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Minor-groove binders are inhibitors of the catalytic activity of DNA gyrases

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Non-intercalating DNA minor-groove binders may effectively inhibit the supercoiling activity of gyrases by influencing the enzyme recognition and cleavage site on DNA. For gyrase from *Streptomyces noursei* a wide range of inhibitory potency for different classes of ligands is observed. This can be explained by a number of structural and binding factors of the ligands competing with the gyrase on the target site of DNA, the mechanism of which is different from the classical gyrase inhibitors.

DNA binding drug; Inhibition; DNA gyrase; *Streptomyces noursei*

1. INTRODUCTION

DNA gyrase is an essential enzyme in the DNA metabolism of prokaryotes, the function of which includes DNA replication, recombination, transcription, chromosome segregation and the maintenance of the nucleoid structure in bacteria [1–5]. The enzyme is also believed to be the target for certain bacterial growth inhibitors, such as coumarins and quinolones [3,4,6,7], though the interference of the latter type of inhibitors has been attributed to the binding with DNA in the enzyme complex [8,9]. The activity of gyrase in vitro is related to its ability to induce DNA supercoiling, catenation or decatenation [2].

It has been established that a number of drugs and organic agents affecting enzymes of DNA metabolism bind in the minor groove of B-DNA by non-intercalative means (for review see [10]), and most of these ligands, such as Nt and Dst-3, prefer AT-rich sequences [10,11]. A new synthetic type of drug, lexitropsins, show a reduced affinity for AT pairs, but may also accept GC pairs [13–15]. Synthetic minor groove binders were designed to develop modified agents reading defined sequences [16]. This includes a series of netropsin dimers containing two netropsin units [17]. The interference of some minor-groove binders with the activity of mammalian topoisomerase II has been reported [18–20]. Recently, we showed that Dst-3 is an inhibitor of gyrase activity [21].

In this report we demonstrate that netropsin and a variety of minor groove binders with different chemical structures are potent gyrase inhibitors, the effectiveness of which depends significantly on the nature and binding ability of the ligand to DNA. The molecular mechanism of interference clearly differs from that of classical gyrase inhibitors.

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Abbreviations: Dst-3, distamycin A; Nt=Nt-PyPy, netropsin containing methylpyrrole (Py); Nt-PyIm, Nt-ImPy, Nt-Im_3, netropsin analogs (lexitropsins) containing methylimidazole (Im) and (or) methylpyrrole (Py); bis-Nt-X (X=5,6,8), dimeric netropsin analogs linked by X methyleneresidues(X=5,6,8); DAPI,4′,6-di-amidino-phenylindole; Hoechst 33258, 2-(4-hydroxyphenyl)-5-[5-(4-methyl-piperazin-1-yl)-benzimidazole-2-yl]benzimidazole; SN-6999, NSC-101327, SN-6132, SN-6131, SN-16814, SN-1871: bisquaternary ammonium heterocycles (structures are given in [10,22]); SDS, sodium dodecylsulfate.

2. MATERIALS AND METHODS

2.1. *Minor groove binding ligands*

Dst-3 was obtained from Sigma (St. Louis, MO); Nt was a highly purified product isolated from *Streptomyces netropsis* [10]. The lexitropsins Nt-ImPy, Nt-Im_3 and bis-Nt-X (X = 5,6,8) have been reported previously [14,23]. DAPI, Chromomycin A_3 and Hoechst 33258 were purchased from Fluka and Serva (Heidelberg). Bisquaternary ammonium heterocyclic compounds were gifts from Dr. B. Baguley (Auckland, New Zealand). DNA binding data of various ligands investigated are described in the reference given in Table I. Structures of ligands are summarized elsewhere [10,22].

2.2. *Enzymes and assay*

Purification of gyrase from *Streptomyces noursei* is reported in a forthcoming paper (J. Störl et al.).

Assay of gyrase-mediated supercoiling of relaxed pBR322 DNA: the reaction mixture (20 μl) contained 40 mM Tris-HCl (pH 7.5), 19 mM KH_2PO_4, 34 mM KCl, 5 mM spermidine, 3.6 mg/ml bovine serum albumin, 100 μg/ml t-RNA, 1.6 mM $MgCl_2$, 1.4 mM ATP, 0.5 μg relaxed DNA plus one unit of gyrase. One unit is defined as the amount of enzyme required to convert 0.5 μg relaxed plasmid DNA into its supercoiled form in 45 min at 30°C. The enzyme reaction was

terminated with 3 μl stopper solution containing 10% SDS, 0.05% bromophenol blue and 50% glycerol. Samples were electrophoresed on 1% agarose, stained with ethidium bromide (0.5 μg/ml) and photographed. The negatives were scanned on a computerized Bio-Rad densitometer. The inhibitory effect of the ligands on gyrase activity was detected from the amounts of closed circular DNA, which were most pronounced.

3. RESULTS AND DISCUSSION

The interference of various non-intercalating minor groove binders with the catalytic activity of DNA gyrases from *S. noursei* and *E. coli* was examined by the enzyme-mediated supercoiling reaction. As an example the gel electrophoretic analysis of the influence of increasing amounts of the AT-specific DNA binding drug Nt on the supercoiling activity of *S. noursei* gyrase is shown in Fig. 1. The enzyme without Nt produces supercoiled DNA (lane 1). Addition of 1 and 2.5 μM Nt (lanes 2,3) has no significant effect whereas concentrations of 5, 10 and 20 μM Nt (lanes 4–6) progressively inhibit the formation of supercoiled DNA. At 30 μM Nt, the enzyme reaction is nearly completely blocked (lane 7).

It was of interest to investigate whether other minor groove binders having different chemical structures show a correlation with their functional effects on gyrase supercoiling activity. The influence of increasing ligand concentrations on gyrase activity for six representative minor groove binders is shown in Fig. 2. Dst-3 most strongly inhibits the enzyme activity followed by Nt, chromomycin A_3, SN-6999 and bis-Nt-6. SN-18071 is ineffective in the concentration range up to 30 μM at which Dst-3, Nt and chromomycin A_3 completely block the gyrase activity (Fig. 2, curves 1–3). SN-6999 or bis-Nt-6 show half of the inhibitory effect (curves 4,5). In the case of SN-18071 IC_{50} is 150 μM (curve 6). This means that SN-18071 is nearly inactive relative to the other minor groove binders (Fig. 2).

The antigyrase activities of the four groups of DNA binding ligands investigated for the *S. noursei* enzyme are summarized in Table I. These effects of minor groove binders are similar for the *E. coli* gyrase (data not shown). As indicated by the IC_{50} and IC_{90} values, Dst-3, Nt and the imidazole-containing lexitropsins as well as DAPI and chromomycin A_3 show most pronounced inhibitory effects whereas Hoechst 33258 and ligands of group 3 and 4 are moderate or weak inhibitors of gyrase activity. All these compounds bind to DNA in the minor groove (for review see [10]). In view of the properties of these ligands one might expect that the antigyrase activity is related to their DNA-binding parameters. However, no direct correlation of the efficiency of ligand-induced modulation of the enzyme activity to their DNA-binding properties could be found. This is exemplified by the following considerations: the AT-specific minor groove binder, Nt, with an overall association constant of $K_a = 2 \times 10^6 \cdot M^{-1}$ for calf thymus DNA [10] and $K_a \sim 10^8 \cdot M^{-1}$ for poly(dA-dT)·poly(dA-dT) [10] shows similar IC_{50} and IC_{90} values as chromomycin A_3, which is highly GC-specific [25] with a K_a

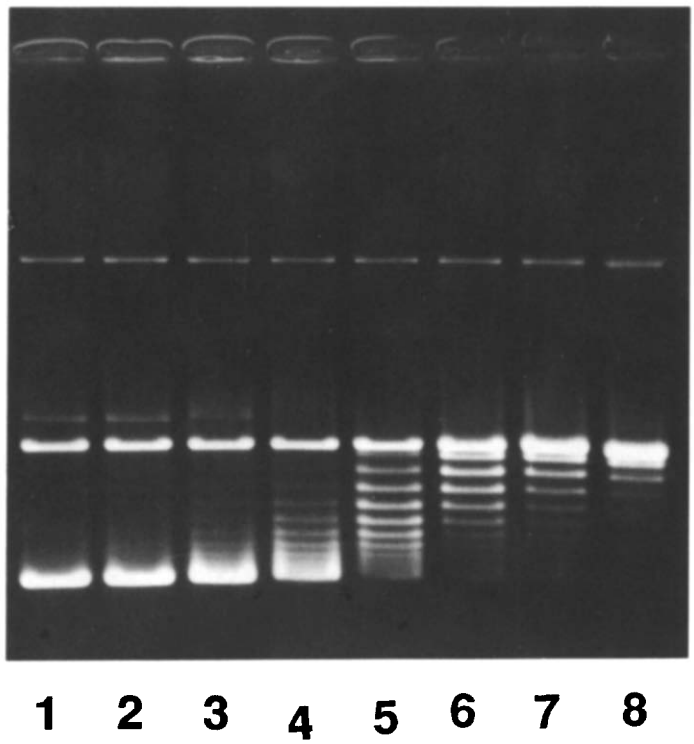

Fig. 1. Effect of netropsin (Nt) on the supercoiling activity of gyrase from *S. noursei*. Lanes: 1, supercoiled pBR322 DNA; 8, relaxed DNA; lanes 2–6: 1, 2.5, 5, 10, 20, 30 μM Nt, respectively.

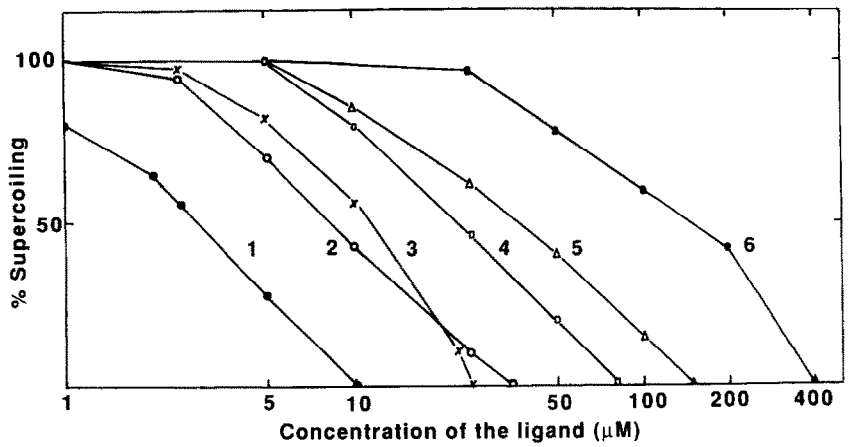

Fig. 2. Inhibitory effect of some minor groove binders on supercoiling activity of gyrase. Attached numbers indicate drugs: 1, Dst-3; 2, Nt; 3, chromomycin A_3; 4, SN 6999; 5, bis-Nt-6; 6, SN-18071.

value for the calf thymus DNA–Me^{2+} complex of $\sim 10^{11} \cdot M^{-1}$ [26]. Dst-3 appears to be the most efficient gyrase inhibitor (Table I); like Nt it prefers AT base pairs, but it may accept one GC pair [28,29]. Dst-3 shows an overall K_a value of $1.2 \times 10^6 \cdot M^{-1}$ for calf thymus DNA and a K_a of $1.8 \times 10^7 \cdot M^{-1}$ for poly(dA-dT)·poly(dA-dT) [10,28], which is one characteristic parameter for its AT-affinity. For Nt-Im_3 a strongly reduced AT-affinity and a high acceptance of GC pairs was demonstrated with a binding constant of $K_a = 1.06 \times 10^6 \cdot M^{-1}$ for calf thymus DNA [13]. As indicated by the IC_{50} and IC_{90} values for Nt-Im_3 the inhibitory effect is comparable with that of Dst-3 or Nt (Table I). These features demonstrate that base pair preference and binding strength alone cannot be regarded as factors responsible for the antigyrase activity of the ligands. On the other hand, it was reported that bidentate binding to DNA of bi-functional netropsin analogs can increase the inhibitory effect of mammalian topoisomerase II [19]. In contrast to this finding we observe the reverse effect for the inhibition of gyrase activity: bis-netropsins show a significantly weaker antigyrase activity com-

Table I

Inhibition of DNA supercoiling activity of gyrase from *S. noursei* by non-intercalating DNA-binding ligands

Group	Ligand	IC_{90} (μM)	IC_{50} (μM)	Base pair affinity		DNA binding references
				AT	GC	
1	Dst-3	8	2.7	++	(+)	[10,12]
	Nt-Im_3	16	8.4	(+)	++	[13,15]
	Nt-PyIm	20	15	+	+	[13,15]
	Nt-ImPy	18	12	+	+	[13,15]
	Nt-PyPy	25	8	++	−	[10,15]
2	DAPI	17	8.8	+	(+)	[10,27]
	Chromomycin A_3	23	12	−	++	[25,26]
	Hoechst 33258	68	33	++	(+)	[10,40]
3	Bis-Nt-5	58	25	++		[17]
	Bis-Nt-6	111	34	++		[17]
	Bis-Nt-8	113	39	++		[17]
4	SN-6999	62	23	++	(+)	[10,22]
	NSC-101327	79	38	+	(+)	[10,22]
	SN-6132	91	46	++		[22,24]
	SN-6131	100	50	+		[22,24]
	SN-18071	350	150	+		[10,22]
	SN-16814	no inhibition	−	+	(+)	[10,22]

IC_{50} and IC_{90}, concentration of the ligand at 50% and 90% inhibition, respectively, of the enzyme-mediated supercoiling.

pared with Nt as indicated by much higher IC_{90} and IC_{50} values (Table I, group 3). Thus, it appears that elongation of the ligand by increasing number of *N*-methylpyrrole units contained in the bis-netropsin analogs does not enhance, but instead lowers, the inhibitory effect on gyrase activity. These different effects of minor-groove binders observed for gyrase (Fig. 2, Table I) and eukaryotic topoisomerase II ([19] and unpublished data of the authors) may reside in the differences in the molecular interaction mechanisms between the two types of enzymes with DNA. While the production of double-strand breaks is a common reaction of both enzymes, the complexes formed with double-stranded DNA involve distinctly different elements in the interaction. Eukaryotic topoisomerase II exists as a homodimer containing sequence regions homologous to the gyr A and gyr B subunits of gyrase [30] and it binds preferentially to negatively supercoiled DNA [30]. Gyrase is a tetramer consisting of gyr A and gyr B subunits as dimers [31], which interact with the DNA target site by wrapping the DNA on the outside of the enzyme forming a gyrase–DNA complex [32]. This distinguishes DNA-bound gyrase from that of eukaryotic topoisomerase II. DNA in this complex could be more readily accessible to smaller minor-groove binders, such as Nt, than to much longer ones, such as bis-netropsins (Table I). Consequently, it seems that coiling of DNA in the complex with gyrase together with the affinity to the DNA target sequence, could influence the competitive interaction with the ligand.

To test that minor-groove binders may directly block the enzyme from binding to its cleavage site, SDS treatment of the gyrase reaction was applied before electrophoresis. SDS rapidly denatures the complex in which DNA has been cleaved by gyrase and in the presence of the inhibitor oxolinic acid cleaved DNA can be detected [33]. By using ciprofloxacin as a reference for the appearance of linear DNA (Fig. 3, lanes 3 and 6), which indicates formation of a cleavable complex, it follows from the absence of linear DNA in the presence of high inhibitory concentrations of Nt and Dst-3 (Fig. 3, lanes 1, 2, 4 and 5) that minor-groove binders prevent formation of a cleavable complex. This feature is illustrated in Fig. 4 by a schematic drawing based on the model of Shen et al. [34]. The formation of this complex mediated by interaction with DNA of gyrase via tyrosine as active site of subunit gyr A is totally blocked by minor-groove binding of Nt. It is plausible that the minor groove plays a role for the access of the enzyme through gyr A on DNA. Most probably, minor-groove binding of Nt and related agents cause a displacement of gyrase from its DNA target region rather than a dislocation (Fig. 4c). Preliminary gel retardation experiments with DNA fragments (Simon, H., unpublished data) support this suggestion. To explain the differences of the inhibitory potency of various minor-groove binders on gyrase activity (Table I), a number of additional factors determining the interaction mechanism have to be considered, such as binding strength, site size, AT- vs. GC-affinity and conformational effects, as well as the geometry and flexibility of the ligands. An essential aspect resides in the affinity of the ligands to the enzyme recog-

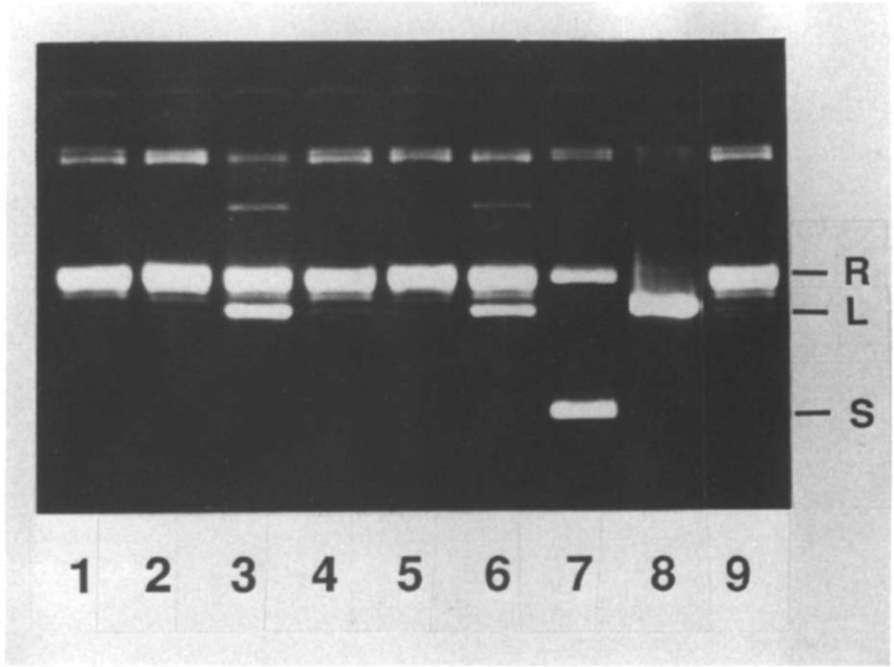

Fig. 3. Comparative inhibitory effects of Nt, Dst-3 and ciprofloxacin on supercoiling activity of gyrase from *E. coli* in the absence of ATP, after SDS treatment. AT 200 μM drug: lanes 1,4: Dst-3; 2,5: Nt; 3,6: ciprofloxacin; 7: in presence of ATP without drug; 8: pBR322 DNA linearized with *Eco*RI; 9. without ATP, without drug; R, relaxed; L, linear; S, supercoiled DNA.

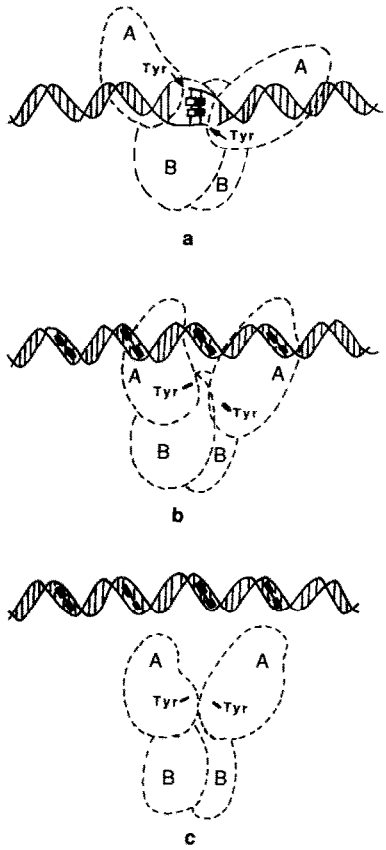

Fig. 4. Schematic model of proposed effects for quinolone and minor groove binders in the inhibition of gyrase on DNA; a, quinolone–DNA binding according to Shen et al. [34]; b, gyrase dislocated and c, displaced by netropsin-minor-groove binding.

nition and cleavage site on DNA. For pBR322 plasmid DNA, several strong gyrase sites have been detected by using oxolinic acid-mediated DNA cleavage in vitro [35,36] and in vivo [33]. Enzyme binding occurs to a DNA stretch of at least 120 bp (or larger) whereas a region of about 40 bp surrounding the cleavage site is most strongly bound [35]. By inspection of the sequences analysed for various strong gyrase cleavage sites and surrounding segments in pBR322 DNA [35–37] it appears that AT and GC pairs are nearly equally distributed in these regions and AT clusters are very rare adjacent to the cleavage site. As an example, the sequence of a representative strong gyrase site, located at position 990 on the pBR322 map [33,35], is as follows: 5′-GAGGCTGGATGGCCTTCCCCATTA. Most of the four base pair staggered cleavage sites of the gyrase contain a higher number of GC pairs, and for in vivo assayed sites it was reported that the position 3′ to a cut requires G [33]. As mentioned above, AT-sequence specificity of the ligand alone cannot account for the competition at the gyrase binding site. Dst-3 binds tightly to AT clusters (see binding data discussed above), but also accepts a GC pair [28,29] and is known to induce conformational changes in DNA, such as allosteric or bending effects [37,38]. This may explain its high inhibitory potency indicated by IC_{50} and IC_{90} values (Table I, Fig. 2). For the antigyrase activity of Nt, which is AT-specific, other factors must be taken into account, such as structural effects; e.g. an important element in minor groove binding for Nt and the related ligands of group 1 (Table I) concerns the high flexibility of the oligopeptide structure allowing a proper isohelical alignment in the groove [10]. Nt also induces a local stiffening of bound regions [39] achieved by hydrogen bonding, electrostatic attraction and van der Waals contacts (for review see [10]). The dicationic nature of Nt seems to be an essential factor since the related monocationic ligand, Dst-2, showed only a poor inhibitory effect on gyrase activity [21]. Interestingly Nt-Im_3, structurally related to Nt, represents a potent antigyrase agent, which is even slightly more active than Nt (Table I). This dicationic lexitropsin permits GC binding besides a reduced AT affinity, which therefore can compete with GC pairs at the gyrase recognition site by formation of hydrogen bonds to G-NH_2 in the minor groove [13,15]. In case of DAPI, which shows an AT preferred binding to DNA [27], its simultaneous intercalative binding ability to a GC pair [27] may be a possible factor contributing to the antigyrase activity. Chromomycin A_3 may also effectively compete with gyrase at the binding and cleavage site due to its high GC-specificity [26] by blocking the G site required at the enzyme cutting site. On the other hand bis-netropsins (Table I, group 3) are very poor inhibitors which may be a consequence of the absence of longer AT-stretches required for bidentate binding to compete with the enzyme at the recognition site. For bisquaternary ammonium heterocyclic ligands (Table I, group 4) their rigidity may be a factor to explain the lower antigyrase activity.

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