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INTERACTION BETWEEN A 3-NITROBENZOTHIAZOLO (3,2-a) QUINOLINIUM ANTITUMOUR DRUG AND DEOXYRIBONUCLEIC ACID

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Abstract—The interaction of 3-nitrobenzothiazolo (3,2-a) quinolinium (NBQ) perchlorate with DNA was studied by u.v.-visible and fluorescence spectrophotometry as well as by hydrodynamic methods. On binding to DNA, the absorption spectrum underwent bathochromic and hypochromic shifts, and the fluorescence was quenched. Binding parameters, determined from spectrophotometric measurements by Scatchard analysis according to an excluded-site model, indicated a binding constant of $2.4 \times 10^5\ M^{-1}$ for calf thymus DNA at ionic strength 0.01. The interaction was markedly suppressed by increasing the salt concentration. Binding to the GC-rich DNA of *Micrococcus lysodeikticus* was weaker than the binding to calf thymus DNA at ionic strength 0.01. NBQ increased the viscosity of sonicated rod-like DNA fragments, producing a calculated increment in length of 2.4 Å/bound drug molecule. It removed and reversed the supercoiling of closed circular duplex plasmid pBR322 DNA by virtue of a helix-unwinding angle estimated as approximately 13°/bound ligand molecule. We conclude that the binding of NBQ to DNA occurs by a mechanism of intercalation, which probably accounts for its reported antitumor activity.

Several years ago the antitumour activities of fagaronine [1] and nitidine [2] against P388 and L1210 murine leukemias were reported. In an attempt to establish a structure–activity relationship, analogues of both alkaloids have been synthesized [3–5] but none has shown interesting activity. Studies on the mechanism of action of the two alkaloids suggested that they inhibit reverse transcriptase activity by interacting with the A:T template-primer [6, 7].

3-Nitrobenzothiazolo (3,2-a) quinolinium (NBQ; Fig. 1) is a new analogue of these substances that was conceived and synthesized in order to investigate possible structure–activity relationships for antitumour activity [8]. NBQ has produced marked cytotoxic effects against KB and HeLa cells in culture and displayed good antitumour activity against P388 leukemia and Ehrlich ascites tumours *in vivo* [8]. In addition, we have found that NBQ inhibits nucleic acid synthesis strongly in both KB and Ehrlich ascites tumour cells *in vitro* (manuscript in preparation).

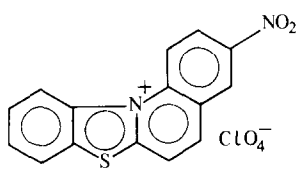

Fig. 1. Structural formula of 3-nitrobenzothiazolo (3,2-a) quinolinium perchlorate.

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NBQ has been shown to stabilize calf thymus DNA against heat denaturation [8].

On the basis of these indications, we have tentatively identified NBQ as a new member of the class of anti-cancer drugs whose activity can be attributed to binding to DNA so as to interfere with its function as a template for nucleic acid synthesis in susceptible cells [9]. This paper reports the results of experiments designed to investigate that hypothesis, using a variety of physicochemical techniques to study the interaction of NBQ with DNA.

MATERIALS AND METHODS

Materials. Calf thymus DNA type 1 (42% GC) and *Micrococcus lysodeikticus* DNA (72% GC) were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. DNA solutions were prepared by dissolving the solid material, normally at 1–2 mg/ml, in SHE buffer and dialyzing overnight at 4° against the same buffer; SHE buffer [10] contains 2 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 10 μM EDTA and an appropriate concentration of NaCl to give ionic strengths (I) of 0.01, 0.1 and 1.0. Buffer solutions were adjusted to pH 7.0 with NaOH. Plasmid pBR322 DNA was a gift from Drs. F. Malpartida and A. Jiménez. It contained approximately 60% closed circular duplex monomer molecules (M_r 2.81×10^6) and 40% nicked circles; a tiny proportion of linear molecules was just visible in a heavily-loaded electrophoresis gel but completely undetectable in the analytical ultracentrifuge. DNA concentrations were determined spectro-

photometrically at 260 nm using a molar extinction coefficient of 6600 M^{-1} cm^{-1}.

3-Nitrobenzothiazolo (3,2-a) quniolinium perchlorate was synthesized by Dr. O. Cox at the Department of Chemistry of the University of Puerto Rico. Drug solutions were freshly prepared by dissolving a few milligrams in SHE buffer. The concentrations of drug solutions were determined spectrophotometrically at 273 nm using a molar extinction coefficient of 26,083 M^{-1} cm^{-1}.

Spectroscopy. Ultraviolet-visible absorption spectra were determined in a Perkin–Elmer model 554 UV-VIS recording spectrophotometer using 10 mm light-path semimicro quartz cuvettes. The parameters of interaction between NBQ and DNA were determined spectrophotometrically using a Beckman 25 double beam spectrophotometer equipped with 10 mm light path quartz cuvettes as previously described [11, 12]. Aliquots of a concentrated DNA solution (5–12 mM) were added, with the aid of a microliter syringe, to a cuvette filled with an NBQ solution (16–25 μM) and thoroughly mixed. Extreme care was taken to ensure that optical reference solutions were prepared in an identical manner.

The binding data were expressed in the form of a Scatchard plot [13]. The variables of r (moles of ligand bound/mole of nucleotides) and c (the molar concentration of free drug) were calculated from the absorption measurements according to the method of Peacocke and Skerrett [14]. $K(O)$, the intrinsic association constant for an isolated site, and n, the number of nucleotides occluded by the binding of a single drug molecule, were computed by an iterative process designed to satisfy Equation 10 of McGhee and Von Hippel [15].

Fluorescence spectra were determined in a Perkin–Elmer MPF-44 fluorescence spectrophotometer. Emission spectra of the drug alone and in the presence of increasing concentrations of DNA were measured according to methods previously described [16, 17].

Viscometry. Viscosity measurements were made according to published procedures [16, 18] using a capillary viscometer (Viscomatic Fica MgW) with a thermostatted bath D40S at 20°. The flow time for water was 71.3 sec. For the viscosity experiments samples of calf thymus DNA were sonicated [18] to fragments having an estimated molecular weight of approximately 500,000 [19, 20].

Analytical ultracentrifugation. Sedimentation coefficients were determined by boundary sedimentation at 36,000 rpm in a Beckman model E analytical ultracentrifuge equipped with u.v. optics and a photoelectric scanner according to standard procedures [12]. They are presented in the form S_{20}, determined directly at 20° and uncorrected for viscosity, buoyancy or DNA concentration. Complexes between NBQ and DNA were prepared by direct mixing of stock solutions (method 1 of Waring [12]) and left to stand for at least 1 hr before loading into the rotor. Each sample was run only once. A least-squares program was used to compute S_{20} values and to verify statistical goodness-of-fit to the measured data. Indicated errors did not exceed 1% of the calculated value.

RESULTS

The ultraviolet-visible absorption spectrum of NBQ was characterised by two maxima at 273 and 363 nm. The addition of increasingly higher concentrations of DNA led to bathochromic and hypochromic changes, as seen in Fig. 2, i.e. the interaction of NBQ with DNA resulted in a strong decrease of the absorption intensity at both peaks, accompanied by a shift towards higher wavelengths. Fine structure evident in the spectrum of the free drug disappeared on binding to DNA. A 38% reduction in absorption was observed at the 363 nm peak maximum in the presence of an excess of DNA at a molar ratio of DNA nucleotide:NBQ (P/D) equal to 32. An isosbestic point was formed at 387 nm, and no further variations were observed at P/D ratios higher than 32.

Figure 3 shows a typical fluorescence spectrum of NBQ with a peak of emission centred around 584 nm when excited at 370 nm. In the presence of increasing concentrations of DNA, the fluorescence was substantially quenched.

Binding of NBQ to calf thymus DNA was measured spectrophotometrically, resulting in the Scatchard plot [13] shown in Fig. 4. Calculations of values of r and r/c were performed using the absorbancies measured at 363 and 340 nm. These two wavelengths were chosen because of the large absorbance change and to verify that the fraction of NBQ bound to DNA was independent of the wavelength of measurement. The theoretical curve was drawn according to the excluded-site model developed by Crothers [21] and McGhee and Von Hippel [15]

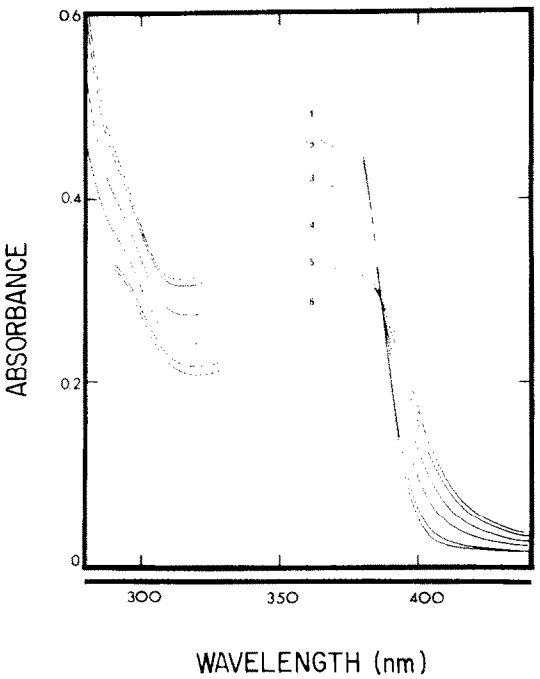

Fig. 2. Absorption spectra of 29.2 μM NBQ in SHE buffer in the absence of DNA (curve 1) or with calf thymus DNA added to yield a nucleotide: drug ratio of 1 (curve 2), 4 (curve 3), 8 (curve 4), 16 (curve 5), or 32 (curve 6).

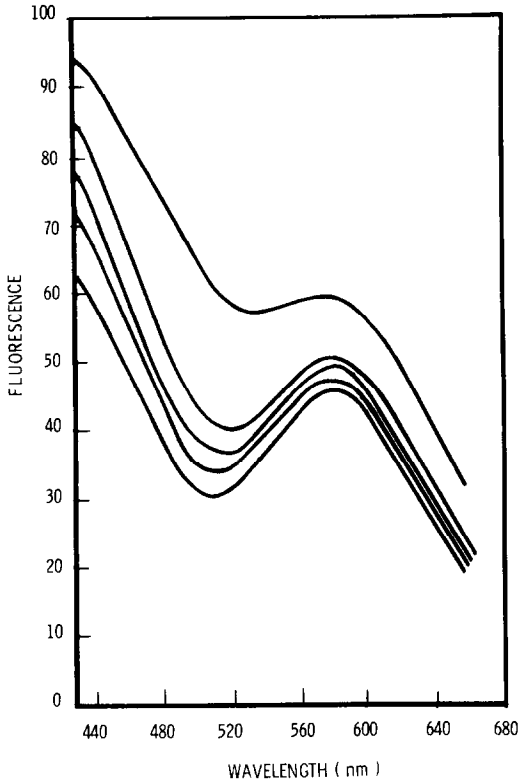

Fig. 3. Fluorescence emission spectra of 27.9 μM NBQ in SHE buffer alone (top curve) or in the presence of calf thymus DNA at nucleotide:drug ratios as follows, reading from the top down: 1.00, 1.79, 4.87 and 5.44 (bottom curve).

giving a $K(O)$ of 2.4×10^5 M^{-1} and the site-size parameter $n = 6.1$ nucleotides.

Previous studies [11, 14] have shown that the ionic strength of the medium often affects the binding of a ligand to DNA. We studied the interaction between NBQ and calf thymus DNA under the same buffer conditions with the ionic strength increased to 0.1 and 1.0 by addition of NaCl. Binding of NBQ was strongly dependent on ionic strength, as can be seen in Fig. 5. The binding parameters at an ionic strength of 0.10 revealed that the association constant was reduced 40-fold to 6.9×10^3 M^{-1} and that the frequency of binding sites was changed. When the ionic strength was raised 100-fold to $I = 1.0$, only a meagre reduction in the absorbance of NBQ was observed even when the molar ratio of DNA:NBQ was increased above 30, demonstrating that the ability of the drug to form a complex with DNA was grossly impaired.

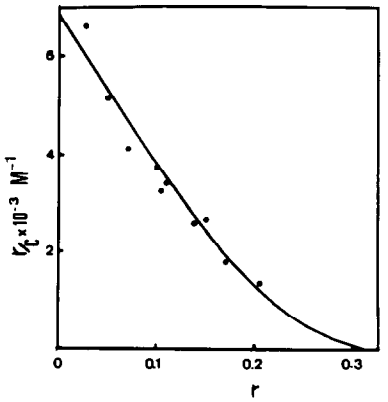

Fig. 5. Scatchard plot of the binding of NBQ to calf thymus DNA at ionic strength $I = 0.10$. The total drug concentration was 26.3 μM $K(O) = 6.9 \times 10^3$ M^{-1} and $n = 3.0$ nucleotides.

Since a number of drugs have been reported to exhibit sequence-specificity in binding to DNA [9] the interaction of NBQ with *M. lysodeikticus* DNA was also studied (Fig. 6). The binding isotherm yielded $K(O) = 1.5 \times 10^5$ M^{-1} and $n = 4.0$ nucleotides. It can be seen that both parameters differ from those measured for calf thymus DNA, revealing that this DNA, which is characterised by a higher content of G plus C (72%), bound NBQ less efficiently.

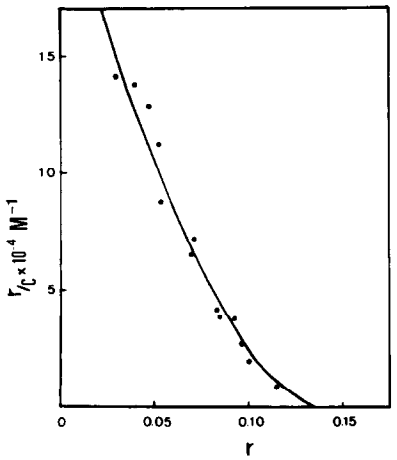

Fig. 4. Scatchard plot of the binding of NBQ to calf thymus DNA in SHE buffer. The total drug concentration was 25 μM. The line drawn corresponds to Equation 10 of McGhee and Von Hippel [15] with $K(O) = 2.4 \times 10^5$ M^{-1} and $n = 6.1$ nucleotides.

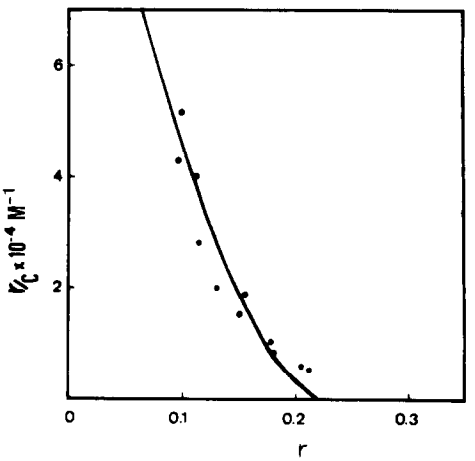

Fig. 6. Scatchard plot of the binding of NBQ to *M. lysodeikticus* DNA in SHE buffer. The total drug concentration was 43.8 μM. $K(O) = 1.5 \times 10^5$ M^{-1} and $n = 4.0$ nucleotides.

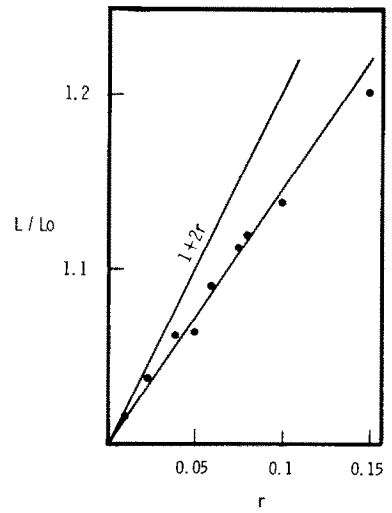

Fig. 7. Effect of NBQ on the relative contour length of sonicated calf thymus DNA fragments. L represents the contour length of fragments with drug bound at the indicated binding ratio r; L_o is the contour length of control drug-free DNA. The line labelled $1 + 2r$ represents the theoretical relation for an idealised intercalation process. The line fitted to the experimental points was computed by the method of least-squares and constrained to pass through the origin (0,1). Its slope corresponds to the relation $L/L_o = 1 + (1.43 \pm 0.05)r$. Each point represents the mean of three experiments.

An increase in viscosity of native DNA is regarded as a diagnostic feature of an intercalation process [9, 19]. We have measured the viscosity changes in short, rodlike DNA fragments. The relative length increase (L/L_o) of the complex formed between NBQ and DNA is shown in Fig. 7. It is evident that binding of NBQ increased the viscosity of DNA, corresponding to an increase in the contour length of the DNA fragments. The measured slope of the plot, 1.43 ± 0.05, falls within 72% of the slope of a theoretical curve for an idealised intercalation process ($1 + 2r$). On this basis we calculate that intercalation of one drug molecule provoked an increase of 2.4 Å in the contour length of DNA. Since the size of these sonicated fragments was significantly greater than the persistence length, the estimated 2.4 Å lengthening is probably best regarded as a lower limit.

The observation of a viscosity enhancement on binding to DNA is insufficient evidence by itself to establish a mechanism of intercalation [9]. Combined with evidence of helix unwinding from the circular DNA-binding test [12] the conclusion is much more secure. In Fig. 8 it can be seen that binding of NBQ to closed circular duplex DNA did indeed remove and reverse the negative supercoiling, consistent with helix unwinding as expected. Also visible is the usual small, monotonic decrease in sedimentation coefficient of the nicked circular DNA molecules, again characteristic of intercalative binding. The equivalence binding ratio [12], corresponding to exact relaxation of the supercoiling of the closed circles, occurred at 0.105 ± 0.025 drug molecules bound per nucleotide, assuming that the binding constant measured for calf thymus DNA is approximately applicable to pBR322 DNA. With that proviso we estimate that each bound NBQ molecule unwound the DNA helix by about 13°, roughly half the value accepted for ethidium bromide the best-characterised classical intercalator [9].

DISCUSSION

The results presented in this paper provide coherent evidence that association of NBQ with DNA can be explained by a mechanism of intercalation. In many ways NBQ behaved as an ideal intercalating drug [9]: on binding to DNA we saw the typical bathochromic and hypochromic shifts in its absorption spectrum; its fluorescence was quenched; the binding constants determined at $I = 0.01$ were within the range 10^5 to 10^6 M^{-1}, commonly reported for such drugs; the interaction was suppressed by raising the salt concentration; and hydrodynamic changes consistent with extension and unwinding of the DNA helix were clearly seen.

The importance of electrostatic forces as regards formation of the complex is emphasised by the results obtained when the ionic strength of the medium was increased. Adding NaCl up to $I = 0.1$ dramatically lowered the intrinsic association constant and simultaneously reduced the number of nucleotides occluded. Furthermore, when a 100-fold increase in the ionic strength was effected, the binding was so weakened as to verge on the undetectable. These results could be explained by competition of the Na^+ ions for the charged phosphate groups of the DNA, consequently limiting the possibility of charge neutralization with the NBQ molecules. The dependence of the binding constant on the ionic strength of the environment seems rather more marked than observed with other intercalating drugs, which may indicate a higher dependence of NBQ binding on electrostatic forces for the formation of the complex with DNA. We considered the possibility that the marked ionic strength-dependence of binding might be artifactual, perhaps arising from aggregation of drug molecules at higher salt concentrations, but found no evidence for such a phenomenon. Moreover, no deviations from Beer's law were noted over the entire range of concentrations employed ($\leq 1.3 \times 10^{-4}$ M).

Binding to DNA showed some dependence on the nucleotide content and/or sequence, as evidenced by the reduction in the association constant for *M. lysodeikticus* DNA which has a higher GC content than calf thymus DNA. These findings would be in accordance with the results previously reported [6, 7] for the natural alkaloids fagaronine and nitidine, which ostensibly display a preference for AT base pairs.

The most solid evidence for the intercalation mode of binding comes from the viscometric and sedimentation experiments. The calculated value for the DNA helix extension of 2.4 Å, although lower than the idealised value expected for the intercalation model of 3.4 Å, lies well within the range of values reported for other intercalating agents, i.e. between 1.8 and 4.5 Å [9, 22]. Likewise, the estimated helix-unwinding angle of 13° lies at the lower end of

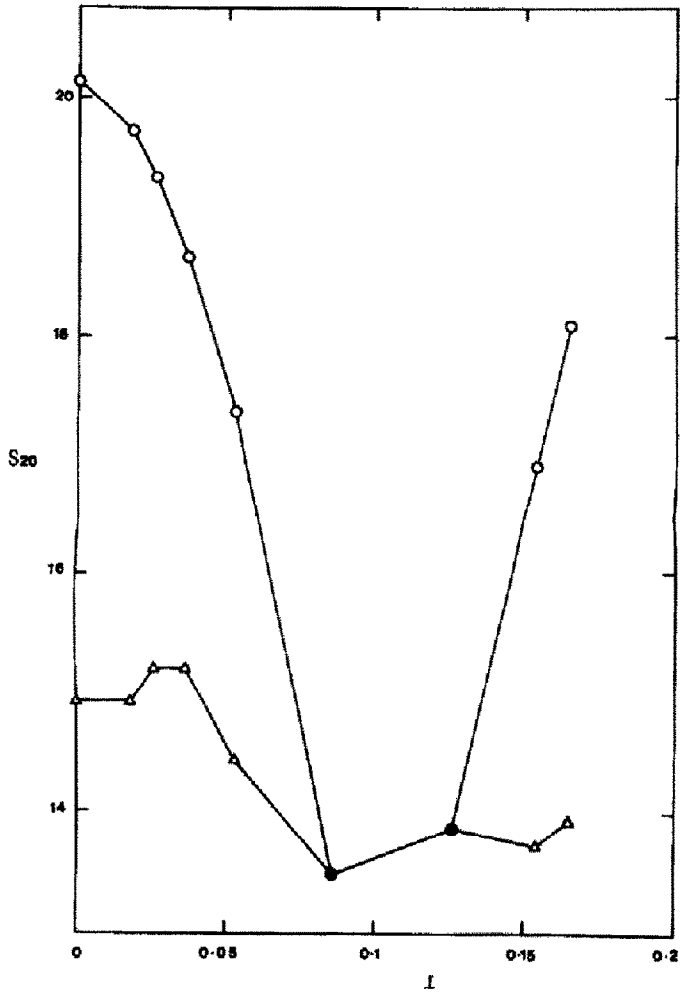

Fig. 8. Effect of NBQ on the sedimentation coefficient of pBR322 DNA. The S_{20} of the closed circular duplex molecules is represented by ○, that of nicked circular molecules by △, and when the two species co-sedimented as a single unresolved boundary the symbol ● is plotted. The abscissa shows the binding ratio (drug molecules bound/DNA nucleotide) computed from the added drug:DNA molar ratio, assuming the binding constant for calf thymus DNA calculated from the data in Fig. 4.

the scale of values determined for established intercalators [9], but in that respect NBQ is in good company: its unwinding angle is barely different from that which characterises the important antitumour anthracycline antibiotics daunomycin and adriamycin [9, 23, 24]. Further experiments to define the range of antitumour efficacy and biochemical basis of action of NBQ are in progress. The results of these studies, and of experiments with analogues containing replacement aromatic heteroatoms, will form the subject of future communications.

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