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Intercalative binding to DNA of antitumour drugs derived from 3-nitro-1,8-naphthalic acid

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ABSTRACT

Two new antitumour drugs, imide derivatives of 3-nitro-1,8-naphthalic acid having different basic side chains linked to the imide nitrogen, have been shown to bind to double-helical DNA by intercalation. At ionic strength 0.01 mol/litre, pH 7, their intrinsic association constants are about 1.45×10^5 M^{-1} and each bound ligand molecule occludes about 3.4 nucleotides of the DNA lattice. They remove and reverse the supercoiling of closed circular duplex PM2 DNA with apparent unwinding angles of 11-12° per bound drug molecule, referred to an assumed unwinding angle of 26° for ethidium. They increase the viscosity of sonicated rod-like DNA fragments, each bound drug molecule producing a calculated increment in length of 2.2 - 2.5 Å. No important differences between the DNA-binding characteristics of the two drugs were detected, though one appears marginally more active than the other in certain biological tests.

INTRODUCTION

Mechanisms of drug binding to DNA attract a good deal of current interest in molecular biology and medicinal chemistry (1-4). An understanding of the precise nature of drug-DNA complexes is considered important for two principal purposes: firstly as a means of explaining the molecular basis of action of chemotherapeutic agents which are believed to owe their selectivity and/or biological effect to interaction(s) at this level; and secondly in monitoring possible toxic, mutagenic, or carcinogenic consequences of exposure to drugs, environmental contaminants, and so on. This paper concerns a study within the former category, i.e. an attempt to elucidate the molecular

basis of action of a new series of antitumour agents.

The drugs in question were synthesised as part of a programme aimed at combining the known antitumour activity of compounds containing a β-nitronaphthalene moiety, as occurs naturally in aristolochic acid (5,6), with other structural entities suspected to be potentially efficacious such as a glutarimide ring and a positively charged tertiary amine side-chain (7,8). Two compounds which originated in this way, code named M-12210 and M-4212 (the latter designated mitonafide by the World Health Organisation) proved particularly active against experimental tumours and several cell lines in vitro (7,8) and have been shown to be selective inhibitors of nucleic acid synthesis in Ehrlich ascites tumour cells (8). Their cytotoxic and biochemical effects are reversible, and thus presumably do not involve covalent binding of either drug (or its metabolites) to a cellular target site, and they have been shown to stabilise double-helical DNA against heat-denaturation (8) — an effect typical of DNA-complexing chemotherapeutic drugs. The structural formulae of these compounds (Figure 1), though lacking the chromophore of three fused six-membered rings characteristic of the most well-established intercalating drugs (1), nevertheless bear some resemblance to the structures of tilorone (9), chloroquine (10-12), quinine (13) and

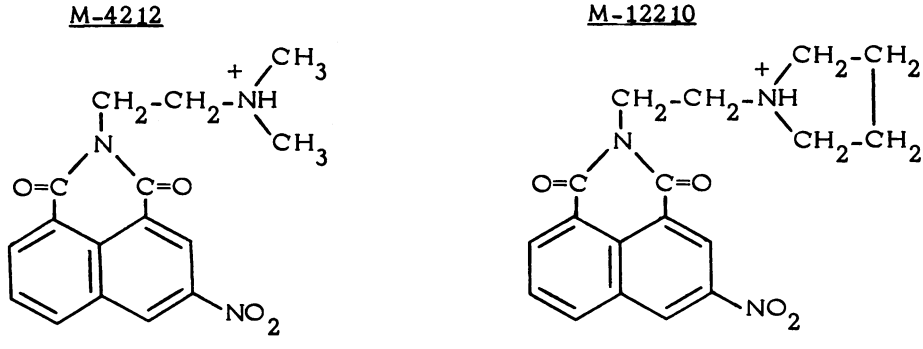

Figure 1. Structural formulae of imide derivatives of 3-nitro-1,8-naphthalic acid

related antimalarial alkaloids (14) which have been reported to be capable of binding to DNA by intercalation. It seemed therefore worth while to quantitate the binding of M-4212 and M-12210 to DNA properly and to investigate the possibility of intercalation using the best-established and most direct techniques presently available, i.e. unwinding of closed circular duplex DNA (11) and enhancement of the viscosity of sonicated rod-like DNA fragments (15,16). By both criteria the drugs proved positive.

MATERIALS AND METHODS

M-4212 and M-12210 were synthesised, purified and characterised as reported elsewhere (7,8). The buffer used throughout (designated 0.01 SHE) contained 2 mM HEPES, 10 μM EDTA, and 9.4 mM NaCl dissolved in glass-distilled water; it was adjusted with NaOH to pH 7.0 at 20°C, giving a resultant ionic strength of 0.01 mol/litre. Drug solutions were always freshly prepared by triturating a few mg of the compound (supplied as the free base) with 0.01 SHE buffer, filtering through two Whatman GF/C glass fibre filters and finally clarifying completely by centrifugation at 5 000 x g for 10 min. The concentration in the supernatant was determined from measurements of the absorbance at 334 nm for M-4212 (E_{334} = 8240 based on a formula weight of 313) and at 335 nm for M-12210 (E_{335} = 8475 based on a formula weight of 339). Stock solutions thus prepared were generally a little less than 1 mM; they were maintained in the dark at room temperature, used to form complexes as promptly as possible, and every effort was made to avoid exposure of experimental solutions to light.

Calf thymus DNA was purchased from Worthington Biochemical Corp. and also from Sigma Chemical Co. as the highly polymerised sodium salt. For spectrophotometric experiments it was dissolved in 0.01 SHE buffer at about 1 mg/ml and lightly blended in a miniature homogenizer at 0°C to reduce the viscosity; subsequently it was

centrifuged at 15 000 rev/min for 15 min to remove all traces of particulate contamination and dialysed overnight at 0-4°C against several changes of buffer. For viscometric experiments the DNA was sonicated to fragments of approx. 0.5 million molecular weight (intrinsic viscosity 3.25 dl/g) as previously described (17). Bacteriophage PM2 DNA, consisting of over 95% closed circular duplex molecules, was prepared by the method of Espejo et al. (18) using strains of virus and host bacteria kindly provided by Dr R. T. Espejo. Samples containing approximately 25% nicked circular molecules (for analytical ultracentrifugation experiments) were generated by repeated freezing and thawing. DNA concentrations were based on an assumed value for $E(P)_{260}$ (the molar extinction coefficient at 260 nm with respect to nucleotides) of 6600.

Absorption spectra were recorded on a Beckman Acta C III double-beam spectrophotometer with 10 mm light path quartz cuvettes. Binding curves were determined by the spectrophotometric procedure of Waring (19) based on the original method of Peacocke and Skerrett (20), using a Gilford 2400-S spectrophotometer with semi-micro quartz cuvettes of 10 mm light path. The wavelength of measurement for both drugs was 335 nm. All solutions contained a constant total drug concentration of 80 μM, while the DNA concentration was varied between 2.3 mM and 79 μM to generate a series of absorbance measurements at different drug/nucleotide ratios. These were converted to values of r (mol. drug bound per mol. of nucleotides) and c (the free drug concentration) and expressed in the form of Scatchard plots (21) which were analysed in terms of equation (10) of McGhee and Von Hippel (22):

$$\frac{r}{c} = K(O)(1 - nr)\left[\frac{1 - nr}{1 - (n - 1)r}\right]^{n - 1}$$

In this equation the intrinsic association constant for binding to an isolated site K(O) is given by the intercept on the ordinate (r/c axis) of a Scatchard plot and n, the parameter of apparent site-size,

represents the number of nucleotides occluded by the binding of a single ligand molecule. Computations were performed as previously described (17).

Sedimentation coefficients were determined by boundary sedimentation at 34 000 rev/min in a Beckman model E analytical ultracentrifuge equipped with u.v. optics and photoelectric scanner according to standard procedures (11). They are presented in the form S_{20}, determined directly at 20°C and uncorrected for viscosity, buoyancy or DNA concentration. Drug-PM2 DNA complexes were formed by method 2 of Waring (11) in which samples may be re-run after addition of fresh increments of drug.

Viscometric experiments were conducted as previously described (17,23) using a simple viscometer having a 10 cm capillary of 0.4 mm bore and a bulb of volume approx. 0.7 ml, thermostated at 20 ± 0.02°C. The flow time for water was 106 sec. The standard DNA concentration employed was 864 μM (O.D.$_{260}$ = 5.7) giving a flow time in the absence of drugs of 117 sec. The data were transformed directly from flow times to values for the relative DNA contour length using the expression (23)

$$\frac{L}{L_o} = \left[\frac{t_C - t_0(V)}{t_D - t_0(V)}\right]^{1/3}$$

where L is the contour length in the presence of drug, L_o is the contour length of the uncomplexed DNA, t_C is the flow time for the complex, t_D is the flow time for pure DNA, and $t_0(V)$ is the flow time for buffer at a given total volume, V, in the viscometer.

RESULTS

Spectrophotometry. In the presence of DNA the long-wavelength absorption peak of M-4212 undergoes bathochromic and hypochromic shifts as commonly seen with DNA-binding drugs (Figure 2). The red-shift is relatively slight, and is better described as a

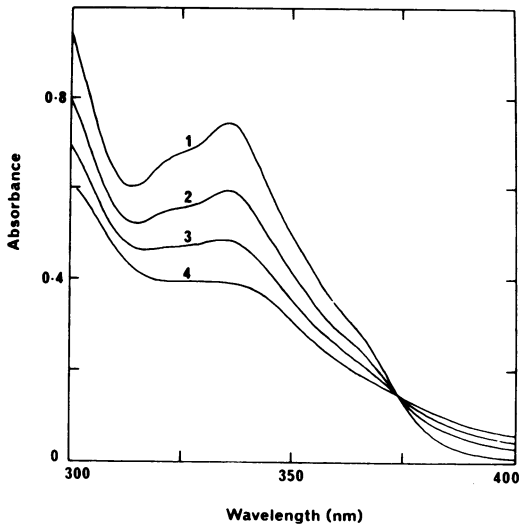

Figure 2. Absorption spectra of M-4212 (89.6 μM in 0.01 SHE buffer) in the absence of DNA (curve 1) or with calf thymus DNA added at a final drug/nucleotide ratio of 0.64 (curve 2), 0.32 (curve 3) or 0.11 (curve 4).

generalised broadening with loss of the shoulders at approx. 320 and 370 nm present in the spectrum of the free drug. The hypochromism, however, is substantial: a 49% reduction occurs in the absorbance at the peak maximum in the presence of excess DNA. More importantly, when the drug/nucleotide ratio is such that only a fraction of the ligand is bound the measured spectra pass clearly through an isosbestic point at 374 nm. This is presumptive evidence that such intermediate spectra represent the sum of contributions from two species of drug — free and bound — and that they may be used to estimate the fraction of the drug in the DNA-bound state by simple proportionality (1,19,20). Similar behaviour was observed with M-12210. For the determination of binding isotherms the peak wavelength of the free drug (335 nm) was chosen, being the wavelength at which the largest change in extinction coefficient occurs on binding to the polymer.

Scatchard plots determined by this means are presented in

Figure 3. The experimental data calculated for the two drugs fall remarkably close together; when analysed to determine the best fit to equation (10) of McGhee and Von Hippel (22) they yielded binding parameters as follows:

M-4212: $K(0) = 1.45 \times 10^5\ M^{-1}$; $\underline{n} = 3.40$ nucleotides
M-12210: $K(0) = 1.44 \times 10^5\ M^{-1}$; $\underline{n} = 3.44$ nucleotides.

These values for the intrinsic binding constant compare reasonably well with estimates for other DNA-binding drugs in the same buffer (24) and the value of $\underline{n}$, the site-size parameter, approaches 4 as predicted for intercalating drugs subject to the phenomenon of neighbour exclusion in their interaction with DNA (22,25).

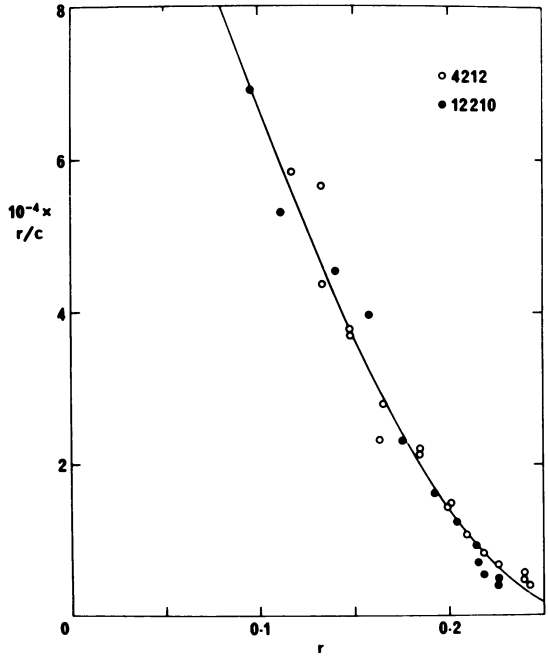

Figure 3. Binding of M-4212 (○) and M-12210 (●) to calf thymus DNA. The line drawn corresponds to equation (10) of McGhee and Von Hippel (22) with $K(0) = 1.45 \times 10^5\ M^{-1}$ and $\underline{n} = 3.4$ nucleotides.

Interaction with closed circular duplex DNA. In Figure 4 it can be seen that both drugs promote removal and reversal of the supercoiling of covalently closed circular DNA in the fashion characteristic of intercalating agents (1,11,12). At the same time they cause the usual small, monotonic decrease in S_{20} of the nicked circular DNA molecules also characteristic of intercalative binding. The equivalence ratios (11) corresponding to exact relaxation of the supercoiling of the closed circles are 0.14 ± 0.03 and 0.13 ± 0.03 for M-4212 and M-12210 respectively. Granted that the data points are (unaccountably) more scattered for M-12210 than for M-4212 it is dubious whether there is any significant difference between the effects of the two drugs, as the overlap in the estimates of error would imply. These numbers can be converted to true equivalence binding ratios and

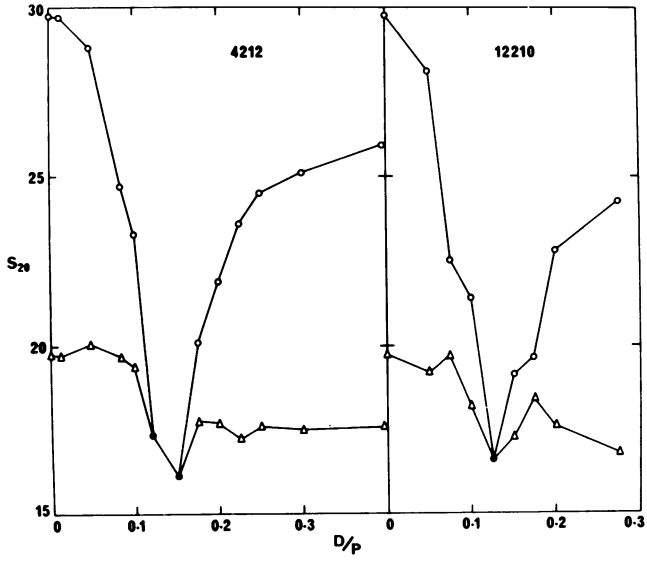

Figure 4. Effects of M-4212 (left) and M-12210 (right) on the sedimentation coefficient of PM2 DNA. The S_{20} of the closed circular duplex molecules is represented by O, that of nicked circular molecules by Δ, and when the two species co-sedimented as a single unresolved boundary the symbol ● is plotted. The abscissa shows the input ratio of added drug to total DNA nucleotides.

employed to estimate relative helix-unwinding angles for the drugs (11) if the binding data shown in Figure 3 are held to be applicable to the interaction with relaxed PM2 DNA. Since calf thymus and PM2 DNAs share the same gross base-composition, 42% guanine plus cytosine (18), this assumption may be considered reasonable. On that basis the values of $\underline{r}$ at equivalence are estimated as 0.118 ± 0.025 for M-4212 and 0.110 ± 0.025 for M-12210. In 0.01 SHE buffer equivalence occurs with ethidium bromide at $\underline{r}$ = 0.051 ± 0.007 (26). Thus binding of M-4212 unwinds the DNA helix by an angle 0.43 ± 0.09 times that caused by binding of ethidium, and the corresponding figure for M-12210 is 0.46 ± 0.10. Taking the unwinding angle of ethidium as 26° (refs. 27,28) leads to estimates of 11.2° ± 2.4° and 12.1° ± 2.8° for the helix-unwinding angle associated with binding of one molecule of M-4212 or of M-12210 respectively.

Interaction with sonicated DNA. The second major diagnostic feature of intercalative drug binding to DNA is extension of the helix, which can be investigated by measuring the effect of binding on the viscosity of sonicated rod-like fragments of double-helical DNA (1,15, 16). For an idealised intercalation process, in which the binding of each ligand molecule extends the helix by 3.4 Å without any other effect, the relative contour length of such fragments is expected to increase according to the relation $L/L_o = 1 + 2\underline{r}$ so that a plot of L/L_o versus $\underline{r}$ would take the form of a straight line having slope = 2. Experimental plots for M-4212 and M-12210 are shown in Figure 5. It is clear that both drugs produce the anticipated enhancement of viscosity, corresponding to an increase in the contour length of the fragments, thus satisfying the qualitative criterion for intercalation. However, the slopes of the experimental plots fall short of the ideal theoretical lines. Additional data for $\underline{r}$ values up to 0.23 were found to deviate even further in the direction of the abscissa. Similar curvature at moderate to high $\underline{r}$ values has been observed in studies with other drugs and variously interpreted as resulting from the onset of "outside" binding, kinking or bending of the helix causing changes in

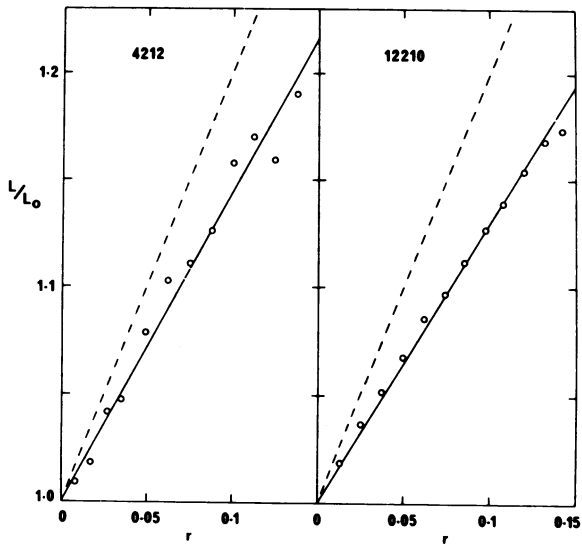

Figure 5. Effects of M-4212 (left) and M-12210 (right) on the relative contour length of sonicated calf thymus DNA fragments. The broken lines correspond to the relation $L/L_o = 1 + 2\underline{r}$. The lines fitted to the experimental points were determined by the method of least squares and constrained to pass through the origin (0,1). Their slopes correspond to $1 + (1.44 \pm 0.07)\underline{r}$ (left; M-4212) and $1 + (1.29 \pm 0.04)\underline{r}$ (right; M-12210).

persistence length, etc. (29-32). Nevertheless, the lines drawn in Figure 5 for $\underline{r}$ values up to 0.15 correspond to length increments of 2.5 Å and 2.2 Å per bound molecule of M-4212 and M-12210 respectively. Again, notwithstanding the low estimates of error implied by the least-squares fitted lines it would be hard to justify attaching any great significance to the difference between the values calculated for the two drugs.

DISCUSSION

The results in all three experimental sections add up to a consistent picture which leaves little room for doubt that these drugs

behave as typical DNA-intercalating agents. The spectral shifts, the magnitudes of the binding constants, the removal and reversal of the supercoiling of circular DNA, and the extension of the helix measured with the sonicated rod-like fragments all follow the classical pattern seen with established intercalating agents such as aminoacridines and ethidium bromide (1). The suggestion from the earlier biological and biochemical studies (7,8) that the action(s) of these drugs in vivo might result from binding to DNA is therefore upheld (but not, of course, proved). Though M-4212 appeared marginally more potent than M-12210 in some biological tests (half as much was required to produce 50% inhibition of growth of HeLa or KB cells in vitro, for example; ref. 7) there is no indication that this is attributable to an enhanced affinity for DNA. Such a finding is not uncommon , however, in studies where the biological activities and DNA-binding characteristics of homologous drugs are compared (see (33) for a recent case in point). Many factors other than the strength of binding to an intracellular target site such as DNA combine to influence the measured potency of a drug in vivo (33,34). It remains possible that M-4212 and M-12210 bind preferentially to different sequences in chromosomal DNA, especially at low binding ratios, and that selective interactions of this type might account for such differences in biological activity as have been detected. Much more detailed studies of DNA-binding, preferably employing more sensitive techniques than the spectrophotometric method used here, would be required to investigate such possibilities.

The strength of interaction between these drugs and DNA, and the character of the intercalated complexes formed, are of some interest in their own right. While it is difficult to draw reliable comparisons between binding constants determined for different drugs under different solvent conditions (and using different theoretical models and definitions) it would appear that the association constant determined here for M-4212 and M-12210 falls not far short of values found for intercalating phenanthridines (24) and is probably a good deal higher than that for

binding of chloroquine to DNA (35). Since the two-ring nitronaphthalene chromophore of the present drugs might reasonably be considered more akin to the quinoline nucleus of chloroquine than to the three-ring phenanthridine system this result may appear surprising.

In respect of their helix-unwinding angles the drugs fall at the low-angle end of the range thus far determined for intercalating agents. In fact, the values of 11.2° and 12.1° are very close to those reported for the antitumour antibiotics daunomycin (11) and adriamycin (12), corrected to the new reference (27,28) of 26° for ethidium, i.e. 11.3° and 11.5° respectively. Again there seems to be a significant difference from chloroquine, which was calculated to have much the same unwinding angle as ethidium (subject to a large margin of error (12)), but the lack of any obvious correlation between the unwinding angles associated with intercalative binding of drugs having differently sized homologous and non-homologous chromophores has been noted before (11,12,24).

Finally, the extension of the DNA helix caused by binding of M-4212 and M-12210, while certainly short of the notional 3.4 Å length increase anticipated for an idealised intercalation model, falls well within the range reported for known intercalating agents, i.e. 1.8 - 4.5 Å (16,30,31,32,36). Using essentially the same viscometric technique values of 2.7 Å were found for proflavine (16) and ethidium (30), 1.8 Å for phenosafranine at low $\underline{r}$ (32), and 4.5 Å for actinomycin D (36). More recently, Hogan et al. (31) using a novel electric dichroism technique have confirmed the values for proflavine and ethidium, revised that for actinomycin to 3.7 Å, and provided an estimate of 2 Å for 9-aminoacridine. It is not yet clear why such a range of values varying from drug to drug, and mostly falling below the idealised estimate, should be observed. Changes in persistence length due to bending or kinking have been considered (29,30,32), but the electric dichroism data suggest that intercalated drugs rarely lie perpendicular to the helix axis and may be tilted by angles up to 28° (31). If so, the intercalation process must provoke substantially more

far-reaching conformational changes in the structure of DNA than mere unwinding and extension along the helix axis, so that theoretical predictions derived from existing simple models may be invalid if not positively misleading.

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