ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 1990, p. 1450–1452
0066-4804/90/071450-03$02.00/0

Vol. 34, No. 7

In Vitro Inhibition of Bacterial DNA Gyrase by Cinodine, a Glycocinnamoylspermidine Antibiotic

MARCIA S. OSBURNE,* WILLIAM M. MAIESE, AND MICHAEL GREENSTEIN

Lederle Laboratories, Medical Research Division, American Cyanamid Company, Pearl River, New York 10965

Received 22 February 1990/Accepted 11 April 1990

Cinodine, a broad-spectrum glycocinnamoylspermidine antibiotic, binds to DNA and irreversibly inhibits bacterial and phage DNA synthesis. Cinodine was found to inhibit the activity of *Micrococcus luteus* DNA gyrase in vitro, but it did not inhibit the activities of two other DNA-binding enzymes, namely, topoisomerase I and *Bam*HI. Although we cannot yet conclude that DNA gyrase is an intracellular target of the drug, in vitro inhibition of the enzyme by cinodine appears to be specific.

Bacterial topoisomerase II (DNA gyrase) is an essential enzyme that is known to be the target of two classes of antibiotics. The coumarins, natural products which include coumermycin A1 and novobiocin, inhibit gyrase probably by competing with ATP for binding to the B subunit of the enzyme (6, 11). The quinolones, synthetic products typified by nalidixic and oxolinic acids, target the A subunit of the enzyme and probably act by interfering with the DNA-rejoining step of the gyrase-mediated, DNA strand-passing reaction (1, 4, 5, 12; for recent reviews of topoisomerase inhibitors, see references 2 and 13).

The broad-spectrum antibiotic cinodine, which is produced by a *Nocardia* species (3, 9), represents a class of antibiotics referred to as the glycocinnamoylspermidines. The three components of cinodine, β, γ_1, and γ_2, are distinguished by the structure of the terminal pentose of the trisaccharide (Fig. 1). In previous studies of its mechanism of action (7), cinodine was shown to cause an immediate and irreversible cessation of DNA synthesis in growing cultures of *Escherichia coli*, whereas RNA synthesis remained unaffected for at least 30 min. Furthermore, equilibrium dialysis studies showed that cinodine was able to bind physically to DNA (7). In the present study, we investigated the mechanism of action of cinodine further, and we found that the drug inhibits bacterial DNA gyrase in vitro.

DNA gyrase activity of *Micrococcus luteus* is inhibited by cinodine. The conversion of relaxed plasmid pBR322 DNA to the supercoiled form by *M. luteus* DNA gyrase was tested in the presence and absence of cinodine. Purified DNA gyrase from *M. luteus* was obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md. DNA gyrase was assayed in vitro for the ability to convert relaxed covalently closed circular DNA to the supercoiled form in the presence of ATP by the standard electrophoretic assay method of Otter and Cozzarelli (10). The relaxed substrate (0.5 μg of plasmid pBR322) was prepared by treating supercoiled plasmid DNA with topoisomerase I isolated from calf thymus (Bethesda Research Laboratories), according to the directions of the manufacturer, followed by three phenol-chloroform extractions (to remove the enzyme) and ethanol precipitation of the DNA. Gyrase assays were carried out in a total volume of 20 μl. Enzyme (1 U) was added to the assay mixture as the last step, to initiate the reaction; the mixture was then incubated at 30°C for 1 h and applied directly to a 0.8% agarose gel. Samples were electrophoresed at 150 V/cm for 2 h, stained with ethidium bromide, and photographed. Gyrase activity was dependent on the addition of ATP (Fig. 2A). Stock solutions of cinodine, which was obtained from Lederle Laboratories, Pearl River, N.Y., were prepared in deionized water at a concentration of 1 mg/ml and stored at −70°C. Thawed stocks were used once and discarded.

Results (Fig. 2A) indicate that cinodine inhibited the supercoiling reaction by DNA gyrase at drug concentrations of between 0.5 and 1 μg/ml. At these concentrations, the amount of DNA appearing in the supercoiled band was noticeably diminished. A second effect of cinodine, altered mobility of DNA, was seen in our assay at cinodine concentrations of 5 to 10 μg/ml and above (Fig. 2A and B). This effect is consistent with the previous demonstration that cinodine physically binds to DNA (7).

In a separate experiment, *M. luteus* DNA gyrase was assayed in the presence and absence of cinodine, and DNA bands were quantitated by scanning of film negatives by densitometry (model CS930 densitometer; Shimadzu). The fraction of DNA appearing in the supercoiled band only is presented in Table 1. Significant inhibition of supercoiling by cinodine was apparent at drug concentrations of 0.5 μg/ml.

To ascertain that cinodine actually inhibited DNA gyrase activity and did not merely bind to supercoiled DNA in such a way as to cause it to comigrate with relaxed DNA, the following control experiment was performed. Relaxed plasmid pBR322 DNA was treated with DNA gyrase from *M. luteus* as described above. After sufficient time for comple-

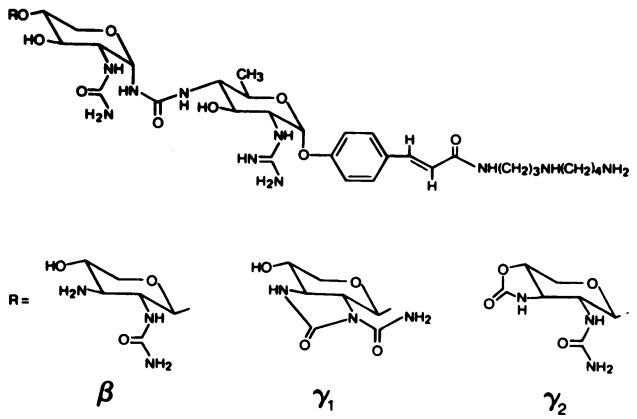

FIG. 1. Structures of the β, γ_1, and γ_2 forms of cinodine.

* Corresponding author.

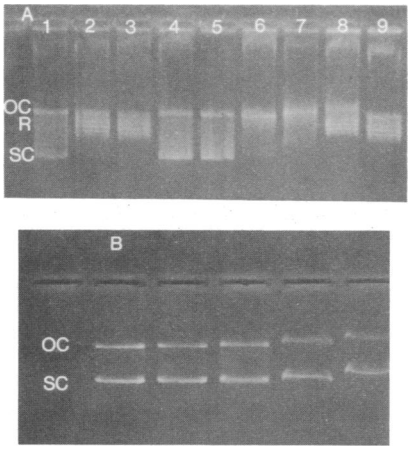

FIG. 2. Effect of cinodine on supercoiling of relaxed plasmid pBR322 DNA by *M. luteus* DNA gyrase. (A) Cinodine present during the supercoiling reaction. Lane 1, complete, no cinodine; lane 2, no cinodine, no ATP; lane 3, no cinodine, no gyrase; lane 4, complete, 0.1 μg of cinodine per ml; lane 5, complete, 0.5 μg of cinodine per ml; lane 6, complete, 1 μg of cinodine per ml; lane 7, complete, 5 μg of cinodine per ml; lane 8, complete, 10 μg of cinodine per ml; lane 9, complete, 25 μg of novobiocin per ml. (B) Cinodine added after the supercoiling reaction. All reactions contained the complete assay mixture and, in addition, from left to right, 0, 1, 2.5, 5, and 10 μg of cinodine per ml, respectively, which was added after the supercoiling reaction was completed. Relaxed (R), supercoiled (SC), and open circular (OC) forms of plasmid DNA are indicated.

tion of the reaction, various concentrations of cinodine were added to the DNA, which was then analyzed on an agarose gel as described above. In this case, cinodine had no apparent effect on supercoiling (Fig. 2B), although, as before, the mobility of DNA was altered at cinodine concentrations of 5 μg/ml and above. Thus, it is highly unlikely that the inhibitory effect of low concentrations of cinodine on the accumulation of supercoiled DNA in the DNA gyrase reaction was due to altered DNA migration resulting from cinodine binding. Rather, inhibition was most likely caused by direct interference with the DNA-supercoiling activity of gyrase.

Effect of cinodine on other DNA-binding enzymes. Because cinodine is known to bind to DNA, we considered the possibility that the drug may interfere with gyrase activity in a nonspecific manner. Therefore, we evaluated the effect of cinodine on the activities of topoisomerase I (Bethesda Research Laboratories) and the restriction endonuclease *Bam*HI (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Both enzymes were used according to the directions of the manufacturer, with 0.5 μg of plasmid pBR322 used as the substrate. Reactions were subjected to electrophoresis through 0.8% agarose gels, as described above for the gyrase assays. There was no apparent inhibition of either topoisomerase I or *Bam*HI at concentrations of cinodine up to 5 μg/ml (Fig. 3 and 4, respectively). Thus, the in vitro inhibition of DNA gyrase by cinodine appears to be specific. Even if inhibition of gyrase by cinodine is due, in part, to binding of the drug to DNA, the drug must bind in such a way as to inhibit gyrase specifically and not to inhibit the other two DNA-binding enzymes. Although we cannot, as yet, conclude that DNA gyrase is an intracellular target of the drug, we have shown that cinodine, a natural product, represents a new class of antibiotic which inhibits DNA gyrase activity in vitro.

TABLE 1. In vitro activity of DNA gyrase from *M. luteus* in the presence of cinodine

Assay mixture	Fraction of DNA converted from relaxed to supercoiled form[a]
Complete, no drug	1.00
Complete + 25 μg of novobiocin per ml	<0.11
Complete + 0.1 μg of cinodine per ml	1.00
Complete + 0.5 μg of cinodine per ml	0.15
Complete + 1 μg of cinodine per ml	<0.11
Complete + 2.5 μg of cinodine per ml	<0.11

[a] Determined by densitometry.

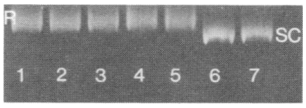

FIG. 3. Effect of cinodine on activity of topoisomerase I. Enzyme activity was assayed as described in the text. Each lane contained 0.5 μg of plasmid pBR322 treated with 1 U of topoisomerase I. Lane 1, complete, no cinodine; lane 2: complete, 0.1 μg of cinodine per ml; lane 3, complete, 0.5 μg of cinodine per ml; lane 4, complete, 1 μg of cinodine per ml; lane 5, complete, 5 μg of cinodine per ml; lane 6, no enzyme; lane 7, 5 μg of cinodine per ml, no enzyme. R, Relaxed plasmid DNA; SC, supercoiled plasmid DNA.

We have made many attempts to isolate cinodine-resistant mutants of *E. coli*. Interestingly, the appearance of resistant mutants is extremely rare. To date we have isolated four spontaneous cinodine-resistant mutants, and these were derived from strain KNK453, a temperature-sensitive *nal* derivative of *E. coli* C (8). We have been unable to isolate mutants from a DNA gyrase wild-type strain. It is possible that a specific type of preexisting alteration in DNA gyrase might be necessary for a cell to express cinodine resistance and retain viability. Furthermore, there may be only a very narrow range of drug concentrations at which a cinodine-resistant gyrase could ensure cell survival in the presence of

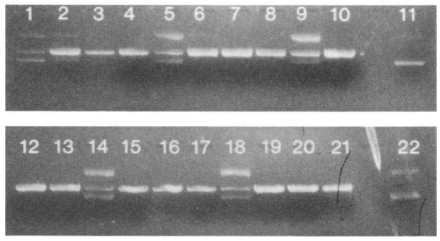

FIG. 4. Effect of cinodine on activity of *Bam*HI. Enzyme activity was assayed over a time course of 15 min. Each lane contained 0.5 μg of plasmid pBR322 DNA digested with 2 U of *Bam*HI. Lanes 1 through 4 represent a time course of cutting for 30 s, 2 min, 5 min, and 15 min, respectively, in the absence of cinodine; lanes 5 through 8 show the same time course as those in lanes 1 through 4, respectively, in the presence of 0.1 μg of cinodine per ml; lanes 9, 10, 12, and 13 show the same time course as those in lanes 1 through 4, respectively, in the presence of 0.5 μg of cinodine per ml; lanes 14 through 17 show the same time course as those in lanes 1 through 4, respectively, in the presence of 1 μg of cinodine per ml; lanes 18 through 21 show the same time course as those in lanes 1 through 4, respectively, in the presence of 5 μg of cinodine per ml; lanes 11 and 22 contain uncut plasmid pBR322 DNA.

the drug. At higher concentrations, even a cell containing a resistant enzyme might be killed because of binding of the drug to DNA. In vitro studies of DNA gyrase and its separated subunits from mutant and wild-type strains may help to elucidate these points.

We thank B. Bachmann for bacterial strains, V. Doelling for help with densitometry, and D. Rothstein and E. Baum for critical review of the manuscript.

LITERATURE CITED

1. **Cozzarelli, N. R.** 1980. DNA gyrase and the supercoiling of DNA. Science **207**:953–960.
2. **Drlica, K., and R. J. Franco.** 1988. Inhibitors of DNA topoisomerases. Biochemistry **27**:2253–2259.
3. **Ellestad, G. A., D. B. Cosulich, R. W. Broschard, J. H. Martin, M. P. Kunstmann, G. O. Morton, J. E. Lancaster, W. Fulmor, and F. M. Lovell.** 1978. Glycocinnamoylspermidines, a new class of antibiotics. III. The structures of LL-BM123 β, γ_1, and γ_2. J. Am. Chem. Soc. **100**:2515–2524.
4. **Gellert, M.** 1981. DNA topoisomerases. Annu. Rev. Biochem. **50**:879–910.
5. **Gellert, M., K. Mizuuchi, M. H. O'Dea, T. Itoh, and J. Tomizawa.** 1977. Nalidixic acid resistance: a second genetic character involved in DNA gyrase activity. Proc. Natl. Acad. Sci. USA **74**:4772–4776.
6. **Gellert, M., M. H. O'Dea, T. Itoh, and J. Tomizawa.** 1976. Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. Proc. Natl. Acad. Sci. USA **73**:4474–4478.
7. **Greenstein, M., J. L. Speth, and W. M. Maiese.** 1981. Mechanism of action of cinodine, a glycocinnamoylspermidine antibiotic. Antimicrob. Agents Chemother. **20**:425–432.
8. **Kreuzer, K. N., and N. R. Cozzarelli.** 1979. *Escherichia coli* mutants thermosensitive for deoxyribonucleic acid gyrase subunit A: effects on deoxyribonucleic acid replication, transcription, and bacteriophage growth. J. Bacteriol. **140**:424–435.
9. **Martin, J. H., M. P. Kunstmann, F. Barbatschi, M. Hertz, G. A. Ellestad, M. Dann, G. S. Redin, A. C. Dornbush, and N. A. Kuck.** 1978. Glycocinnamoylspermidines, a new class of antibiotics. II. Isolation, physicochemical and biological properties of LL-BM123 β, γ_1, and γ_2. J. Antibiot. **31**:398–404.
10. **Otter, R., and N. R. Cozzarelli.** 1983. *Escherichia coli* DNA gyrase. Methods Enzymol. **100**:171–180.
11. **Sugino, A., N. Higgins, P. Brown, C. Peebles, and N. Cozzarelli.** 1978. Energy coupling in DNA gyrase and the mechanism of action of novobiocin. Proc. Natl. Acad. Sci. USA **75**:4838–4842.
12. **Sugino, A., C. Peebles, K. Kreuzer, and N. Cozzarelli.** 1977. Mechanism of action of nalidixic acid: purification of *Escherichia coli nalA* gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. Proc. Natl. Acad. Sci. USA **74**:4767–4771.
13. **Wang, J. C.** 1987. Recent studies of DNA topoisomerases. Biochim. Biophys. Acta **909**:1–9.