Coralyne Binds Tightly to Both T·A·T- and C·G·C$^+$-Containing DNA Triplexes†

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ABSTRACT: Coralyne is a DNA-binding antitumor antibiotic whose structure contains four fused aromatic rings. The interaction of coralyne with the DNA triplexes poly(dT)·poly(dA)·poly(dT) and poly[d(TC)]·poly[d(GA)]·poly[d(C$^+$T)] was investigated by using three techniques. First, T_m values were measured by thermal denaturation analysis. Upon binding coralyne, both triplexes showed T_m values that were increased more than those of the corresponding duplexes. A related drug, berberinium, in which one of the aromatic rings is partially saturated, gave much smaller changes in T_m. Second, the fluorescence of coralyne is quenched in the presence of DNA, allowing the measurement of binding parameters by Scatchard analysis. The binding isotherms were biphasic, which was interpreted in terms of strong intercalative binding and much weaker stacking interactions. In the presence of 2 mM Mg^{2+}, the binding constants to poly(dT)·poly(dA)·poly(dT) and poly[d(TC)]·poly[d(GA)]·poly[d(C$^+$T)] were 3.5×10^6 M^{-1} and 1.5×10^6 M^{-1}, respectively, while the affinity to the parent duplexes was at least 2 orders of magnitude lower. In the absence of 2 mM Mg^{2+}, the binding constants to poly[d(TC)]·poly[d(GA)]·poly[d(C$^+$T)] and poly[d(TC)]·poly[d(GA)] were 40×10^6 M^{-1} and 15×10^6 M^{-1}, respectively. Thus coralyne shows considerable preference for the triplex structure but little sequence specificity, unlike ethidium, which will only bind to poly(dT)·poly(dA)·poly(dT). Further evidence for intercalation of coralyne was provided by an increase in the relative fluorescence quantum yield at 260 nm upon binding of coralyne to triplexes as well as an absence of quenching of fluorescence in the presence of $Fe[(CN)_6]^{4-}$. Third, coralyne promoted intermolecular triplex formation between plasmids containing a pyr·pur tract and a single-stranded polypyrimidine. The ability of coralyne to bind to triplexes may relate to its *in vivo* activity and also suggests ways of designing triplex-binding drugs with sequence specificity.

Drugs which bind to duplex DNA have been studied extensively (Jain & Sobell, 1972; Muller & Crothers, 1975; Muller & Gautier, 1975; Gale et al., 1981). One motivation for this work is that many of these compounds have proven to be useful antitumor agents. As well, DNA-binding drugs have been used to probe the structure and function of nucleic acids. For example, our understanding of the topology of circular DNA owes much to the intercalative properties of ethidium (Bauer & Vinograd, 1970). Drugs binding to higher ordered structures have received less attention, but there are some recent reports of interactions with triplexes of the pyr·pur·pyr class.

The antibiotic netropsin binds to triplexes in the minor groove but destabilizes them relative to duplexes (Park & Breslauer, 1992). Alternatively, other minor groove ligands, such as the polyamines spermine and spermidine, strongly favor triplex formation (Hampel et al., 1991). In general, minor groove binding drugs are expected to interact primarily through ionic bonds with the phopshate backbone. Intercalators, on the other hand, require strong stacking interactions and are therefore planar aromatic molecules, although many are positively charged as well. Ethidium binds well to T·A·T triplexes but not to those containing a high percentage of C·G·C$^+$ base triads presumably because of repulsion between the two positive charges (Morgan et al., 1979; Lee et al., 1984; Scaria & Shafer, 1991). The interaction with poly(dT)·poly(dA)·poly(dT) occurs via intercalation (Scaria & Shafer, 1991). Similarly, an ellipticine derivative stabilizes T·A·T triplexes but not C·G·C$^+$ triplexes (Mergny et al., 1992).

We reasoned that a large intercalator might bind more tightly to triplexes than to duplexes because the surface area for potential stacking interactions is greater in a triplex. One such drug is coralyne, which has four fused aromatic rings compared to only three found in ethidium (Figure 1). Coralyne is a member of the berberine group of alkaloids and possesses significant antitumor activity (Zee-Cheng & Cheng, 1974). It has been shown to intercalate into duplex DNA, but it also stacks on the outside of the helix particularly at low ionic strengths (Wilson et al., 1976). The related alkaloid berberinium also intercalates, with insertion of rings A and B, but shows little stacking presumably because the saturated ring gives a kink to an otherwise planar molecule (Davidson et al., 1977).

In this paper we have studied the binding of coralyne to both T·A·T and C·G·C$^+$ triplexes. The results demonstrate that coralyne has a higher affinity for triplexes than for duplexes, especially in the presence of 2 mM Mg^{2+}, and unlike ethidium, coralyne shows little sequence preference.

MATERIALS AND METHODS

Drugs. Coralyne chloride, berberinium chloride, and Hoechsst 33258 were purchased from Sigma. Stock solutions were made up in water and protected from light. Concentrations were determined by direct weighing.

Nucleic Acids. Synthetic duplexes and poly[d(TC)] were prepared as described previously (Lee et al., 1979). Poly(dT) was purchased from Pharmacia. The plasmid pTC45 contains an insert of $T(CT)_{22}$ in the multiple cloning site of p913 (Pulleyblank et al., 1985). The triplex poly(dT)·poly(dA)·poly(dT) was prepared by incubating the duplex with the appropriate concentration of poly(dT) in 2 mM Mg^{2+} and 10 mM Tris-HCl, pH 8. The triplex poly[d(TC)]·poly[d(GA)]·poly[d(C$^+$T)] was prepared by incubating the duplex with the appropriate concentration of poly[d(TC)] in 10 mM

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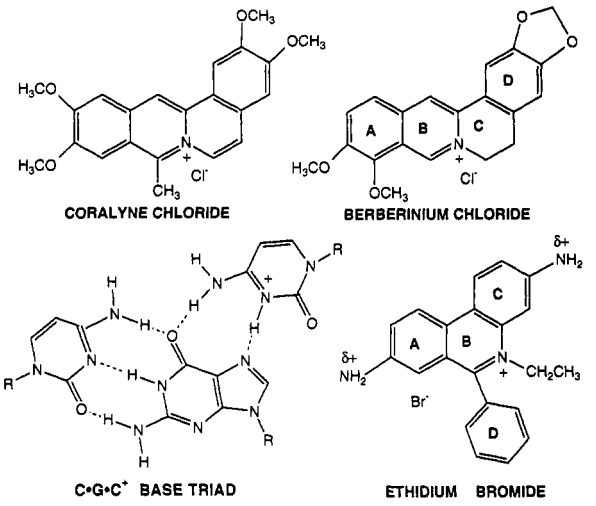

FIGURE 1: Structures of coralyne, berberinium, and ethidium. Ring C of berberinium is partially saturated, which gives a kink to the molecule. The exocyclic amino groups of ethidium carry significant positive charge because of delocalization of the unpaired electrons into the ring nitrogen (Wakelin & Waring, 1974). A C·G·C$^+$ base triad is drawn to the same scale.

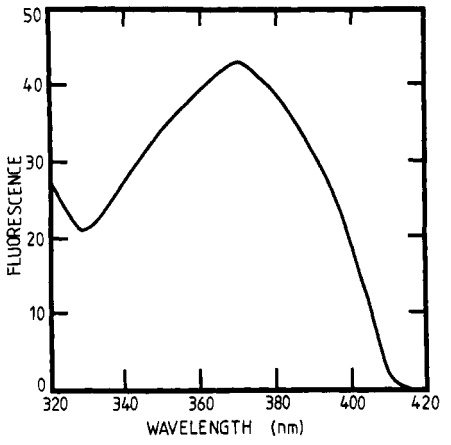

FIGURE 2: Fluorescence emission spectrum of coralyne. The fluorescence of 4 μM coralyne was measured as a function of wavelength after excitation at 300 nm.

sodium acetate, pH 5. The concentrations were calculated from published extinction coefficients, and triplex formation was monitored by the ethidium fluorescence assay (Morgan et al., 1979).

Thermal Denaturation Profiles. T_m measurements were made at 260 nm on a Gilford 600 spectrophotometer equipped with a thermoprogrammer at a heating rate of 0.5 °C/min. Poly(dT)·poly(dA)·poly(dT) was melted in 10 mM Tris HCl, pH 8, with 5 mM NaCl and 2 mM Mg^{2+}. The buffer for poly[d(TC)]·poly[d(GA)]·poly[d(C$^+$T)] was 10 mM HEPES, pH 7, with 5 mM NaCl.

Binding Constants. The UV absorption spectrum of coralyne shows a major peak at 300 nm (data not shown). Excitation at this wavelength in a Turner Model 430 spectrofluorometer gives good fluorescence on the 100× scale with a maximum emmission at 370 nm (Figure 2). Addition of an excess of duplex or triplex DNA quenches the fluorescence by as much as 70%. This forms the basis of a rapid method for measuring binding isotherms and other intercalative properties (see below).

Briefly, serial aliquots of a concentrated solution of the DNA were added to 2 mL of 2 μM coralyne in the standard buffers at pH 5 or 8 with or without 2 mM Mg^{2+}. The fluorescence of bound coralyne was estimated from a plot of fluorescence against DNA concentration after extrapolation to infinite DNA concentration. Even at the highest DNA concentration the inner filter effect ($e^{a/2}$, where a is the absorbance) was calculated to be less than 1.005 (Le Pecq & Paoletti, 1967). The fraction of coralyne bound to DNA and free in solution was then calculated at each DNA concentration to yield r, the number of drug molecules bound per base pair (or base triad), and c, the free coralyne concentration, as described previously (Lee et al., 1982; Latimer & Lee, 1991; Scaria & Shafer, 1991). Binding parameters were estimated from a Scatchard plot of r/c versus r according to the theory of McGhee and von Hippel (1974).

Fluorescence Quantum Yield. Excitation spectra of 2 μM coralyne between 240 and 310 nm were recorded at an emission wavelength of 370 nm. Where necessary, a correction for the inner filter effect was applied (Le Pecq & Paoletti, 1967). For both the duplex and the triplex derived from poly(dA)·poly(dT) the D/P value (drug to base pair or base triplet ratio) was set at 0.1, and for both the duplex and the triplex derived from poly[d(TC)]·poly[d(GA)] the D/P value was set at 0.5. These D/P values correspond to the tight binding region of the binding isotherms (see below). The ratio $Q = q_b/q_f = I_bE_f/I_fE_b$ was calculated at each wavelength, where q is the quantum efficiency, I is the fluorescence intensity, E is the molar extinction coefficient for coralyne, and the subscripts refer to bound (b) or free (f) drug. The ratio Q_λ/Q_{310} was calculated and plotted against wavelength (Le Pecq & Paoletti, 1967; Scaria & Shafer, 1991).

Fluorescence Quenching. The quencher $K_4Fe[(CN)_6]^{4-}$ (Barton et al., 1986) was effective at concentrations in the micromolar range, and therefore no correction was applied for changes in ionic strength. The experiments were performed in the standard pH 5 or 8 buffers at the same D/P values as for the quantum yield experiments described above with excitation at 300 nm and emmission at 370 nm. The ratio I_0/I was plotted as a function of the concentration of $Fe[(CN)_6]^{4-}$, where I is the fluorescence intensity and I_0 is the intensity in the absence of quencher.

Triplex Formation in Plasmids. pTC45 (0.5 μg) was incubated with poly[d(TC)] (0.1 μg) with or without coralyne in 10 μL of pH 5–8 buffers (25 mM sodium acetate, pH 5; 56 mM MES, pH 6; 56 mM BES, pH 7; or 33 mM HEPES, pH 8) with 1 mM EDTA for 2 h at 50 °C. An aliquot was loaded onto a 1% agarose gel and electrophoresed at 50 V for 4 h in a buffer of 40 mM sodium acetate, pH 5, and the gel was stained with ethidium.

RESULTS

One of the simplest methods for detecting binding to DNA is thermal denaturation analysis. In 2 mM Mg^{2+} poly(dT)·poly(dA)·poly(dT) gives two transitions: the triplex melts to a duplex, which in turn melts to single strands as the temperature is increased further (Scaria & Shafer, 1991). The effect of coralyne on these transitions is shown in Figure 3. Upon addition of 2 μM coralyne only one transition is observed with an increased T_m. The hyperchromicity of this transition corresponds to the melting of a triplex to single strands, and no independent duplex melt is observed. Therefore, in the presence of coralyne the T_m of the triplex is higher than the T_m of the duplex. With 20 μM coralyne the T_m of the triplex is increased by 16 °C. In contrast, 20 μM berberinium (Figure 3) only increases the T_m by 4 °C, and one transition is again observed. Thus, although the trend is the same, the binding of berberinium to triplexes is much weaker than that of coralyne.

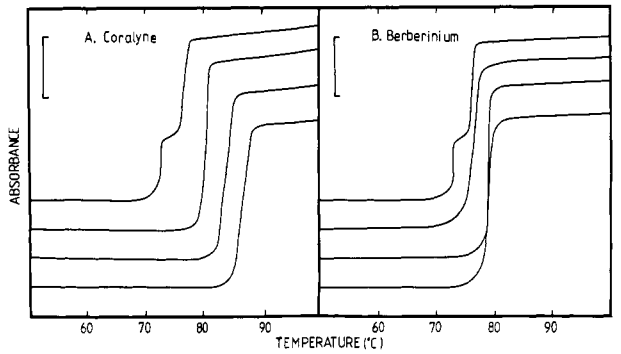

FIGURE 3: Thermal denaturation profiles for poly(dT)·poly(dA)·poly(dT) at pH 8 in 2 mM Mg^{2+} with (A) coralyne and (B) berberinium. The curves are (from left to right) 0, 4, 10, and 20 μM added drug. The bar represents a hyperchromicity of 20%.

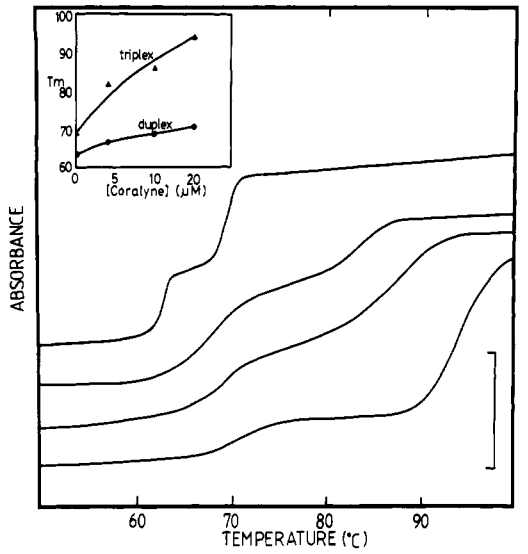

FIGURE 4: Thermal denaturation profiles for poly[d(TC)]·poly[d(GA)] at pH 7 with increasing concentrations of coralyne (0, 4, 10, and 20 μM from left to right). The bar represents a hyperchromicity of 20%. Note that the second transition is due to the triplex. The inset shows the T_m values for both duplex and triplex as a function of the coralyne concentration.

At pH 7 and low ionic strength, poly[d(TC)]·poly[d(GA)] also gives two transitions upon melting, but in contrast to the triplex denaturation described above, the higher T_m is due to the triplex (Lee et al., 1984). The dismutation is probably not a single step since presumably some of the duplex must melt before the triplex can form, e.g.,

$$\text{duplex} + \text{duplex} \underset{T_{m1}}{\Leftrightarrow} \text{duplex} + \text{ssPyr} + \text{ssPur} \Rightarrow$$

$$\text{triplex} + \text{ssPur} \underset{T_{m2}}{\Leftrightarrow} \text{single strands}$$

As the pH is lowered, the T_m of the duplex (T_m1) remains unchanged at 64 °C (see Figure 4), while that of the triplex (T_m2) increases dramatically (Lee et al., 1979, 1984). Also, ethidium binds to the duplex and increases the T_m but does not bind the triplex, whose T_m is unchanged. Thus the duplex and triplex melt independently (Lee et al., 1979, 1984). This makes the estimation of relative binding constants very difficult (McGhee, 1976). A pH of 7 was chosen for these experiments since both duplex and triplex transitions can be conveniently monitored simultaneously. Figure 4 shows the effect of coralyne on these T_m values. The T_m of the triplex is increased by as much as 25 °C with 20 μM coralyne, whereas the T_m of the duplex only increases by 7 °C. Thus coralyne binds well to both triplex and duplex forms of poly[d(TC)]·poly[d(GA)]. As well, the hyperchromicity due to the duplex decreases as the coralyne concentration increases. Similar effects were noted previously with spermine, which was shown to promote the dismutation of duplex to triplex (Hampel et al., 1991; see also Figure 10).

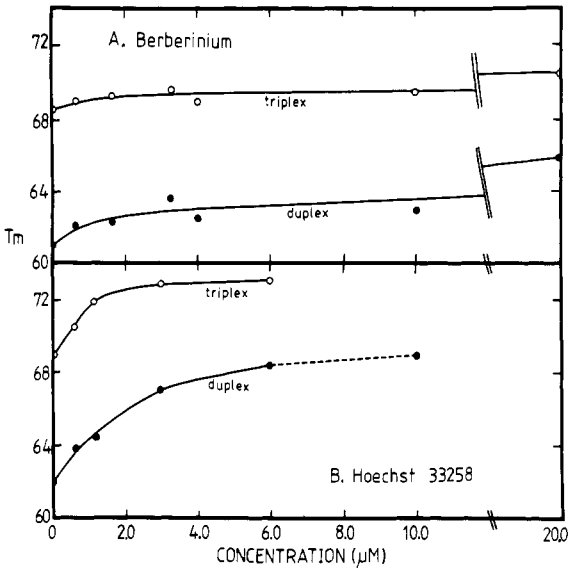

FIGURE 5: T_m of poly[d(TC)]·poly[d(GA)] at pH 7 with increasing concentrations of (A) berberinium and (B) Hoechst 33258. Above 6 μM Hoechst 33258 the two transitions merged (dotted line).

The effect of berberinium on the thermal behavior of poly[d(TC)]·poly[d(GA)] is shown in Figure 5A, and that of Hoechst 33258, which is a minor groove binder similar to netropsin, is shown in Figure 5B. As with poly(dT)·poly(dA)·poly(dT), berberinium gives only small changes in T_m of both the duplex and the triplex. With Hoechst 33258, the T_m of the duplex is increased by 6 °C at the highest concentration tested. (At higher concentrations the two transitions merge.) This small increase is not unexpected since the drug only binds tightly to stretches of three or four contiguous A·T base pairs (Muller & Gautier, 1975). The increase observed for the triplex is even less (4 °C), suggesting that Hoechst 33258 has no preference for the triple helix.

It was discovered that coralyne fluoresces with excitation at 300 nm and emmission at 370 nm. Moreover, this fluorescence is quenched in the presence of DNA, which allows a rapid and sensitive method for the estimation of binding parameters. Coralyne is known to dimerize in solution with a dimerization constant of the order of 10^4 M^{-1} at low ionic strength (Davidson et al., 1977). However, one advantage of this fluorescence method is that experiments can be performed at coralyne concentrations in the micromolar range. Therefore, dimerization can be ignored. A Scatchard plot for the binding of coralyne to poly(dT)·poly(dA)·poly(dT) in 2 mM Mg^{2+} is shown in Figure 6. The intercept on the r/c axis gives the binding constant, K (McGhee & von Hippel, 1974), which is estimated to be 3.5×10^6 M^{-1}. However, the binding isotherm is L-shaped, which is characteristic of drugs which have two modes of binding (Gale et al., 1981; Stone & Bradley, 1961). This was not unexpected since coralyne is known to intercalate and stack to the outside of duplex DNA (Wilson et al., 1976). At high drug to DNA ratios (high r), the curve is nearly flat and r values greater than 1 can be acheived. This part of the isotherm is therefore due to the stacking interactions, which have a lower binding constant than the intercalative mode. It should be noted that the presence of two modes of binding does not invalidate the estimation of the binding constant from the intercept on the r/c axis (McGhee & von

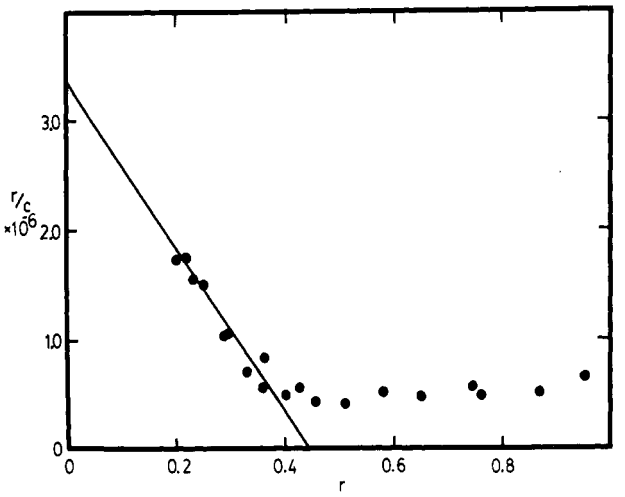

FIGURE 6: Scatchard plot for the binding of coralyne to poly(dT)·poly(dA)·poly(dT) at pH 8 in 2 mM Mg^{2+}. As shown by the line through the points at low r values, the intercept on the r/c axis gives the binding constant, K, and the intercept on the r axis is equal to $1/(2n - 1)$, where n is the number of base triads occluded by the binding of 1 drug molecule. The points at high r values represent stacking to the outside of the helix and are quite characteristic of many intercalators.

Table I: Summary of Binding Constants Derived from Scatchard Plots

DNA	buffer, pH	binding constant ($\times 10^{-6}$ M^{-1})
T·A·T	2 mM Mg^{2+}, pH 8	3.5
T·A	2 mM Mg^{2+}, pH 8	<0.01
T·A	pH 8	5
TC·GA·C⁺T	2 mM Mg^{2+}, pH 5	1.5
TC·GA·C⁺T	pH 5	40
TC·GA	2 mM Mg^{2+}, pH 8	<0.01
TC·GA	pH 8	15

Hippel, 1974). An estimate of n, the number of base triads occluded by the intercalation of one drug molecule, can be made by extrapolating the line at low r to the r axis as shown in Figure 6. This intercept of 0.42 is equal to $1/(2n - 1)$ (McGhee & von Hippel, 1974), which yields an n value of 1.7 base triads suggesting intercalation at every other base triad, i.e., neighbor exclusion. Binding of coralyne to poly(dT)·poly(dA)·poly(dT) cannot be measured in the absence of Mg^{2+} because the triplex is not stable.

The binding of coralyne to the parent duplex poly(dA)·poly(dT) was also studied. In the presence of 2 mM Mg^{2+} the fluorescence quenching was 10-fold lower even at the highest DNA concentrations. Therefore no binding isotherm could be generated, and it was estimated that the binding constant was less than 10^4 M^{-1}. In the absence of 2 mM Mg^{2+} good quenching was acheived, and a binding constant of 5×10^6 M^{-1} was estimated. These results are summarized in Table I.

For poly[d(TC)]·poly[d(GA)] in the presence of 2 mM Mg^{2+} the binding constant was again too low to be measured by fluorescence quenching, and a value of less than 10^4 M^{-1} was estimated. For the triplex poly[d(TC)]·poly[d(GA)]·poly[d(C⁺T)] in the presence of 2 mM Mg^{2+}, significant fluoresence quenching of coralyne was observed and a binding constant of 1.5×10^6 M^{-1} was calculated (see Table I). Thus the binding of coralyne to triplexes in the presence of 2 mM Mg^{2+} is at least 2 orders of magnitude stronger than to the related duplex.

Binding isotherms for the interaction of coralyne with the duplex and the triplex derived from poly[d(TC)]·poly[d(GA)]

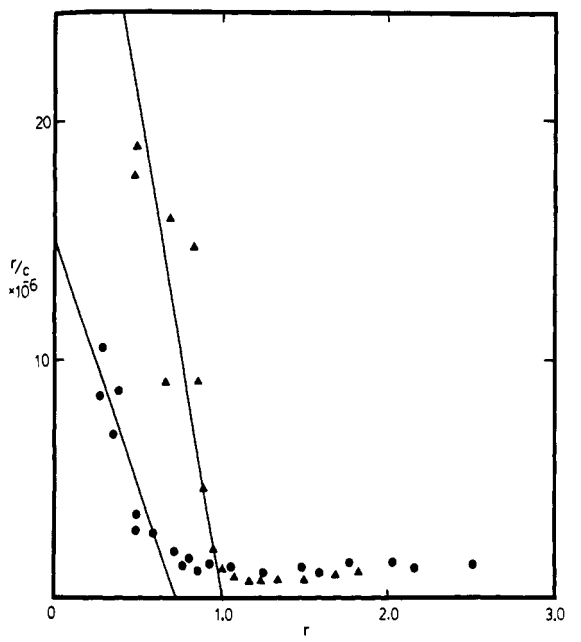

FIGURE 7: Scatchard plots for the binding of coralyne to poly[d(TC)]·poly[d(GA)] at pH 8 (●) and to poly[d(TC)]·poly[d(GA)]·poly[d(C⁺T)] at pH 5 (▲). The lines drawn through the points at low r values give intercepts on the r/c axis equal to the binding constant, K, and intercepts on the r axis equal to $1/(2n - 1)$. Again the L-shaped curves are characteristic of intercalators which also stack to the outside of the helix at high drug concentrations.

in the absence of 2 mM Mg^{2+} are shown in Figure 7. Although the pH values are different (8 and 5, respectively), the ionic strengths are comparable. As expected from the T_m measurements (Figure 4), the binding constant to the triplex is higher than to the duplex. Values of 40×10^6 M^{-1} and 15×10^6 M^{-1} were estimated from the intercept on the r/c axis for the triplex and the duplex, respectively. However, it is difficult to make accurate extrapolations when the binding constant is so high, and the value for the triplex may be a considerable underestimate. The intercepts on the r axis for the duplex and the triplex are 0.7 and 1.0, respectively [compared to only 0.42 for poly(dT)·poly(dA)·poly(dT)], from which n values of 1.2 and 1 can be calculated. Thus in this case the n value for intercalation is closer to 1 base pair or base triad, suggesting that there is no neighbor exclusion for the duplex and the triplex derived from poly[d(TC)]·poly[d(GA)]. As well, the flat part of the isotherm extends to r values greater than 2, showing that stacking interactions are even more pronounced at low ionic strength.

Additional evidence for intercalation was provided by examination of the fluorescence quantum yield as a function of wavelength. Energy transfer from the DNA to the bound drug only occurs efficiently at wavelengths around 260 nm and only if the chromophore is stacked against the base pairs (Le Pecq & Paoletti, 1967; Scaria & Shafer, 1991). The ratio Q_λ/Q_{310} is plotted against wavelength in Figure 8. The increase in relative quantum yield at 260 nm is 5–7-fold for the triplex and the duplex of poly(dA)·poly(dT) [D/P = 0.1] and 2–3-fold for the triplex and the duplex of poly[d(TC)]·poly[d(GA) [D/P = 0.5]. The ratio of coralyne to base pair or base triplet (D/P) was chosen to correspond to the tight binding region of the Scatchard plots (Figures 6 and 7)(since the binding constant is so high, D/P is equivalent to r). The duplex poly[d(TC)]·poly[d(GA)] was also examined at a D/P value of 0.1, and Q_{260}/Q_{310} was calculated to be 3.5 (data not shown). Thus the efficiency of energy transfer is lower for G·C base pairs or C·G·C⁺ base triads than for A·T

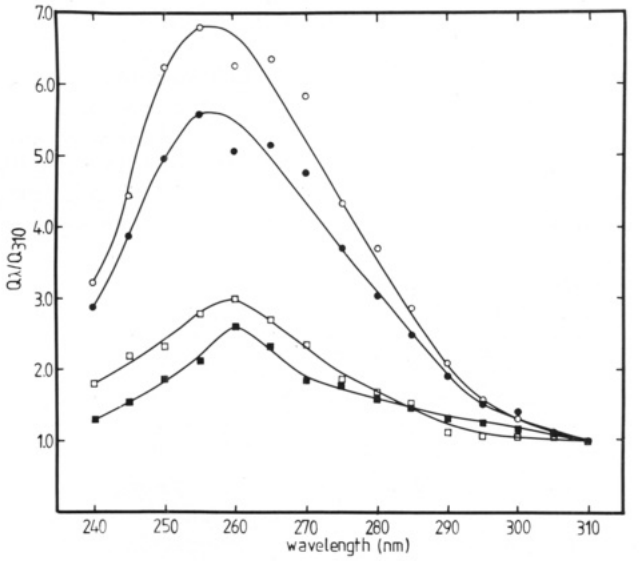

FIGURE 8: The relative fluorescence quantum yield ($Q\lambda/Q_{310}$) of bound coralyne as a function of wavelength: poly(dA)·poly(dT) at pH 8 (○), poly(dT)·poly(dA)·poly(dT) at pH 8 in 2 mM Mg^{2+} (●), poly[d(TC)]·poly[d(GA)] at pH 8 (□), and poly[d(TC)]·poly[d(GA)]·poly[d(C^+T)] at pH 5 (■).

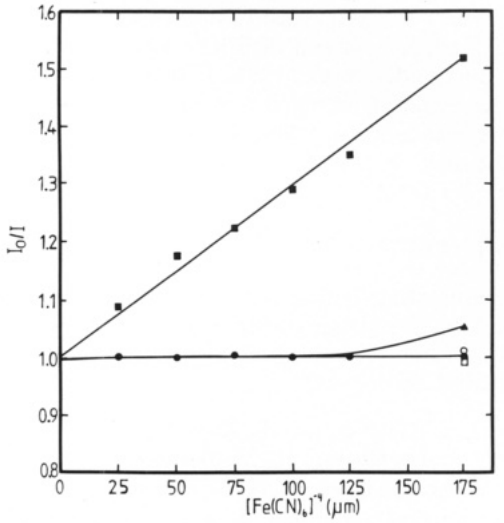

FIGURE 9: Stern–Volmer plots for the $Fe[(CN)_6]^{4-}$ quenching of the fluorescence of 2 μM coralyne at pH 8 or 5, with or without 2 mM Mg^{2+} (■) and of 2 μM coralyne bound to poly(dA)·poly(dT) at pH 8 (○); poly(dT)·poly(dA)·poly(dT) at pH 8 in 2 mM Mg^{2+} (●); poly[d(TC)]·poly[d(GA)] at pH 8 (□); or poly[d(TC)]·poly[d(GA)]·poly[d(C^+T)] at pH 5 (▲).

base pairs or T·A·T base triads; but in all cases an increase in energy transfer provides good evidence for the intercalation of coralyne. Sequence- and structure-dependent differences in the efficiency of energy transfer have also been observed with ethidium (Le Pecq & Paoletti, 1967; Scaria & Shafer, 1991).

In a further series of experiments, the fluorescence of bound coralyne was investigated in the presence of the quenching agent $Fe[(CN)_6]^{4-}$. An anionic quencher should not be able to penetrate the negatively charged helix, and if the coralyne is buried within the helix, little change in fluorescence would be expected (Lakowixz, 1983; Barton et al., 1986). Stern–Volmer plots for the quenching of coralyne by $Fe[(CN)_6]^{4-}$ are shown in Figure 9. Free coralyne is quenched efficiently, while coralyne bound to the duplex or triplex forms of poly(dA)·poly(dT) and poly[d(TC)]·poly[d(GA)] is essentially unaffected even at the highest concentration of quencher.

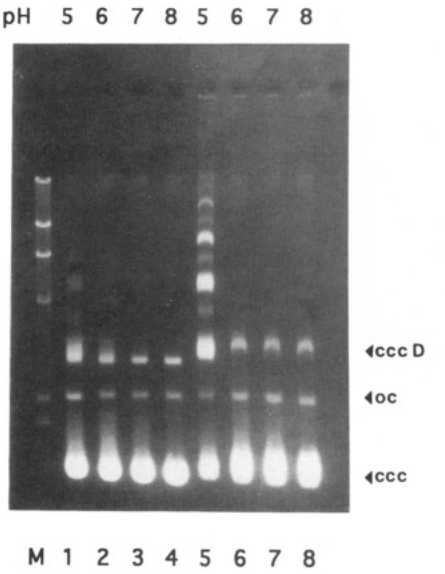

FIGURE 10: Coralyne enhancement of triplex-mediated plasmid dimerization. pTC45 was incubated with poly[d(TC)] at the pH values shown without coralyne (lanes 1–4) or with 38 μM coralyne (lanes 5–8). The plasmid was then loaded onto a 1% agarose gel and electrophoresed at pH 5 for 2 h. Lane M is phage λ DNA cut with *Hin*dIII as a molecular weight marker. The arrowheads point (from top to bottom) to covalently closed circular dimers (cccD), open circles (oc), and covalently closed circular monomers (ccc). In lane 1 dimers and multimers are just visible, but the presence of coralyne enhances their formation considerably (lane 5).

Therefore, at these low *r* values, the coralyne is in a protected environment. This is not compatible with stacking to the outside of the helix.

Since coralyne binds more tightly to triplexes than duplexes, it should promote triplex formation in plasmids containing pyr·pur tracts. When pTC45 is incubated with single-stranded poly[d(TC)] at low pH, multimers of the plasmid are observed on agarose gels because the poly[d(TC)] is linking the pyr·pur tracts together via triplex formation (Lee et al., 1989). Other polypyrimidines such as poly[d(TTC)] do not promote multimer formation because the sequence is not complimentary to that of the insert in the plasmid (Lee et al., 1989). Figure 10, lane 1, shows that under these conditions multimers are beginning to form at pH 5. In the presence of coralyne (lane 5) this process is enhanced, and the majority of the plasmid now migrates as dimers, trimers, and higher multimers. In Figure 11 the concentration dependence is investigated (lanes 2–5); the figure shows as well controls with no poly[d(TC)] (lane 6) and the plasmid without the insert (lane 7). Even 9 μM coralyne is effective in promoting multimerization, and both poly[d(TC)] and the plasmid with the insert are required. In contrast, ethidium did not promote multimerization, and berberinium only showed a small effect at 50 μM, the highest concentration tested (data not shown). These results are therefore consistent with the thermal denaturation profiles.

DISCUSSION

The binding of coralyne to duplex DNA via both intercalation and stacking interactions has been known for some time (Wilson et al., 1976). The results presented here demonstrate that coralyne will also bind to triplexes. In contrast, berberinium is thought to bind only by intercalation (Davidson et al., 1977), and as judged from the T_m values of Figures 3 and 5, this binding is much weaker than coralyne binding to both duplexes and triplexes. Therefore, strong binding requires the large unsaturated ring system of coralyne. Hoechst 33258

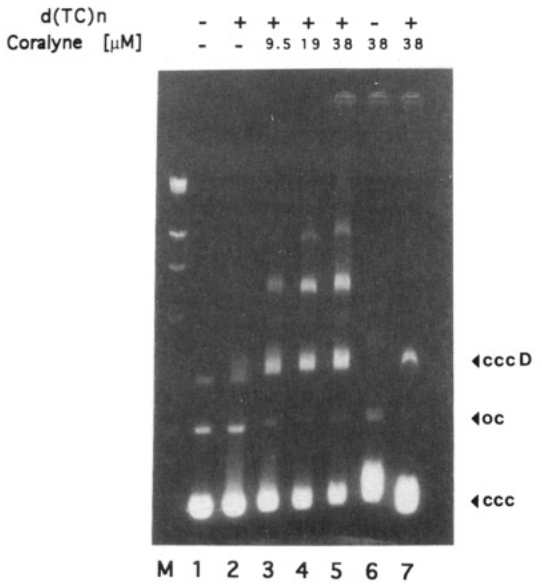

FIGURE 11: Effect of coralyne concentration on triplex-mediated plasmid dimerization. pTC45 (lanes 1–6) was incubated with poly[d(TC)] at pH 5 with increasing concentrations of coralyne (as shown at the top of the gel). The plasmid was then loaded onto a 1% agarose gel and electrophoresed at pH 5 for 2 h. Lane M is phage λ DNA cut with *Hin*dIII as a molecular weight marker. The arrowheads point (from top to bottom) to covalently closed circular dimers (cccD), open circles (oc), and covalently closed circular monomers (ccc). Lane 6 is a control with no poly[d(TC)], and lane 7 is a control with p913 which lacks the pyr·pur insert.

gives larger increases in T_m than berberinium but smaller increases than coralyne (Figure 5). As well, it destabilizes the triplex relative to the duplex in agreement with previous results for netropsin, a related drug which also binds in the minor groove (Park & Breslauer, 1992). However, it is difficult to estimate binding constants from T_m data (McGhee, 1976), and therefore Scatchard analysis was also performed.

In all cases, the plots of r/c against r were highly curved to an extent which could not be due solely to neighbor exclusion. Nor could the curvature be due to sequence preferences because, at least in the case of poly(dT)·poly(dA)·poly(dT), there is only one type of base triad (McGhee & von Hippel, 1974). The shapes of these curves are similar to those of other intercalators, such as the acridines or proflavine, which also stack weakly to the outside of the helix (Gale et al., 1981; Stone & Bradley, 1961). Further evidence for intercalation at low r values was provided by the studies on fluorescence energy transfer (Figure 8) and the lack of quenching by an anionic quencher (Figure 9). Therefore, triplexes can accommodate intercalation and stacking of chromophores in much the same way as duplexes. Ethidium does not stack well to the outside of the helix because of interference from the phenyl group, which is out of the plane of the chromophore (Wakelin & Waring, 1974). Addition of a phenyl group to coralyne might be expected to have a similar effect.

In the presence of 2 mM Mg^{2+}, coralyne has an affinity for triplexes which is at least 2 orders of magnitude greater than for duplexes. Scaria and Shafer (1991) reported binding constants for ethidium of 3×10^3 M^{-1} to poly(dT)·poly(dA) in 100 mM NaCl and 9×10^4 M^{-1} to poly(dT)·poly(dA)·poly(dT) in 5 mM Mg^{2+}. Thus, although the buffers are slightly different, ethidium has a similar preference for the triplex but a somewhat reduced affinity compared to coralyne (Table I). In the absence of 2 mM Mg^{2+}, coralyne also has a higher affinity for the C·G·C$^+$ triplex, but the degree of preference is difficult to determine accurately for binding constants above 10^7 M^{-1}.

Since ethidium will bind to T·A·T triplexes by intercalation, it was not unexpected that coralyne should do the same. However, the strong binding to C·G·C$^+$ triplexes was not anticipated since, like ethidium, coralyne also carries a positive charge on the aromatic ring (Figure 1). One difference between coralyne and ethidium is that in the latter the charge is delocalized onto the two amino groups, one of which by necessity will be placed in close proximity to the protonated cytosine during intercalation (Wakelin & Waring, 1974). This charge repulsion may tend to destabilize the intercalation of ethidium with C·G·C$^+$ triplexes. In coralyne, on the other hand, less charge will be delocalized onto the methoxy groups so that there is no preference for T·A·T over C·G·C$^+$ triplexes. If charge repulsion is indeed a factor in determining the specificity of these drugs, then it may be possible to design new intercalators with preference for C·G·C$^+$ triplexes by including functional groups that might interact favorably with the protonated cytosine. In the long term, multi-intercalators could be prepared with specificity for a long triplex sequence (Wakelin et al., 1979; Markovits et al., 1979).

Because the effective intracellular concentration of Mg^{2+} is probably in the millimolar range, the affinity of coralyne for triplexes would be at least 100-fold higher than for duplex DNA. If triplexes have a significant role *in vivo* (Lee et al., 1987; Burkholder et al., 1991; Ulrich et al., 1992), then coralyne would be expected to have a significant effect on their prevalence and stability. Thus the antitumor activity of coralyne may be due to interactions with triplexes and not with duplex DNA.

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