NB-506, an indolocarbazole topoisomerase I inhibitor, binds preferentially to triplex DNA

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Abstract A novel competition dialysis method was used to study the structural selectivity of the nucleic acid binding of NB-506, a promising indolocarbazole anticancer agent. A pronounced preference for NB-506 binding to the DNA triplex poly [dA]:(poly[dT])$_2$ was observed among potential binding to 12 different nucleic acid structures and sequences. Structures included in the assay ranged from single-stranded DNA, through a variety of right-handed DNA duplexes, to multistranded triplex and tetraplex forms. RNA and left-handed Z DNA were also included in the assay. The preferential binding to triplex was confirmed by UV melting experiments. The novel and unexpected structural selectivity shown by NB-506 may arise from a complementary shape between its extended aromatic ring system and the planar triplex stack.

Key words: DNA; RNA; Intercalation; Dialysis; Anticancer drug; Triplex DNA

1. Introduction

DNA intercalating agents have a long history in the treatment of human disease and of cancer in particular [1]. For over three decades the intercalating anthracycline antibiotics doxorubicin and daunorubicin have been the lead clinical drugs of choice against a number of neoplastic diseases. Actinomycin, another intercalating antibiotic discovered in the early 1950s, continues to be used extensively worldwide as an antitumor agent. Mitoxantrone, which is particularly useful for the treatment of breast cancers, is also a typical DNA-intercalating agent. Other potent anticancer agents that intercalate into DNA include amsacrine, bisantrene, amonafide, nogalamycin, and porphyrins [2].

In recent years, novel DNA-intercalating agents endowed with potent anticancer activities have been discovered. One of the most promising series is the indolocarbazoles, typified by the antitumor antibiotic BE-13793C produced by a strain of *actinomycetes* [3] and its glycosylated analog rebeccamycin isolated from a culture of *Saccharotrix aerocolonigenes* [4]. Water-soluble derivatives of these two antibiotics have been developed recently. The most active compound in the series is the compound 6-*N*-formylamino-12,13-dihydro-1,11-dihydroxy-13-(-D-glucopyranosyl)-5*H*-indolo[2,3-*a*]pyrrolo-[3,4-*c*]-carbazole-5,7-(6*H*)-dione (NB-506, Fig. 1) [5]. The carbohydrate moiety of NB-506 and related derivatives not only significantly increases the water-solubility of the drugs, but also enhances their interaction with double stranded DNA [6,7]. NB-506 has shown remarkable efficacy against a variety of human tumor xenografts, including lung and colon cancers, and metastatic cells [5,8–10]. Recently the drug has entered clinical trials. The results of the first phase I study are promising and exciting clinical developments for this compound are expected in the near future [11].

The exceptional antitumor activity of NB-506 is attributed to the capacity of the drug to intercalate into DNA and to inhibit topoisomerase I [12]. NB-506 converts topoisomerase I into a cell poison by trapping covalent DNA-topoisomerase I complexes, thereby enhancing the formation of persistent DNA breaks responsible for cell death. In that respect, the mechanism of action of NB-506 closely resembles that of another series of antitumor agents recently introduced in the clinic, the camptothecins [13]. Other aspects of the mechanism of action of NB-506, including its interaction with nucleic acids, have not been characterized in any detail. Recently we showed that NB-506 intercalates preferentially into GC-rich sequences of B-DNA [14]. But apart from B-DNA, it is not known if NB-506 can interact with other nucleic acids structures such as single strand polymers, RNA, Z DNA, DNA triple helices, and tetraplexes. This issue is addressed here.

DNA is a highly adaptable molecule that can exist within cells in a variety of conformations and structures not restricted to the canonical Watson–Crick B-form. DNA mainly exists as an antiparallel double-stranded helix in the cell, but it can form triplex and quadruplex structures into which an intercalating drug like NB-506 may bind. In the past few years, a variety of triplex-selective intercalators have been designed (e.g. BePI and derivatives, naphthyl quinolines, anthraquinone) [15,16] and more recently a few intercalating agents capable of interacting with G-quadruplexes have been reported [17–19]. NB-506, which possesses a large six-ring planar chromophore, could stack over base triplets and tetrads, in addition to interacting with duplex DNA.

In most drug–DNA binding studies, the interaction of the test drug with only one or two DNA substrates are studied, due to the labor involved in conducting proper equilibrium binding studies. To better understand how small molecules can interact selectively with different nucleic acids structures, ideally one would like to compare the binding of the drug to a variety of nucleic acid structures simultaneously. A suitable method for this purpose was recently devised, a competition dialysis method that provides a rigorous, thermodynamically sound indication of structural selectivity [20].

The present competition dialysis method evolved from a

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technique first used by Mueller and Crothers to explore the base specificity of DNA intercalation reactions [21,22]. In the new application [20], we have devised a suitable buffer in which a variety of DNA and RNA structures are stable. In the competition dialysis experiment, equal volumes of these DNA samples (at identical concentrations) are dialyzed against a common dialysate solution containing the ligand under study. After equilibrium dialysis is attained (usually in 24 h or less), the amount of ligand bound to each DNA is measured by absorbance. Since all of the DNA samples are in equilibrium with the same free ligand concentration, the amount of bound ligand is directly proportional to the association constant for ligand binding to a particular structure. Comparison of the amount of bound ligand to each DNA sample provides a rapid, thermodynamically rigorous indication of the structural selectivity of the ligand under study. The advantages of the new method are many, including its speed, its sound thermodynamic basis, and its relative ease.

The competition dialysis method has been successfully applied to determine the structural specificity of NB-506. This novel approach has allowed us to monitor simultaneously the binding of the indolocarbazole derivative to a variety of DNA substrates ranging from single stranded synthetic polymers to much more complex three- and four-stranded structures. Unexpectedly, we discovered that NB-506 exhibits a remarkable preference for the poly dA:(poly dT)$_2$ triple helical polymer. The triplex-stabilizing effect of NB-506 was confirmed by thermal denaturation studies. So far as we know, this is the first example of an uncharged intercalating agent shown to stabilize triplex DNA.

2. Materials and methods

2.1. Drug

Dr. Tomoko Yoshinari (Banyu Pharmaceuticals, Japan) kindly provided a sample of NB-506. The chemical synthesis of the drug has been reported [23]. The drug was first dissolved in dimethylsulfoxide (DMSO) at 10 mM and then further diluted with water. The stock solution was kept at −20°C and freshly diluted to the desired concentration immediately prior to use.

2.2. Nucleic acids

DNA samples from *Clostridium perfringens* (Lot 86H4010), *Micrococcus lysodeikticus* (Lot 108H4017) and calf thymus (Lot 95H9526) were purchased from Sigma (St. Louis, MO, USA) and were sonicated, phenol-extracted and purified as previously described [24]. Poly dA (Lot 7067836021), poly dT (Lot 8017834021), poly dA:poly dT (Lot 8097860021), poly (dAdT) (Lot 8067870021) and poly (dGdC) (Lot 8107910021) were purchased from Pharmacia Biotech (Piscataway, NJ, USA). Poly A:poly U (Lot 10H4005) was purchased from Sigma (St. Louis, MO, USA). Synthetic polynucleotides were used without further purification. Solutions containing the poly dA:(poly dT)$_2$ triplex were prepared by mixing poly dA:poly dT with poly dT in a 1:1 mol ratio, heating to 90°C, and slowly cooling to room temperature. Tetraplex DNA ([5′ $T_2G_{20}T_2$]$_4$) was prepared by heating [5′ $T_2G_{20}T_2$] (from Research Genetics, SW Huntsville, AL, USA) to 90°C for 2 min, slowly cooling to room temperature, and then equilibrating for 48 h at 4°C before use. Left-handed, Z DNA was prepared by bromination of poly (dGdC) as previously described [25].

2.3. Competition dialysis assay

Full details of the competition dialysis assay may be found in [20]. A brief description follows. For each competition dialysis assay, 200 ml of the dialysate solution containing 1 μM ligand concentration was placed into a beaker. A volume of 0.5 ml of each of the nucleic acid samples was pipetted into a separate 0.5 ml Spectro/Por DispoDialyzer® unit (Spectrum, Laguna Hills, CA, USA). All nucleic acid samples were at an identical concentration of 75 μM. (Nucleic acid concentration is expressed in terms of the monomeric unit for each polymer, i.e. nucleotides for single strands, base pairs for duplex forms, triplets for the triplex and tetrads for the tetraplex). All 12 dialysis units were then placed in the beaker containing the dialysate solution. The beaker was covered with parafilm, wrapped in foil, and allowed to equilibrate with continuous stirring for 24 h at room temperature (20–22°C). At the end of the equilibration period, DNA samples were carefully removed to microfuge tubes, and were made to a final concentration of 1% (w/v) sodium dodecyl sulfate (SDS) by the addition of appropriate volumes of a 10% (w/v) stock solution. The total concentration of NB-506 (C_t) within each dialysis unit was then determined spectrophotometrically using an extinction coefficient of 14 000 M^{-1} cm^{-1} (at 330 nm). An appropriate correction for the slight dilution of the sample resulting from the addition of the stock SDS solution was made. The free ligand concentration (C_f) was determined spectrophotometrically using an aliquot of the dialysate solution, although its concentration usually did not vary appreciable from the initial 1 μM concentration. The amount of bound drug was determined by difference, $C_b = C_t - C_f$.

2.4. UV melting studies

UV DNA melting curves were determined using a Cary 3E UV/Visible spectrophotometer (Varian, Palo Alto, CA, USA), equipped with a thermoelectric temperature controller. Solutions of DNA (final concentration 2.0×10^{-5} M) were prepared by direct mixing with aliquots from ligand stock solution, followed by incubation for 12 h at 24°C to ensure equilibration. Samples were heated at a rate of 1°C min^{-1}, while continuously monitoring the absorbance at 260 nm. Primary data were transferred to the graphics program Origin (Microcal, Northampton, MA, USA) for plotting and analysis.

3. Results and discussion

Fig. 1 shows the results obtained from the competition dialysis procedure. Data are presented as a bar graph, in which the amount of NB-506 bound to each nucleic acid sample is plotted. The striking result to emerge is the strong preference of NB-506 for the triplex DNA sample included in the assay, poly dA:(poly dT)$_2$. Under the ionic conditions of this assay, NB-506 binding to triplex is at least four-fold greater than to any other nucleic acid conformation, with duplex *M. lysodeikticus* DNA representing the next most preferred form. The amount bound plotted in Fig. 1 is directly proportional to the binding constants for the interaction of NB-506 with the various nucleic acid structures studied. In the competition dialysis experiment, all nucleic acid structures are maintained at identical concentrations. Since all nucleic acid structures in the assay are in equilibrium with the same free ligand concentration, the amount bound depends only on the binding constant. We previously measured the binding constant (K) for the interaction of NB-506 with calf thymus DNA by fluorescence titration under the same buffer conditions as used in the competition dialysis experiment, and found $K = 1.5 \times 10^4$ M^{-1} [14]. From the data obtained in Fig. 1, $K_{app} = 0.8 \times 10^4$ M^{-1} may be calculated for the binding of NB-506 to calf thymus DNA, a value in reasonable agreement with the more complete titration data.

The pronounced preference of NB-506 for triplex DNA was confirmed by the UV melting studies shown in Fig. 2. In the absence of NB-506, melting of poly dA:(poly dT)$_2$ is biphasic. The triplex → duplex+poly dT transition is centered at $T_m = 42.5$°C, with melting of the remaining poly dA:poly dT duplex at $T_m = 75.2$°C. Upon addition of increasing molar ratios of NB-506, the T_m for the triplex transition is systematically increased up to a maximal shift of over 30°C. In marked contrast, melting of the duplex is largely unaffected

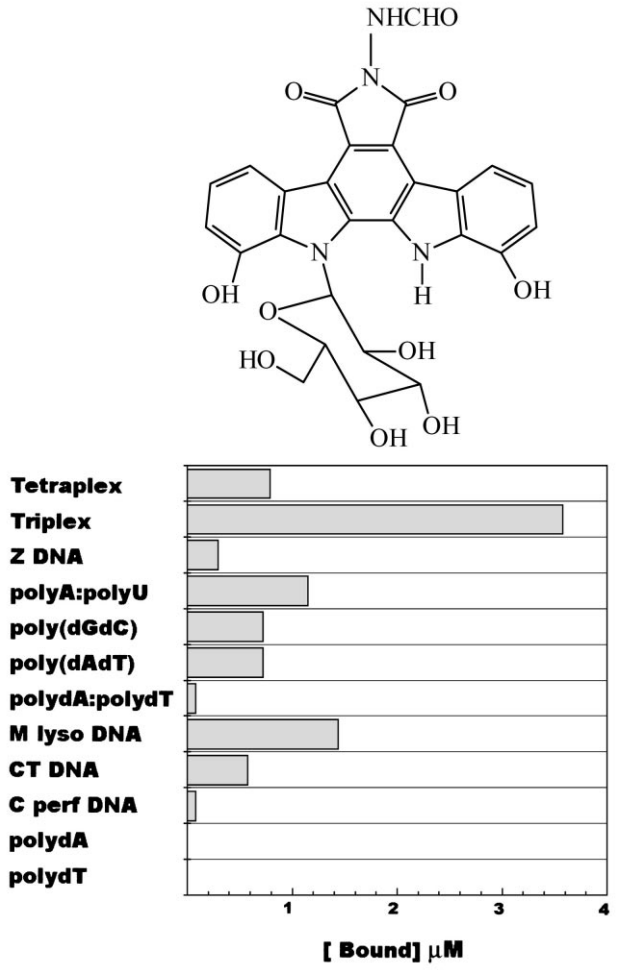

Fig. 1. Top: Structure of NB-506. Bottom: Results of competition dialysis experiment using NB-506. The amount of NB-506 bound to the various nucleic acid structures listed in the table is shown as a bar graph. The free ligand concentration in the experiment was 1 μM, and the total concentration of each nucleic acid conformational form was 75 μM (in terms of the monomeric unit of each polymer).

by addition of NB-506. Note in Fig. 1 that NB-506 binds very poorly to poly dA:poly dT. The melting studies are fully consistent with the results obtained with the competition dialysis method. Poor NB-506 binding to poly dA:poly dT is also consistent with the results of recent DNase I footprinting studies [14].

A variety of compounds has now been discovered that bind with some preference to triplex DNA [26–28]. Competition dialysis results were recently presented for some triplex selective compounds [20]. The relative preference for triplex exhibited by NB-506 compares favorably with that shown by the compounds BePI and coralyne, and its affect on triplex melting is similar. The compound berberine was discovered to be more triplex selective that either BePI or coralyne in the competition dialysis procedure. Both NB-506 and berberine dramatically stabilize triplex DNA against thermal denaturation, but have essentially no effect on the melting of duplex. Comparatively, the overall binding of NB-506 to all forms of nucleic acid was observed to be weaker than was observed for BePI, coralyne and berberine, consistent with its comparatively smaller duplex DNA binding constant [14]. The weaker affinity of NB-506 must arise, in part, from its lack of charge, and consequently a near zero contribution to the binding free energy from the polyelectrolyte effect [29,30]. To our knowledge, the data of Figs. 1 and 2 represent the first demonstration of the binding of an uncharged ligand to triplex DNA.

Inspection of the structure of NB-506 reveals a region homologous with large portions of BePI, a compound known to bind preferentially to triplex DNA. Fig. 3 shows an overlay of the structures of NB-506 and BePI, showing the region of maximal homology. The crescent shaped fragment common to both compounds represents a potential pharmacophore for selective triplex binding. Rigorous development of this pharmacophore hypothesis will, of course, require much more extensive testing of a large number of additional compounds

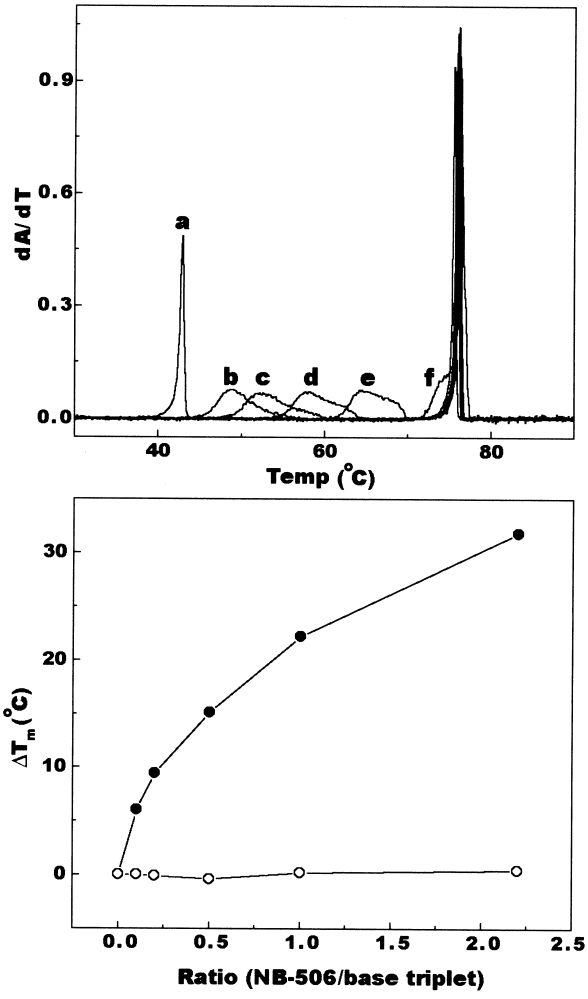

Fig. 2. Results of thermal denaturation studies of the triplex poly dA:(poly dT)$_2$ in the presence of NB-506. Top: Differential UV melting curves are shown at different molar ratios of added NB-506. The molar ratios (NB-506/triplet) for the curves are: a: 0; b: 0.1; c: 0.2; d: 0.5; e: 1.0 and f: 2.2. In the absence of NB-506, the triplex → duplex+poly dT transition is centered at $T_m = 42.5$°C. Melting of the poly dA:poly dT duplex is centered at $T_m = 75.2$°C. NB-506 increases the triplex melting transition, but has little effect on duplex melting. All of the curves for duplex melting in the presence of NB-506 very nearly superimpose, and are centered around 75°C. Bottom: Plot of ΔT_m (the difference in the apparent T_m in the presence and absence of added NB-506) versus the molar ratio of added NB-506. Data for triplex melting are shown as the solid circles, while open circles show data for duplex melting.

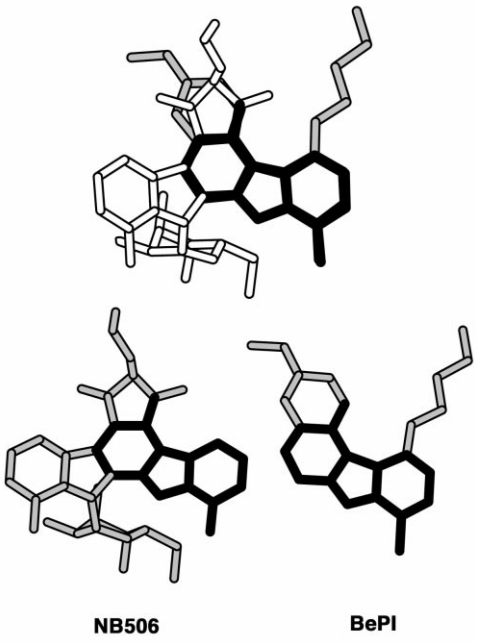

Fig. 3. Identification of a potential triplex pharmacophore for triplex binding. The top image shows NB-506 (white) overlaid upon the structure of the known triplex binding agent BePI (gray). The region of maximal overlap is shown in black, and represents a potential pharmacophore for triplex binding. The bottom pair of images shows the structures of the two compounds in isolation with the common structural feature highlighted in black.

containing the molecular fragment. The ease and speed of the competition dialysis approach will facilitate that testing process.

It is of interest to note that the known triplex binders BePI and coralyne also function as weak topoisomerase I inhibitors [31,32]. The basis of a possible correlation between triplex binding and topoisomerase I inhibitions is certainly unclear. Perhaps the inhibitor binding site within the DNA-topoisomerase I complex shares structural similarities to triplex DNA.

If the homologous structural fragment within NB-506 shown in Fig. 3 intercalates into triplex DNA in a manner similar to the binding mode of BePI, we can speculate that NB-506 binding would be stabilized by additional molecular interactions of its carbohydrate moiety within one of the grooves found in the triplex structure. We previously have determined that the carbohydrate contributes 1.5 kcal mol^{-1} to the DNA binding free energy of rebeccamycin (the parent molecule of NB-506) [7]. A similar favorable contribution to NB-506 binding to triplex would not be unreasonable.

The competition dialysis method yields additional significant information about the interaction of NB-506 with nucleic acids. Its binding to natural DNA samples (Fig. 1) reveals a positive correlation between the amount of NB-506 bound and the GC content of the DNA. That observation is a clear demonstration of preferential binding to GC base pairs. In previous DNase I footprinting studies, we observed preferential binding of NB-506 to GC and GT dinucleotide sites [14]. The competition dialysis results independently confirm such a GC preference.

Little or no binding to single-stranded DNA or to left-handed Z DNA was observed. Previous linear dichroism studies demonstrated the NB-506 intercalated into a variety of right-handed duplex DNA samples [14]. Single-stranded DNA would not offer an appropriate intercalation site. Lack of binding to left-handed Z DNA would be consistent with the behavior of many intercalators, which inevitably show a strong preference for right-handed, B form DNA [33–35].

Finally, we note that NB-506 interacts to some extent with RNA (as represented by poly A:poly U) and with a parallel-stranded tetraplex DNA, $(5'\ T_2G_{20}T_2)_4$. The strength of these interactions is comparable to that observed for the interaction of NB-506 with duplex calf thymus DNA.

NB-506 is a potent topoisomerase I inhibitor [12]. The structural preferences demonstrated here by the competition dialysis method might be unrelated to the mechanism by which NB-506 inhibits the enzyme. The mechanisms of action of most anticancer agents are pleiotropic, and no single mechanism can in general account for all aspects of a given agent's cytotoxicity. A variety of experimental approaches has been used to show that triplex DNA exists in the cell [36]. While the biological role of triplex DNA in vivo is not clear, a variety of models was proposed for its use as a regulatory element in transcription or replication [36]. Our data suggests that triplex DNA would represent an additional target for NB-506, although such interactions may not be associated with the drug's effect on tumor cells.

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