Selective inhibition of DNA gyrase in vitro by a GC specific eight-ring hairpin polyamide at nanomolar concentration

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Abstract The influence of an eight-ring hairpin DNA minor groove binder on the gyrase mediated DNA supercoiling and cleavage reaction step of the enzyme was investigated. The results demonstrate that supercoiling is affected by the hairpin polyamide in the millimolar concentration range while the enzyme catalyzed cleavage of a 162 bp fragment of pBR322 containing a single strong gyrase site is effectively inhibited at nanomolar concentration. As demonstrated by footprint analysis the latter effect is caused by a specific binding of the hairpin forming polyamide to the enzyme recognition site (GGCC), which indicates that the gyrase activity to produce a double strand break is blocked at this site. The pyrrole–imidazole hairpin polyamide is the most potent inhibitor of the gyrase mediated cleavage reaction compared to other known anti-gyrase active DNA binding agents.

Key words: DNA gyrase; Inhibition; Minor groove binder

1. Introduction

DNA gyrase is an essential enzyme that controls the topological state of DNA in prokaryotic cells. Only the bacterial gyrase is able to produce negative supercoiling of DNA in the presence of ATP [1–3]. The reaction cycle of DNA supercoiling mediated by the enzyme involves generation of transient DNA double strand breaks, an ATP dependent passage of a DNA segment through the cleaved site and a religation step [3,4]. DNA gyrase is a selective target for antibacterial agents, such as the most studied quinolone and coumarin antibiotics [4–7]. Quinolone drugs (e.g. ciprofloxacin) affect the protein subunit GyrA and coumarins (e.g. novobiocin) act on GyrB [3,6,7].

Drugs that bind specifically to the DNA substrate have been shown to also inhibit gyrase [8–11]. It was demonstrated that the GC specific minor groove binder chromomycin A_3 interferes with the gyrase–DNA binding and cleavage reaction on a DNA fragment from pBR322 [9].

Minor groove binding polyamides containing *N*-methylimidazole (Im) and *N*-methylpyrrole (Py) amino acids were designed to increase the affinity to a defined DNA sequence [12–18]. Pairing rules were put forward in which an Im/Py pair targets GC and Py/Im targets CG [12–16]. Py–Im polyamides, covalently linked by γ-aminobutyric acid (γ) to form eight-ring hairpin structures specifically bind to 6 bp target regions at nanomolar concentrations [12–14].

In this paper we report the anti-gyrase activity in vitro of a hairpin polyamide of sequence composition ImImPyPy-γ-ImImPyPy which according to the pairing rules for minor groove recognition targets the sequence 5′-GGCC-3′ [14–16]. We report that the synthetic DNA binding ligand **1**, targeted to the DNA cleavage sequence of the gyrase, inhibits the enzyme in the nanomolar concentration range.

2. Materials and methods

The eight-ring hairpin polyamide ImImPyPy-γ-ImImPyPy-β-Dp (**1**) has been described earlier [14] (Fig. 1).

Isolation and purification of DNA gyrase, based on the method of Staudenbauer and Orr [19], was used for *Streptomyces* gyrase according to a modified procedure [20].

pBR322 DNA was purified and relaxed by topoisomerase I as described previously [20]. The 162 bp fragment from pBR322 (Fig. 2) was prepared by digestion with *Bgl*I and *Sau*3a [9].

Supercoiling of pBR322 DNA and the cleavage reaction of the 162 bp fragment (performed in 50 mM Tris pH 7.5, 55 mM KCl, 5 mM dithiothreitol, 4 mM $MgCl_2$ and 5% glycerol) have been described elsewhere [9,20].

Reactions were carried out after equilibration with polyamide **1** for 6 min and 16 h as indicated in Fig. 3.

For footprinting, a 103 bp region comprising a sequence (with the single specific gyrase site) from the 162 bp fragment (see shaded sequence part in Fig. 2) was synthesized and cloned into the *Sma*I site of pUC19. DNase I footprinting was performed with an *Eco*RI/*Pst*I fragment from the pUC19 containing the cloned region. The ^{32}P-labelled DNA fragment (2 μl) was incubated with 4 μl of ligand **1** at room temperature for 30 min, and then digested with 2 μl DNase I in buffer (20 mM NaCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$). The reaction was stopped after 4 min using 3 μl 80% formamide containing 0.1% bromophenol blue, 0.1% xylenecyanol and 10 mM EDTA. Samples were heated at 90°C (3 min) and chilled on ice. Gel electrophoresis (8% polyacrylamide plus 8% urea, Tris-borate–EDTA buffer, pH 8.3) was carried out for 2 h at 1500 V, then treated with 10% acetic acid for 15 min, dried at 80°C and subjected to autoradiography at −70°C with an intensifying screen. Digestion products were assigned by co-electrophoresis of sequencing standards.

3. Results

3.1. Influence of the hairpin forming minor groove binder on DNA gyrase activity.

As evidenced earlier, minor groove binders may inhibit the gyrase mediated reaction of DNA supercoiling by influencing the binding of the enzyme at or around the cleavage and religation site [9,10]. The design of an eight-ring hairpin poly-

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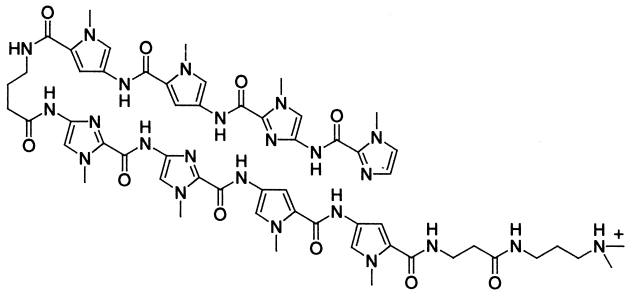

ImImPyPy-γ-ImImPyPy-β-Dp (**1**)

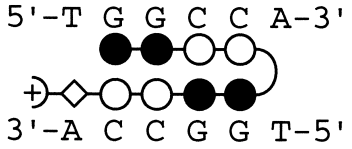

Fig. 1. Structure of the eight-ring hairpin polyamide ImImPyPy-γ-ImImPyPy-β-Dp **1**. γ: γ-aminobutyric acid linker; β: β-alanine residue; Dp: ((dimethylamino)propyl)amide.

amide, which can recognize six base pairs containing four contiguous GC pairs (GGCC) identical with a strong gyrase site prompted us to examine whether this type of minor groove binder offers a basis to enhance the potency and selectivity for an anti-topoisomerase agent. The inhibitory effect of the hairpin polyamide (Fig. 1) was detected by the DNA supercoiling and cleavage assay. The results are summarized in Fig. 3 for gyrase from *Streptomyces noursei* and *E. coli*.

From Fig. 3 it appears that the *E. coli* gyrase is more sensitive to polyamide **1** than the *S. noursei* enzyme. DNA supercoiling mediated by both gyrase species is significantly less affected by the hairpin polyamide (curves 3–6) than the cleavage reaction (curves 1 and 2). For the cleavage reaction the 162 bp fragment (Fig. 2) was used to directly examine the influence of polyamide **1** on a single gyrase recognition site. In the case of the pBR322 plasmid several gyrase cleavage sites consisting of different sequences have been identified [21], and therefore the enzyme may react with sequences which do not contain specific binding sites for polyamide **1**. This explains the large differences in the inhibition curves of DNA supercoiling from those of the cleavage reaction detected with the

162 bp fragment from pBR 322:

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TGGCGGCCGA CGCGCTGGGC TACGTCTTGC TGGCGTTCGC GACGCGAGGC
ACCGCCGGCT GCGCGACCCG ATGCAGAACG ACCGCAAGCG CTGCGCTCCG

TGGATGGCCT TCCCCATTAT GATTCTTCTC GCTTCCGGCG GCATCGGGAT
ACCTACCGGA AGGGGTAATA CTAAGAAGAG CGAAGGCCGC CGTAGCCCTA

GCCCGCGTTG CAGGCCATGC TGTCCAGGCA GGTAGATGAC GACCATCAGG
CGGGCGCAAC GTCCGGTACG ACAGGTCCGT CCATCTACTG CTGGTAGTCC

GACAGCTTCA AG
CTGTCGAAGT TC
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Fig. 2. Sequence of the 162 bp fragment from pBR322 containing a single strong gyrase cleavage site (arrow).

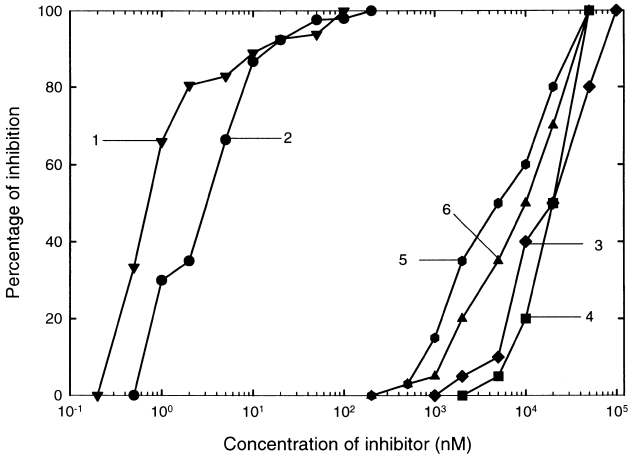
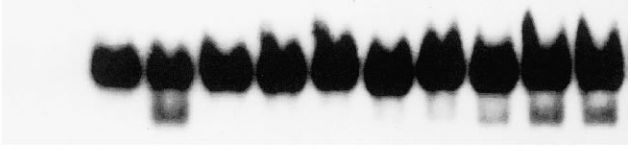


Fig. 3. Inhibitory effect of the hairpin polyamide **1** on the DNA gyrase activity. *E. coli* enzyme: curve 1, cleavage reaction after preformation of the ligand complex (6 min and 16 h); curve 3, supercoiling preformation of the complex for 6 min; curve 5, supercoiling preformation of the complex for 16 h. *S. noursei* enzyme: curve 2, cleavage reaction after preformation of the complex (6 min and 16 h); curve 4, supercoiling preformation of the complex for 6 min; curve 6, supercoiling preformation of the complex for 16 h.

162 bp fragment containing a single enzyme reaction site, which coincides with the recognition sequence of **1**. Fig. 4 displays the gel analysis of the effect of **1** on the cleavage of the 162 bp fragment. The initial inhibition of the enzyme catalyzed cleavage is visible at 5 nM (lane 8) but was already detectable at 1 nM polyamide **1** by densitometry to be about 30% if lane 10 is compared with the reference of lane 2.

3.2. Footprinting

Footprint analysis on the 103 bp fragment shown in Fig. 5 directly demonstrates that polyamide **1** binds to the gyrase cleavage site GGCC and at higher ligand concentration upstream of another single base pair mismatch site GGCG. The



	1	2	3	4	5	6	7	8	9	10
gyrase	-	+	+	+	+	+	+	+	+	+
ligand 1 (nM)	-	-	200	100	50	20	10	5	2	1
cleavage (%)	-	100	0	2.3	2.3	7.6	13.3	33.6	65	70.1
inhibition (%)	-	0	100	97.7	97.7	92.4	86.7	66.4	35	29.9

Fig. 4. Gel electrophoretic analysis of the inhibitory effect of **1** on the gyrase mediated cleavage reaction upon addition of increasing ligand concentration. Gyrase from *S. noursei*; substrate 162 bp fragment from pBR322; in the presence of 100 μM ciprofloxacin required to prevent religation of the cleaved fragment. For reaction conditions see Section 2. The cleavage assay contained 1 ng ^{32}P-labelled fragment.

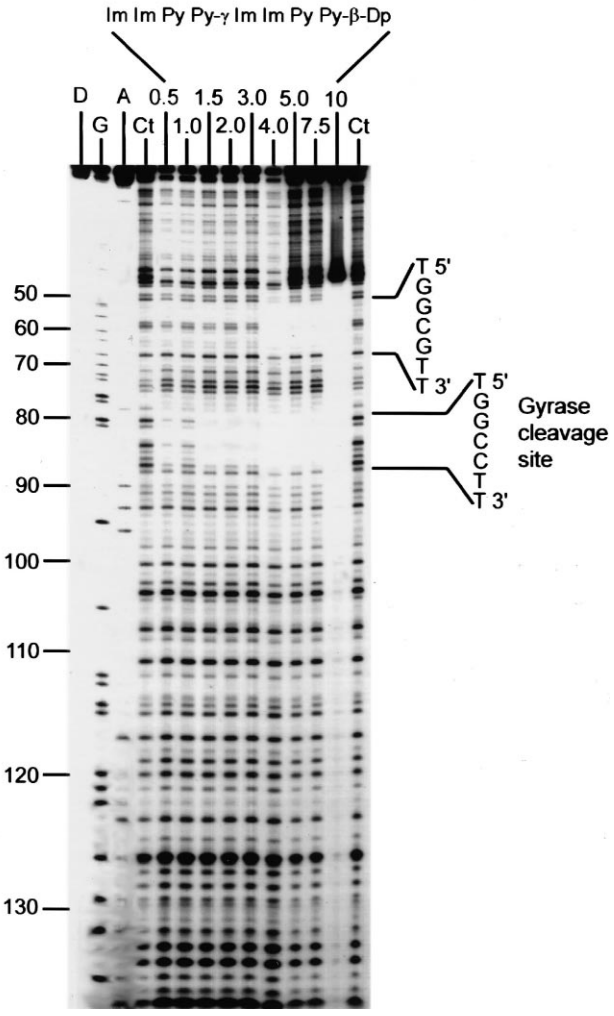


Fig. 5. DNase I footprinting of **1** on an *Eco*RI-*Pst*I fragment from pUC19 containing a cloned region from pBR322 (see Section 2). Protected sequences by the ligand are given on the right side. D, uncleaved DNA; Ct, control (DNase I digestion without ligand); G and A, dimethylsulfate–piperidine and chloroformate–piperidine markers for guanine and adenine, respectively.

result directly indicates that the inhibition of the gyrase mediated cleavage reaction is primarily caused by a specific blocking of the enzyme recognition site through **1**.

4. Discussion

Our present studies demonstrate that an eight-ring hairpin polyamide, which recognizes a GC rich six base pair DNA sequence, effectively inhibits the bacterial DNA gyrase activity in vitro. This inhibitory potency of polyamide **1** is observed to be most pronounced for the enzyme mediated cleavage reaction when a 162 bp fragment with a single specific gyrase site is used as a substrate, whereas the supercoiling reaction is much less affected. The inhibition of the cleavage step occurs in the nanomolar concentration range, which is three to four orders in magnitude lower than that observed for supercoiling (Fig. 3). This can be reasonably explained by the presence of numerous gyrase cleavage sites present in the pBR322 plasmid [21], which differ in their sequence (the enzyme recognition site is degenerate). A specific inhibitory effect can be detected by the cleavage assay using a DNA substrate containing a single gyrase site. It is interesting to compare the anti-gyrase activity of some DNA minor groove binders on the gyrase mediated cleavage reaction. As may be seen in Table 1 the hairpin polyamide **1** exhibits the lowest IC_{50} value at 3 nM for *S. noursei* gyrase in the cleavage assay, which is 10-fold less than that of the netropsin analog, 20-fold less than chromomycin A_3 and more than 300-fold less than distamycin.

Table 1
Comparison of the inhibitory potency on the cleavage reaction of *S. noursei* gyrase of the GC specific eight-ring hairpin polyamide **1** with the minor groove binders bis-netropsin (Nt-trans), chromomycin A_3 (CHR) and distamycin (Dst)

	Polyamide **1**	Nt-trans	CHR[a]	Dst[b]
IC_{50} (nM)	3	30	60	100

[a]From [10].
[b]From [9].

The IC_{50} value is even lower for *E. coli* gyrase showing an IC_{50} value of 0.7 nM (Fig. 3, curve 1).

Our data demonstrate that the hairpin forming polyamide **1** has the strongest anti-gyrase effect when compared with other DNA minor groove binders acting on a specific sequence of the DNA substrate.

It has been previously observed that chromomycin A_3 as a GC specific agent also has a profound inhibitory influence on the gyrase mediated cleavage reaction in vitro [9]. The inhibitory potency of **1** is found to be significantly higher than that of chromomycin A_3. This can be mainly ascribed to a highly specific interaction of **1** with the GGCC recognition site of the enzyme (Fig. 2) as evidenced by our footprinting data (Fig. 5). The strong effect of ligand **1** can be best explained by the hairpin formation of the polyamide upon binding to the GGCC enzyme recognition site which directly blocks the double strand breakage reaction catalyzed by the GyrA subunit of the DNA gyrase. Moreover, the high equilibrium association constant of $K_a = 9.7 \times 10^9\ M^{-1}$, which was found for the sequence TGGCCA [14], strongly supports this interpretation.

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