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NOVOBIOCIN AND NALIDIXIC ACID TARGET PROTEINS IN YEAST

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SUMMARY

Novobiocin (and its related drug, coumermycin A_1) and nalidixic acid are specific inhibitors of DNA gyrase in bacteria. These drugs inhibit many enzymatic activities in yeast; such as DNA polymerase activity in crude extracts, in vitro 2-μm plasmid DNA replication, purified DNA polymerase I and II, and topoisomerase I. Therefore, the inhibition by these inhibitors in yeast is not specific for a particular enzyme.

INTRODUCTION

Specific inhibitors of DNA replication are very useful for understanding the roles of various enzymes in complex DNA replication (1). They are particularly important in eukaryotes whose genetics has not been well ascertained. Novobiocin (and its related drug, coumermycin A_1) and nalidixic acid (and its derivative, oxolinic acid) are specific inhibitors of in vivo DNA replication and more specifically, of DNA gyrase in bacteria (see ref. 2 for a review). DNA gyrase purified from either novobiocin or nalidixic acid resistant bacteria is much less sensitive than the wild-type enzyme and therefore appears to be the target protein of these drugs (3-5).

Recently, these inhibitors have been used to search for activities similar to DNA gyrase in eukaryotes (6-8). Semi-in vitro DNA replication utilizing intact rat liver mitochondria for incorporation of nucleotides into mitochondrial DNA, is sensitive to novobiocin and nalidixic acid (6). It was concluded that DNA gyrase activity is present in the mitochondria. However, the direct supporting biochemical evidence is still missing.

A new type of topoisomerase (topoisomerase II), which requires ATP for activity, has been purified from Drosophila eggs (9), and other organisms (10,11). Interestingly,

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the enzyme is sensitive to novobiocin, but not to nalidixic acid (9,11). While these results were in press, we observed that in vitro repication of yeast plasmid 2-μm DNA (12,13) is also sensitive to novobiocin and nalidixic acid. This observation led us to further analyze the target proteins of novobiocin and nalidixic acid in yeast.

In this paper, we show that several enzymatic activities, including purified DNA polymerase I and II and topoisomerase I in yeast, are similarly inhibited by novobiocin, but not by nalidixic acid. This indicates that novobiocin inhibition is not specific for a particular enzyme reaction in yeast.

MATERIALS AND METHODS

Chemicals: Coumermycin A_1 was a gift from Dr. K. Price (Bristol Laboratories). Novobiocin, nalidixic acid and all nucleotides were purchased from Sigma Chemicals; DEAE-Sephacel was from Pharmacia Chemicals; hydroxylapatite (Bio-Gel HTP) was from Bio-Rad Laboratories; [methyl-^{3}H]dTTP (75.2 Ci/mmole) was from New England Nuclear Co.

DNAs: Escherichia coli plasmid pBR322 DNA and the chimeric plasmid pJDB36 DNA consisting of yeast 2-μm DNA inserted into pMB9 (14) were purified as published (15). Covalently closed, relaxed pBR322 DNA was prepared as before (4) using yeast topoisomerase I (see below). Activated calf thymus DNA was prepared by treatment of native DNA with pancreatic DNase I as published (16).

Enzymes: Yeast DNA polymerase I and II were purified from yeast Saccharomyces cerevisiae (S. cerevisiae) A364A cells, grown to mid-logarithmic phase as published (17). Topoisomerase I (18) was also purified from S. cerevisiae A364A. The cells (6×10^{11}) were grown in 6-L YPD medium (19), collected by centrifugation at room temperature and resuspended in 100 ml of 10% sucrose, 50 mM Tris-HCl, pH7.5. The cell suspension was quickly frozen in liquid N_2 and stored until use. After thawing to room temperature, the suspension was made 10 mM in EDTA, 5 mM in spermidine, and 500 μg/ml Zymolyase 60,000 (Kirin Brewery Co., Ltd., Japan), successively, and incubated at 0°C for 30 min. Then, 0.15 M KCl, 0.5 mM phenylmethanesulfonylfluoride (PMSF) and 0.1% Brij 58 were successively added into the cell suspension, and cell lysis was accomplished with an additional 10 min incubation at 30°C. The cell lysate was chilled to 0°C for 10 min, centrifuged at 35,000 rpm for 20 min in a Spinco SW41 rotor. The subsequent steps were performed at 4°C, unless otherwise indicated. Solid $(NH_4)_2SO_4$ was slowly added to the supernatant with stirring, to 50% saturation, over a period of 20 min or more. After stirring for an additional 20 min the sample was centrifuged at 12,000 rpm for 20 min in a Sorvall SS34 rotor. The precipitates were resuspended in 10 ml of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol and 0.1 mM PMSF (Buffer A), dialyzed against Buffer A, and applied to a DEAE-Sephacel column (5.0 x 8.8 cm) equilibrated with Buffer A. The column was washed exhaustively with Buffer A, and topoisomerase was eluted with a 1.7 liter linear gradient of 0-0.5 M NaCl in Buffer A. The active fractions were pooled and precipitated as before with $(NH_4)_2SO_4$ added to 50% saturation, then resuspended in 12 ml of 25 mM KPO_4, pH 6.8, 10 mM 2-mercaptoethanol, 10% glycerol, and 0.1 mM PMSF (Buffer B). The enzyme was dialyzed against Buffer B and applied to a 2.3 x 8.8 cm hydroxylapatite column equilibrated with Buffer B. A 120 ml linear gradient of 25-500 mM KPO_4, pH 6.8, containing 10 mM 2-mercaptoethanol, 10% glycerol, and 0.1 mM PMSF was run through the column. The enzyme was eluted at about 0.35 M KPO_4. The active fractions were pooled,

dialyzed against 30% polyethylene glycol 6,000, then 50% glycerol containing 50 mM Tris-HCl, pH7.5, 1 mM EDTA, and 0.1 mM PMSF and stored at -20°C.

Topoisomerase I from rat liver nuclei was purified as described (10). DNA polymerase α from calf thymus and DNA polymerase γ from calf liver were purchased from Worthington Biochemicals.

Enzyme assays: In vitro replication of yeast 2-μm DNA was investigated using the chimeric plasmid pJDB36 as a template in crude extracts of S. cerevisiae cells as described (12,13). This plasmid consists of the 2-μm DNA inserted into pMB9. The reaction mixture (50 μl) contained 35 mM Hepes buffer, pH 7.8, 10 mM $MgCl_2$, 1 mM spermidine, 1 mM DPN, 5 mM ATP, 100 μM of each of the four dNTP's (dTTP was labelled with ^3H, specific activity 500 cpm/pmole), 1 μg pJDB36 DNA, 100 μg/ml bovine serum albumin, and crude extracts of yeast containing 1-5 mg protein per ml.

The reactions of DNA polymerase I and II were performed as described (17).

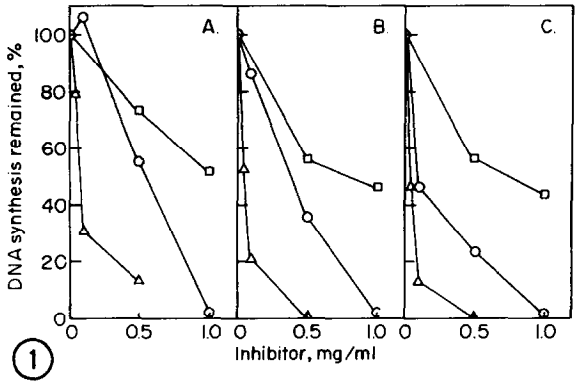
The reaction mixture (17 μl) for topoisomerase I contained 0.4 μg of native supercoiled pBR322 DNA, 50 mM Tris-HCl, pH 7.8, 150 mM KCl, 1 mM dithiothreitol, and enzyme. Topoisomerase II activity was measured as reported (9). Both reactions were incubated at 30°C for 30 min and terminated by addition of 5 μl of 20% sodium dodecylsulfate, 20% glycerol, and 0.05% bromophenol blue. The products were analyzed by electrophoresis through 1% agarose gels as previously described (4). The gels were stained with ethidium bromide and photographed over long wave UV irradiation. Relaxation of the native pBR322 DNA was quantitated by densitometric tracing of the photograph negatives (21).

RESULTS AND DISCUSSION

DNA gyrase introduces negative supercoils into a covalent closed circular double-stranded DNA in the presence of ATP. This enzyme is the target protein of both novobiocin (and its related drug, coumermycin A_1) and nalidixic acid (and its derivative, oxolinic acid) in bacteria (ref. 2 for a review). These characteristics have been exploited to search for similar enzymatic activity in various eukaryotic organisms. In one such study, it was shown that in vitro replication of mitochondrial DNA from rat liver is sensitive to both novobiocin and nalidixic acid, implying that the mitochondria possess a DNA gyrase activity similar to bacteria (6). However, a direct enzymological evidence is still missing. Moreover, a DNA-binding protein, called HM, in yeast mitochondria, and nicking-closing enzyme can generate negative supercoils in a relaxed circular DNA (22) in a manner similar to histones or histone-like proteins and a nicking-closing enzyme (23) suggesting no need for a DNA gyrase activity in yeast mitochondria. However, a newly purified topoisomerase (topoisomerase II) from Drosophila melanogaster eggs (9), and other systems (10,11, and our unpublished results) which requires

ATP for activity, is sensitive to novobiocin but not to nalidixic acid (9,11, and our unpublished results). These observations encouraged us to search for enzymatic activities sensitive to novobiocin and nalidixic acid.

As shown in Fig. 1B, our in vitro replication system of 2-μm yeast plasmid DNA which mimics in vivo replication (12,13) exhibited sensitivity to both novobiocin and nalidixic acid as was in vitro replication system of mitochondrial DNA from rat liver (6). The inhibition is less severe, however, with nalidixic acid. This observation suggested that either DNA gyrase and/or topoisomerase II might perticipate in 2-μm plasmid DNA replication. Similarly, Fig. 1A shows that DNA polymerase activity was sensitive to novobiocin and nalidixic acid in crude extracts from yeast when activated calf thymus DNA was used as a template-primer. Fig. 1C also shows that the sensitivity to novobiocin increased when the purified DNA polymerase I of yeast was used, instead of crude cell extracts. In all cases, coumermycin A_1 was roughly an order of magnitude more potent than novobiocin. These results suggest that the target of novobiocin and nalidixic acid in yeast was at least in part DNA polymerase I, instead of topoisomerase II, since we could detect no significant topoisomerase II and DNA gyrase activities in yeast crude extracts (data not shown). However, novobiocin inhibition was not specific for DNA polymerase I reaction. DNA polymerase II, which is associated with 3'→5' exonuclease activity and which is immunologically distinguished from DNA polymerase I (7) was similarly inhibited by novobiocin and coumermycin A_1 (data not shown). Furthermore, DNA polymerases from other organisms, such as DNA polymerase α from calf thymus and Drosophila melanogaster cell line, and DNA polymerase γ from calf liver, also were as sensitive to novobiocin as yeast DNA polymerase I and II (data not shown). Surprisingly, topoisomerase I (nicking-closing enzyme) of yeast (18) was also inhibited by novobiocin and coumermycin A_1, but not by nalidixic acid (Fig.2 and 3), although the preparation of topoisomerase I did not contain any detectable topoisomerase II activity (Fig. 3). Topoisomerase I from rat liver nuclei was also as sensitive to novobiocin as yeast enzyme (data not shown). Novobiocin sensitivity of yeast topoisomerase I was not identical to that of topoisomerase II from Drosophila melanogaster and Xenopus



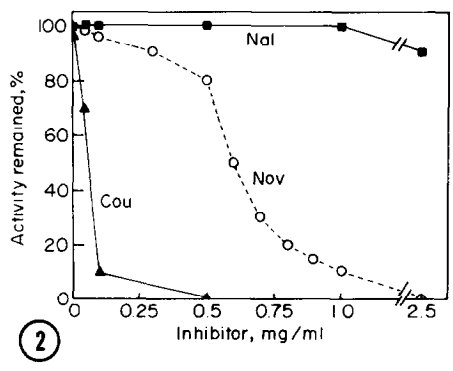


Fig. 1. Antibiotic sensitivity of in vitro DNA synthesis in yeast S. cerevisiae. DNA synthesis activity was measured in the presence of various concentrations of nalidixic acid (-□-), novobiocin (-O-), or coumermycin A_1 (-Δ-). Templates used were activated calf thymus DNA (A and C) or the chimeric plasmid pJDB36 DNA (B) in either crude extracts from S. cerevisiae A364A (A and B) or with purified yeast DNA polymerase I (C). The three figures were standardized with 100% activity representing 150, 40, and 85 pmoles of incorporated nucleotides per 50 µl assay in A, B, and C, respectively.

Fig. 2. Antibiotic sensitivity of topoisomerase I of yeast. Enzyme activity was measured as in Materials and Methods in the presence of various concentrations of nalidixic acid (-■-), novobiocin (-O-), or coumermycin A_1 (-▲-).

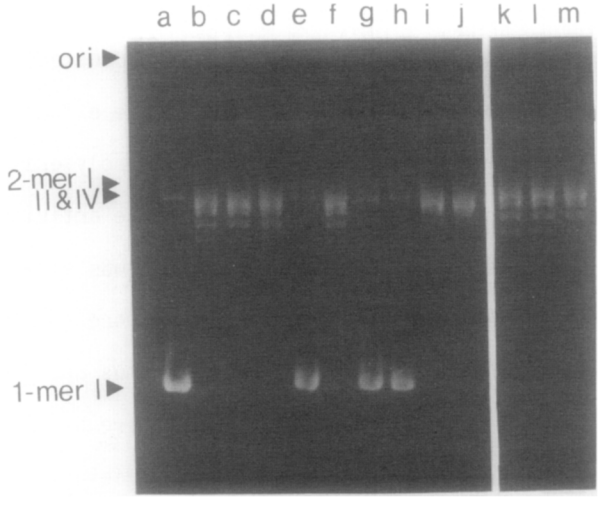


Fig. 3. Electrophoretic analysis of yeast topoisomerase I reactions. In lanes a-j, relaxation of native pBR322 supercoiled DNA was measured as in Materials and Methods. Lane a contains native pBR322 DNA with no enzyme. Lanes b-j all contain 5 units of topoisomerase I plus b) no drug; c-e) 100, 500, and 1000 µg/ml novobiocin, respectively; f-h) 10, 50, and 100 µg/ml coumermycin A_1, respectively; and i-j) 0.5 and 1.0 mg/ml nalidixic acid, respectively. Monomeric and dimeric forms of supercoiled pBR322 DNA are shown as 1-mer I and 2-mer I, respectively, and II and IV represent the nicked and fully relaxed closed circular molecular forms.
Lanes k-m represent an assay for topoisomerase II catenation activity in the preparation of topoisomerase I, measured as reported (9). Relaxed pBR322 DNA was incubated in a reaction mixture containing ATP and k) no enzyme; l) 5 units; and m) 10 units of yeast topoisomerase I. No catenated forms at the top of the gel were observed.

laevis (9,11). However, the sensitivity of the activity to novobiocin and coumermycin A_1 was reduced by the addition of ATP as that of topoisomerase II from Drosophila (9), even though ATP is not required for topoisomerase I activity. Perhaps the enzyme contains an ATP binding site, despite the lack of an ATP requirement, resulting in an allosteric inhibition against the binding of novobiocin. Alternatively, the ATP may bind competitively to the site for novobiocin in the enzyme, although structural similarities between these two molecules are not apparent. Kinetic studies are presently underway to test these two possibilities.

In summary, several enzymatic reactions in yeast are inhibited by novobiocin and coumermycin A_1, but are relatively resistant to nalidixic acid. These observations preclude the determination of particular target enzymes based strictly on drug sensitivities, without the isolation and study of specific drug resistant mutants. For example, it has been reported that relatively higher concentrations of novobiocin and coumermycin A_1 cause a secondary inhibition of DNA and RNA polymerases in E. coli (24), in addition to the primary inhibition of DNA gyrase (2). Hence the sensitivity of topoisomerase II to these drugs as observed in higher eukaryotes may not be specific. Further investigation will be necessary to resolve these questions.

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