymatic relaxation, what they found was generally in good agreement with what we found in our present experiments.

Hoechst 33258 has been known as a minor groove binding drug, and one of the interesting questions has been “can such a groove binding drug cause an unwinding of the DNA duplex?” Our answer is now “yes, Hoechst 33258 does unwind DNA duplex, but not through groove binding, but through another binding mode.”

Chen et al.[21] once suggested an unwinding of DNA duplex by Hoechst 33258. However, they used topoisomerase I for relaxing plasmid. Therefore, it is probable that what they found was not the effect of unwinding but was merely an effect of the inhibitory action of this drug. Colson et al.[22] suggested that Hoechst 33258 interacts with DNA through a mode other than the groove binding, and they named it “non-classical intercalation”. Because the unwinding angle we found ($1.1 \pm 0.1^\circ$) is much smaller than what were found for aclacinomycin A ($8^\circ$), daunomycin ($12^\circ$), ethidium bromide ($15^\circ$), or chromomycin A$_3$ ($11.8 \pm 1^\circ$) bindings,[6,7] the second mode of interaction of Hoechst 33258 would not be a “genuine” intercalation. The elucidation of this mode of interaction is one of our next targets.

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**References**