Effect of Pyrimido[1,6-a]Benzimidazoles, Quinolones, and Ca$^{2+}$ on the DNA Gyrase-Mediated Cleavage Reaction

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The quinolones inhibit the A subunit of DNA gyrase in the presence of Mg$^{2+}$ by interrupting the DNA breakage and rescaling steps, and the latter step is also retarded without quinolones if Mg$^{2+}$ is replaced by Ca$^{2+}$. Pyrimido[1,6-a]benzimidazoles have been found to represent a new class of potent DNA gyrase inhibitors which also act at the A subunit. To determine alterations in the DNA sequence specificity of DNA gyrase for cleavage sites in the presence of inhibitors of both classes or in the presence of Ca$^{2+}$, we used DNA restriction fragments of 164, 85, and 71 bp from the pBR322 plasmid as model substrates. Each contained, at a different position, the 20-bp pBR322 sequence around position 900, where DNA gyrase preferentially cleaves in the presence of quinolones. Our results show that pyrimido[1,6-a]benzimidazoles have a mode of action similar to that of quinolones; they inhibit the rescaling step and influence the DNA sequence specificity of DNA gyrase in the same way. Differences between inhibitors of both classes could be observed only in the preferences of DNA gyrase for these cleavage sites. The 20-bp sequence appeared to have some properties that induced DNA gyrase to cleave all three DNA fragments in the presence of inhibitors within this sequence, whereas cleavage in the presence of Ca$^{2+}$ was in addition dependent on the length of the DNA fragments.

The prokaryotic DNA gyrase (EC 5.99.1.3) belongs to the class of topoisomerase II proteins and is able to catalyze the ATP-dependent negative supercoiling of double-stranded closed circular DNA (23). The active enzyme is a tetramer consisting of two A subunits and two B subunits. It is thought that in the initial stage of the supercoiling process the enzyme binds to double-stranded DNA and that about 120 bp is wrapped around the tetrameric protein in a single positive supercoil. There have been several proposals for the existence of specific sites for the interaction of DNA gyrase with DNA. In Escherichia coli and Salmonella typhimurium, there is a family of repetitive extragenic palindromic sequences, and there is evidence that DNA gyrase binds preferentially to these sites (30). It has been proposed that the par locus in the pSC101 plasmid represents a DNA gyrase binding site (29), and in bacteriophage Mu a sequence that is required for efficient replicative transposition is proposed as a strong DNA gyrase binding site (20). After binding, DNA gyrase cleaves each strand at sites separated by 4 bp and transiently forms a covalent phosphotyrosine bond between the 5'-phosphate groups of the cleaved DNA and Tyr-122 of the A subunits (8, 28). A segment of DNA is translocated through the break and presumably through at least part of the protein complex. There are data indicating that DNA gyrase binding to DNA is sufficiently stable to allow processive supercoiling before the enzyme dissociates from the DNA (16). At some point in the reaction, an ATP molecule binds to each B subunit and is hydrolyzed. Binding of ATP promotes a conformational change of the tetramer, and it is thought that this change brings the DNA segment to be translocated close to the double-stranded DNA break (21). The hydrolysis of ATP is required for further catalytic cycles.

For the DNA supercoiling reaction, DNA gyrase requires the divalent cation Mg$^{2+}$ in addition to ATP. When Mg$^{2+}$ is replaced by Ca$^{2+}$, DNA gyrase efficiently cleaves double-stranded DNA, and this cleavage reaction does not require the binding and hydrolysis of ATP. However, subsequent denaturation of the enzyme is necessary to release cleaved DNA (22).

The quinolones, a major group of DNA gyrase inhibitors, interrupt the DNA gyrase-mediated DNA cleavage and rescaling cycle at the cleavage step (2, 3, 23). Addition of a protein denaturant, such as sodium dodecyl sulfate (SDS), to reaction mixtures containing linear, relaxed, or supercoiled DNA in the presence of quinolones releases double-stranded cleaved DNA (26, 27). Cleavage in the presence of quinolones does not require ATP and occurs at preferred sites. In vivo analysis of cleavage sites in the pBR322 plasmid generated in the presence of oxolinic acid has suggested a consensus sequence (Fig. 1). Also shown in Fig. 1 is the site at position 900 which is preferentially cleaved (12, 18). Mutations at this site can reduce or abolish the cleavage activity of DNA gyrase (4). It is thought that cleavage sites generated in the presence of quinolones represent the physiological sites of action of DNA gyrase, and this reaction has been taken as a model for the double-stranded cleavage event during supercoiling (5, 11, 14–16). Shen et al. (25) have proposed that the quinolones bind to the 4-bp single-stranded DNA region at the cleavage site. They

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FIG. 1. Consensus sequence and preferred cleavage site in pBR322.

\[
\begin{align*}
\text{G} & \quad \text{G} \\
5' - \text{GRYCTNYNGY} - 3' & \quad \text{T} \\
990 \\
5' - \text{GGGCTTCCCAT} - 3' &
\end{align*}
\]

preferred cleavage site in pBR322

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proposed that binding of the quinolones to DNA occurs via hydrogen bonding between donors on the DNA (in particular guanine residues) and the carbonyl and carboxyl side chains common to all quinolones. Furthermore, binding is thought to be cooperative, with four or more quinolone molecules interacting with each other by ring stacking. Interaction with DNA gyrase is proposed to occur via the group at C-7 on the quinolones (25). Palù et al. (19) propose that Mg$^{2+}$ acts as a bridge between the phosphogroups of the DNA and the carbonyl and carboxyl moieties of the quinolones. Additional stabilization may arise from stacking interactions between the rings of the quinolones and DNA bases (19). Furthermore, analysis of mutants highly resistant to quinolones showed point mutations in the \textit{gyrA} gene, and these data suggest that the primary target of the quinolones may be subunit A (17, 31).

Pyrimido[1,6-\(a\)]benzimidazoles were recently designed and synthesized and have been found to represent a new class of potent DNA gyrase A subunit inhibitors (9). Preliminary results showed that they also interrupt the DNA gyrase-mediated DNA cleavage and resealing cycle. The present investigations were undertaken to address the questions of whether DNA gyrase cleaves at the same sites in the presence of inhibitors of both classes or in the presence of Ca$^{2+}$ and what role the sequence around position 990 in the pBR322 plasmid plays. As model substrates, we used DNA restriction fragments of 164, 85, and 71 bp from the plasmid pBR322, each containing the 20-bp pBR322 sequence around position 990 at a different position, and we compared the cleavage sites generated by DNA gyrase in the presence of inhibitors of both classes or Ca$^{2+}$.

**MATERIALS AND METHODS**

**PCR.** The pBR322 DNA sequence from positions 921 to 1107 was amplified by PCR. The oligonucleotides used were 5'CCATTATCGCCGGCATGCGG-3' and 5'GGACGCGCATCCTGAAGCTG-3' and were synthesized by Genosys Biotechnologies, Inc. PCR was carried out in 10 mM Tris-HCl (pH 8.3)–50 mM KC$_2$–15 mM MgCl$_2$–0.1% (wt/vol) gelatin with 2.5 U of \textit{Taq} DNA polymerase (AmpliTaq; Perkin Elmer), 10 ng of DNA template, 0.2 mM (each) dATP, dCTP, dGTP, and dTTP, and the primers at 0.5 \(\mu\)M each. The reaction mixture was incubated for 40 cycles of 1 min at 95°C, 1 min at the annealing temperature of 45°C, and 1 min at 72°C. The PCR product was purified by chloroform extraction and by the Prep-A-Gene DNA purification method (Bio-Rad).

**Cloning, isolation, and labeling of DNA fragments.** pUC18 plasmid was digested with \textit{Sma} I (Boehringer) and dephosphorylated with calf intestinal alkaline phosphatase (Boehringer). The PCR product was phosphorylated with ATP by the activity of bacteriophage T4 polynucleotide kinase (Boehringer) and ligated into the \textit{Sma} I site of pUC18 (Fig. 2). Competent \textit{E. coli} HB101 cells were prepared by the calcium chloride method and transformed with the construct described above (24). Plasmid DNA from the transformants was prepared with \textit{MidiQiagen} according to the manufacturer’s instructions and digested with \textit{Bgl} I, \textit{Msp} I, or \textit{Hin} fI (Boehringer), and the restriction fragments were separated on an native 10% polyacrylamide or MetaPhor agarose gel (Fig. 2). The labeled 164-, 85-, and 71-bp fragments were cut out, and the DNA was eluted from the gels slices by incubation in 2 ml of 0.5 M ammonium acetate (pH 8.0)–1 mM EDTA at 37°C for 16 h on a shaker. The buffer was removed, the gel slices were incubated for an additional 5 h at 37°C in 1 ml of the same elution buffer, and the

![FIG. 2. Cloning and isolation of the 164-, 85-, and 71-bp DNA fragments. The pBR322 DNA sequence from positions 921 to 1107 was amplified by PCR, and the PCR product was ligated into the \textit{Sma} I site of pUC18 plasmid. The 205-bp \textit{Bgl}–\textit{HindIII} fragment from this construct was isolated, the 5-final ends were labeled with [\(\gamma\)-\textit{32P}]ATP, and the DNA fragment was digested with \textit{DpnI}, \textit{MspI}, or \textit{Hin} fI. The restriction fragments were separated on a gel, and the labeled 164-, 85-, and 71-bp fragments were cut out and eluted from the gel.](http://aac.asm.org/)

## Table 1. Activities of quinolones and pyrimido[1,6-\(a\)]benzimidazoles towards \textit{E. coli} DNA gyrase in the DNA supercoiling assay

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>IC$_{50}$ µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fleroxacin</td>
<td><img src="http://aac.asm.org/" alt="Fleroxacin" /></td>
<td>4.0</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td><img src="http://aac.asm.org/" alt="Ciprofloxacin" /></td>
<td>0.25</td>
</tr>
<tr>
<td>Ro 42-6990</td>
<td><img src="http://aac.asm.org/" alt="Ro 42-6990" /></td>
<td>1.5</td>
</tr>
<tr>
<td>Ro 47-3559</td>
<td><img src="http://aac.asm.org/" alt="Ro 47-3559" /></td>
<td>0.7</td>
</tr>
<tr>
<td>Ro 46-7864</td>
<td><img src="http://aac.asm.org/" alt="Ro 46-7864" /></td>
<td>8.0</td>
</tr>
</tbody>
</table>

*Note: The table shows the IC$_{50}$ values in µg/ml for different quinolones and pyrimido[1,6-\(a\)]benzimidazoles.*
two buffer samples were combined. The DNA was recovered by ethanol precipi-
tation with 3 volumes of ethanol for 1 h at ~80°C and washed with 70% ethanol, and the DNA was dissolved in 50 μl of TE buffer (10 mM Tris-HCl [pH 8.0]–1 mM EDTA). The 164-bp fragment was sequenced by the Maxam-Gilbert se-
quencing method as indicated in reference 24.

Expression and purification of the A and B subunits of DNA gyrase. Hallett et al. constructed plasmids which overproduce the E. coli DNA gyrase A and B subunits, and these plasmids were introduced into the K-12 derivative JM109, resulting in strains which were named JMlacA and JMlacB (7). The DNA gyrase A and B subunits were purified essentially as described by Hallett et al., with the following modifications. After being harvested, the cell pellets were resuspended in 50 mM Tris-HCl (pH 7.6)–10% sucrose (1 ml/g of cells) and frozen at ~70°C. After thawing, the cell suspension was adjusted to 1 μg of RNAse per ml, 1 μg of DNase per ml, and 20 μg of lysozyme per ml and incubated for 30 min at room temperature. The cells were disrupted at 60 MPa with a French press and centrifuged at 100,000 × g for 60 min at 4°C. Cell extracts were dialyzed against TE buffer (50 mM Tris-HCl [pH 8.0]–1 mM EDTA), and about 10 ml was applied to a heparin-Sepharose column (16 by 100 mm) (Pharmacia). After being washed with TE buffer, the column was eluted with a 60-mI linear gradient of 0.0 to 1.0 M NaCl in TE buffer. Fractions containing the subunits were identified by SDS-PAGE, pooled, dialyzed against TE buffer, and loaded onto a fast protein liquid chromatography Mono Q HR 5/5 column (5 by 50 mm) (Pharmacia). After a washing with TE buffer, the column was eluted with a 60-mI linear gradient of 0.0 to 0.6 M NaCl in TE buffer. Fractions containing the subunits were identified by SDS-PAGE, pooled, and dialyzed against 50 mM Tris-HCl (pH 8.0)–100 mM KC1–5 mM dithiothreitol–1 mM EDTA–10% glycerol, frozen in liquid nitrogen, and stored at ~80°C (1). Subunits A and B were estimated to be >95 and >90% pure, respectively, as judged by scanning SDS-polyacrylamide gels. The DNA gyrase A2B2 complex was reconstituted immediately before use by incubation of equimolar quantities of each DNA gyrase subunit for 30 min at 25°C, diluted to the appropriate concentration with water, and instantly added to the DNA supercoiling assay buffer. The specific activity of the DNA gyrase A2B2 complex was 2 × 107 U/mg. One unit of DNA supercoiling activity is defined as the amount required to convert 150 fmol of pUC18 plasmid to fully supercoiled DNA in 30 min at 37°C in the DNA supercoiling assay.

DNA supercoiling assay. Relaxed pUC18 plasmid (7.5 mM) was incubated in a total volume of 20 μl with E. coli DNA gyrase (about 10 nM, or 1.3 U) in 40 mM Tris-HCl (pH 8.0)–25 mM KC1–4 mM MgCl2–2.5 mM spermidine–1.4 mM ATP for 30 min at 37°C. The compounds to be tested were included in this reaction mixture at concentrations of 0.01 to 100 μM. Reactions were terminated by the addition of 5 μl of loading buffer (20% [wt/vol] Ficol, 2.0% SDS, and 0.04% [wt/vol] bromophenol blue), and the samples were electrophoresed on 12% sequencing gels containing 7 M urea, and electrophoresed in 90 mM Tris-borate–2 mM EDTA. In lanes 25 to 29, the inhibitors were incubated together with Mg2+ in the absence of the DNA gyrase. F, fleroxacin; C, ciprofloxacin; 1, Ro 42-6890; 2, Ro 47-3359; 3, Ro 46-7864.

FIG. 3. Determination of the cleavage sites on the 164-bp DNA fragment generated by DNA gyrase in the presence of Ca2+ or A-subunit inhibitors. The fragment, labeled at the 5′ end of the top strand, was incubated at 37°C for 10 min in a total volume of 10 μl with 1.3 pmol of DNA gyrase in 35 mM Tris-HCl (pH 8.0)–24 mM KC1–2 mM spermidine. CaCl2 or MgCl2 was added at 4 mM, and the inhibitors were included at concentrations from 0.1 to 100 μM. Reactions were stopped by the addition of EDTA to a final concentration of 8 mM and addition of 1% SDS. When MgCl2 was subsequently added, the addition of SDS was omitted and the final MgCl2 concentration was 8 mM. These samples were then incubated for a further 10 min at 37°C, and reactions were stopped with EDTA (final concentration, 16 mM) and 1% SDS. Proteinase K was added to a final concentration of 300 μg/ml, and the samples were digested for 2 h at 37°C. The DNA was purified by phenol-chloroform extraction and ethanol precipitation and redissolved in 5 μl of TE buffer (pH 8.0). A 5-μl volume of loading buffer (50% formamide, 0.05% bromophenol blue, 0.03% xylene cyanol, and 5 mM EDTA) was added, and the samples were heated for 4 min at 90°C, chilled on ice, loaded onto a 12% sequencing gel containing 7 M urea, and electrophoresed in 90 mM Tris-borate–2 mM EDTA. The gels were stained for 45 min in an aqueous solution of ethidium bromide (0.5 μg/ml), and the bands were visualized by transillumination with UV light and photographed. For the determination of 50% inhibitory concentrations (IC50), the photos were scanned with a Macintosh OneScanner and the amount of supercoiled DNA was quantitated by integration over the whole band with the program Scan Analysis from Biosoft.

Analysis of cleavage products on sequencing gels. Stock solutions of the subunit A inhibitors (Table 1) at 1 mM were made as follows: fleroxacin was dissolved in 25% ethanol and 15 mM NaOH, ciprofloxacin and Ro 42-6890 were dissolved in H2O, and Ro 47-3359 and Ro 46-7864 were dissolved in 5 mM HCl. The stock solutions were diluted with H2O and added to the reaction mixtures as indicated in the figures.

The 164-, 85-, and 71-bp DNA fragments, labeled with [γ-32P]ATP at the 5′ ends on the top strands, were incubated at 37°C for 10 min in a total volume of 10 μl with 1.3 pmol of DNA gyrase in 35 mM Tris-HCl (pH 8.0)–24 mM KC1–2 mM spermidine. CaCl2 or MgCl2 was added at 4 mM, and the inhibitors were added at a concentration of 0.1, 1.0, 10, or 100 μM. Reactions were stopped by
the addition of EDTA to a final concentration of 8 mM and addition of 1% SDS. When MgCl₂ was subsequently added, the addition of SDS was omitted and the final MgCl₂ concentration was 8 mM. These samples were then incubated for a further 10 min at 37°C, and the reactions were stopped with EDTA (final concentration, 16 mM) and 1% SDS. Proteinase K was added to a final concentration of 500 µg/ml, and the samples were digested for 2 h at 37°C. The DNA was purified by phenol-chloroform extraction and ethanol precipitation and redissolved in 5 µl of TE buffer (pH 8.0). A 5-µl volume of loading buffer (50% formamide, 0.05% bromophenol blue, 0.03% xylene cyanol, and 5 mM EDTA) was added, and the samples were heated for 4 min at 90°C, chilled on ice, loaded on a 12% sequencing gel containing 7 M urea, and electrophoresed in 90 mM Tris-borate–2 mM EDTA (1× TBE buffer [24]). Reaction products were visualized by autoradiography using Agfa Curix RPI films with CAWO Fast Tung-state intensifying screens.

FIG. 4. Determination of the cleavage sites on the 164-, 85-, and 71-bp DNA fragments generated by DNA gyrase in the presence of Ca²⁺ or A-subunit inhibitors. The DNA fragments were incubated as indicated in Fig. 3. Only the results obtained with the highest inhibitor concentration are shown. Reactions were stopped and DNA was purified as described for Fig. 3. (A) Sequencing gel with the 164-bp fragment. F, fleroxacin; C, ciprofloxacin; 1, Ro 42-6890; 2, Ro 47-3359; 3, Ro 46-7864. (B) Sequencing gel with the 85-bp DNA fragment. (C) Sequencing gel with the 71-bp DNA fragment. (D) Sequencing gel with all three fragments and sequence analysis of the 164-bp DNA fragment (lanes G and GA). The sequence around position 990 of the pBR322 plasmid is shown on the left, and DNA cleavage occurs at the site indicated by the arrow. Only the results obtained with the three Ro compounds are shown.
RESULTS

The inhibitory activity of pyrimido[1,6-a]benzimidazoles in a DNA supercoiling assay with *E. coli* DNA gyrase was similar to that of quinolones (Table 1) (9). We have found that inhibitors of this class also interrupt the DNA gyrase-mediated DNA cleavage and resealing cycle, since cleaved DNA was released after addition of SDS to reaction mixtures containing linearized pBR322 in the presence of pyrimido[1,6-a]benzimidazoles (6).

To determine the sequence specificity of DNA gyrase in the presence of pyrimido[1,6-a]benzimidazoles, quinolones, or Ca\(^{2+}\), we chose as model substrates 164-, 85-, and 71-bp DNA restriction fragments from the pBR322 plasmid (Fig. 2). Each contained the 20-bp sequence originating from position 990 of pBR322 plasmid that is preferentially cleaved in the presence of quinolones (12). These DNA fragments, labeled at the 5′ ends of the top strands, were incubated with DNA gyrase in the presence of either Ca\(^{2+}\) or Mg\(^{2+}\) and increasing concentrations of inhibitors. After incubation, the protein was denatured with SDS and digested with proteinase K, and the DNA was purified and loaded onto a denaturing gel (Fig. 3 and 4). To determine the positions of the cleavage sites, the 164-bp DNA fragment was sequenced (Fig. 4D).

DNA gyrase alone, without any divalent cation in the reaction buffer, did not cleave the DNA fragments, and neither did the inhibitors together with Mg\(^{2+}\) in the absence of the enzyme. Figure 3 shows as an example the results obtained with the 164-bp DNA fragment (Fig. 3, lanes 1 and 25 to 29). In the presence of Mg\(^{2+}\), uncleaved DNA or only small amounts of cleaved DNA could be observed (Fig. 3, lane 4; Fig. 4A through C, lanes 2). This was also the case when reactions were carried out in the presence of Ca\(^{2+}\) as a first step and then stopped with EDTA and further incubated with Mg\(^{2+}\) (Fig. 3, lane 3). These results confirm earlier observations obtained with supercoiled, relaxed, or linear pBR322 or pUC18 plasmids (6). DNA gyrase retained its religation activity after carrying out the cleavage reaction in the presence of Ca\(^{2+}\), since termination of the cleavage reaction by EDTA and subsequent addition of Mg\(^{2+}\) induced the enzyme to perform the resealing reaction and to release uncut DNA.

DNA gyrase cleaved the 164-bp fragment in the presence of Ca\(^{2+}\) mainly at the expected site within the 20-bp sequence (Fig. 3, lane 2; Fig. 4A, lane 1, and D, lane 3). The optimal Ca\(^{2+}\) concentration for this cleavage reaction was between 1 and 6 mM (6). This DNA fragment was efficiently cleaved in the presence of Ca\(^{2+}\) and is likely to reflect the length of DNA required by DNA gyrase to form a DNA-protein complex, since it is known that about 120 bp is needed to wrap around the DNA gyrase (13, 21).

As in the presence of Ca\(^{2+}\), the 164-bp fragment was also preferentially cleaved at the expected site within the 20-bp sequence in the presence of inhibitors of both classes and Mg\(^{2+}\) (Fig. 3, lanes 3 to 24; Fig. 4A, lanes 3 to 7, and D, lanes 5 to 7). However, the DNA fragment was also cleaved, though less efficiently, at several additional sites. These additional weak cleavage products were in principle the same for each inhibitor, and some of them were also visible after cleavage reactions carried out in the presence of Ca\(^{2+}\) but only after a longer exposure of the gels (Fig. 4D). The activities of the different inhibitors in the DNA supercoiling assay were reflected in their potencies in promoting DNA cleavage. At a 100 μM concentration of each inhibitor, DNA gyrase cleaved each DNA fragment efficiently. Figure 3, lanes 5 to 24, shows as an example the results obtained with the 164-bp DNA fragment.

DNA gyrase cleaved the 85-bp fragment in the presence of Ca\(^{2+}\) only weakly at the expected site within the 20-bp sequence but much more strongly at a site about 16 bp downstream (Fig. 4B, lane 1, and D, lane 13). It is known that a 34-bp sequence containing the 20-bp preferred cleavage sequence is not a cleavage substrate, but if this sequence is extended with flanking sequences in either direction, cleavage will occur (4, 13). Cleavage in the presence of Ca\(^{2+}\) at this downstream site can be explained by an asymmetric wrapping of the DNA fragment around the enzyme. Presumably, 70 bp of the 5′ end was wrapped around one site of the tetramer and about 15 bp was wrapped around the other. As a consequence, DNA gyrase cleaved not within the 20-bp sequence but within a sequence which is about 15 bp from the 3′ end of the fragment. Cleavage at the 5′ end of the DNA fragment would lead to a labeled cleavage product of about 15 bp, and in fact, DNA fragments of about 15 and 20 bp could be detected (Fig. 4D, lane 13).

In the presence of the inhibitors (except Ro 42-6890), the DNA fragment was also cleaved at the site about 16 bp downstream and, in addition, was cleaved strongly at two sites within the 20-bp sequence around the preferred cleavage site (Fig. 4B, lanes 3 to 7, and D, lanes 15 to 17). With this DNA fragment, differences between quinolones and pyrimido[1,6-a] benzimidazoles could be observed only in the relative preferences for these cleavage sites. In this context, it is worth
mentioning that Ro 42-6890 differs from the other pyrimido[1,6-\(a\)]benzimidazole inhibitors, since it does not chelate divalent cations but nevertheless inhibits the DNA gyrase-promoted supercoiling reaction as well as the quinolones and the other pyrimido[1,6-\(a\)]benzimidazoles do (Table 1) (10). In addition, this 85-bp DNA fragment was cleaved more frequently at additional sites in the presence of inhibitors than in the presence of Ca\(^{2+}\), although less efficiently. However, these additional weak cleavage sites appeared to be the same for each inhibitor.

DNA gyrase only weakly cleaved the 71-bp fragment in the presence of Ca\(^{2+}\) (Fig. 4C, lane 1, and D, lane 8). This fragment is presumably too short to be positioned onto the enzyme and therefore cannot be cleaved at the expected site (13). In the presence of each inhibitor, however, the fragment was efficiently cleaved by DNA gyrase at apparently the same sites within the 20-bp sequence as observed with the 85-bp DNA fragment, although cleavage was not clearly visible at the site 16 bp downstream, which is presumably too close to the 3' end to be efficiently cleaved or detected (Fig. 4C, lanes 3 to 7, and D, lanes 10 to 12). However, especially with Mg\(^{2+}\) alone, an increased number of fragments cleaved close to the 3' end of the 71-bp DNA fragment could be observed (Fig. 4C, lane 2, and D, lane 9). It is possible that the short 3' end oligonucleotides remained unbound or not correctly bound to the enzyme after the cleavage reaction and that therefore the religation reaction could not be accomplished.

**DISCUSSION**

Quinolones interrupt the DNA cleavage and resealing cycle at the cleavage step, and we have found that pyrimido[1,6-\(a\)]benzimidazoles, a new class of DNA gyrase inhibitors, have probably the same mode of action (6). In addition, a quinolone-resistant *E. coli* gyrA mutant and a quinolone-resistant *Staphylococcus aureus* mutant were also resistant to pyrimido[1,6-\(a\)]benzimidazoles (data not shown). Furthermore, it was shown that cleavage induced in the presence of Ca\(^{2+}\) occurs at the same sites as those obtained in the presence of oxolinic acid but with different relative efficiencies (23). To determine the DNA sequence specificities of pyrimido[1,6-\(a\)]benzimidazoles, quinolones, and Ca\(^{2+}\) in DNA gyrase-mediated cleavage reactions, we used as model substrates DNA restriction fragments of 164, 85, and 71 bp from the plasmid pBR322, each containing at a different position the 20-bp sequence around position 990 at which DNA gyrase preferentially cleaves in the presence of quinolones.

Our results show that the enzyme cleaves mainly the 164-bp DNA fragment at the expected site within the 20-bp sequence, regardless of the presence of the inhibitors or Ca\(^{2+}\) (Fig. 3 through 5). These results support our earlier observations that both inhibitor classes, the quinolones and the pyrimido[1,6-\(a\)]benzimidazoles, have in principle the same mode of action and interrupt the DNA cleavage and resealing cycle at the cleavage step (6, 9). In addition, they show that both inhibitor classes and Ca\(^{2+}\) have the same sequence specificity within this DNA fragment. Whether the pyrimido[1,6-\(a\)]benzimidazoles promote DNA gyrase to cleave strongly outside the DNA region studied remains to be determined. However, in contrast to the Ca\(^{2+}\)-induced cleavage reaction, the DNA fragment was also cleaved, though less efficiently, at several additional sites in the presence of inhibitors. Therefore, it appears that DNA gyrase cleaves more frequently in their presence and at sites which are not recognized or only weakly recognized by the enzyme in the presence of Ca\(^{2+}\).

In the presence of Ca\(^{2+}\), the 85-bp and the 71-bp DNA fragments were cleaved only weakly and with some inaccuracy within the 20-bp sequence. This may reflect the length of DNA required by DNA gyrase to form a cleavable DNA-enzyme complex in the absence of inhibitors (13, 21). Since the 85-bp and the 71-bp fragments contain only about 55 bp in one

![Diagram of DNA cleavage sites](http://aac.asm.org/)

**FIG. 5.** Schematic representation of the cleavage sites on the 164-, 85-, and 71-bp DNA fragments generated by DNA gyrase in the presence of Ca\(^{2+}\) or subunit A inhibitors. The sequences of the DNA fragments are shown. The sequence around position 990 of the pBR322 plasmid (boxes), the sites cleaved by DNA gyrase in the presence of Ca\(^{2+}\) or of subunit A inhibitors (arrows), and the major cleavage sites (large arrows) are indicated.
direction from the expected cleavage site and about 30 and 16 bp, respectively, in the other direction, one can speculate that these DNA arms cannot be positioned onto the enzyme and that, therefore, cleavage at the expected site cannot occur. The 85-bp fragment was cleaved in the presence of Ca$^{2+}$ or inhibitors at a site about 16 bp downstream from the expected site (Fig. 4 and 5), possibly because of an asymmetric binding of the DNA fragment to the enzyme in which about 70 bp of the 5' end is wrapped around one site of the enzyme and about 15 bp of the 3' end is wrapped around the other. As a consequence, DNA gyrase cleaved within a sequence which is about 15 bp from the 3' end of the DNA fragment. The segment around this cleavage site shares some homology with the 20-bp sequence. The consensus sequence is 5'-ATGPYYYYYYYY PYY-3', but it remains to be determined whether it is this sequence which plays a role in determining the cleavage site. The 71-bp DNA fragment contains only the bases 5'-ATG-3' of this consensus sequence, so cleavage at this site presumably cannot be detected. If about 70 bp of the 3' end of the DNA fragment is wrapped around the enzyme, cleavage of the 85- and 71-bp DNA fragments would lead to a labeled cleavage product of about 15 bp, and in fact DNA fragments of about 15 and 20 bp could be detected (Fig. 4D). In summary, it seems that probably the sequence and obviously also the length of the DNA fragments are critical for cleavage reactions carried out in the presence of Ca$^{2+}$.

In contrast to the results obtained in the presence of Ca$^{2+}$, both classes of A-subunit inhibitors resulted in cleavage of the 85- and 71-bp DNA fragments within the 20-bp sequence around position 990 (Fig. 4 and 5). This indicates that these DNA fragments, despite their insufficient length in principle, were accepted as substrates for cleavage reactions in the presence of inhibitors and that they were positioned onto the enzyme in such a way that cleavage could occur at the expected site. It is possible that the inhibitors interact with DNA at inhibitor-specific positions, thus determining cleavage sites by forming a ternary complex between DNA gyrase, DNA, and inhibitors. It may be the primary sequence or the secondary DNA structure which induces the formation of such ternary complexes. Hence, the sites where DNA gyrase cleaves in the presence of inhibitors need not necessarily represent physiological sites of action of DNA gyrase.

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