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Berenil acts as a poison of eukaryotic topoisomerase II

José Portugal*

Departamento de Biología Molecular y Celular, Consejo Superior de Investigaciones Científicas, J. Girona-Salgado, 18–26, 08034 Barcelona, Spain

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Abstract

The ability of the anti-trypanosomal drug berenil to interfere with the activities of eukaryotic type II topoisomerases is evaluated using two different types of reactions catalyzed by the enzyme: the relaxation of naturally supercoiled DNA and the decatenation of kinetoplast DNA (kDNA). The results indicate that berenil acts as an inhibitor of the catalytic activity, but the inhibiting ability appears to be smaller than for other minor-groove binding ligands.

Key words: Berenil; Topoisomerase II; kDNA; Supercoiled DNA

1. Introduction

Berenil is a synthetic drug that is effective against animal trypanosomiasis [1,2]. The pharmacological effect of berenil in vivo has been related [1] to its preference for kinetoplast DNA (kDNA). It is well established that berenil (Fig. 1) interacts preferentially with adenine plus thymine-rich DNAs, binding in the minor groove to sequences which are about four base-pairs long, with a certain preference for alternating AT-sites [3–6]. It has been described that minor-groove binding ligands might be topoisomerase II poisons [7,8]. However, more detailed studies are needed not only to determine whether berenil can act in the same way as other AT-binding ligands, but also to find out if a potential target for this drug could be a topoisomerase involved in the topological manipulation of DNA in the mitochondria, where the kDNA is located, and whether the effect on the topoisomerase would be, therefore, responsible for the pharmacological and toxic effects of the drug. Various topoisomerase activities have been detected in the mitochondria, one of which is a type II topoisomerase which requires ATP and decatenates closed circular kDNA [9], thus sharing functions with the eukaryotic type II topoisomerases. The possibility has been raised that an unusual topoisomerase activity in trypanosomal mitochondria might explain why berenil, and other antitrypanosomal drugs, preferentially attack the mitochondrial DNA [8].

In this study, the effects of berenil are characterized using two different types of reaction catalyzed by eukaryotic topoisomerase II: the relaxation of naturally supercoiled DNA and the decatenation of kDNA.

2. Materials and methods

2.1. Materials

Berenil was kindly provided by Hoechst-Iberica (Spain) and was freshly prepared as a 1 mM stock solution in Tris-HCl, pH 7.4 containing 20 mM NaCl, and diluted to the required concentration just before use. Calf thymus type II topoisomerase and catenated kinetoplast DNA were purchased from TopoGen, Inc. (Columbus, USA). Plasmid pBR322 was purchased from Boehringer Mannheim. The pBR322 sample was analyzed in 1% agarose and was found to contain about 80% of supercoiled DNA.

2.2. Relaxation of pBR322

Reactions contained 0.125 μg of pBR322 DNA in 50 mM Tris-HCl, pH 7.8, 1 mM ATP, 1 mM dithiothreitol, 50 mM KCl, 5 mM $MgCl_2$, 30 μg/ml bovine serum albumin and 1 Unit of enzyme in a final volume of 20 μl. Reactions were performed in the absence or presence of berenil, at the concentrations indicated in the figures, and terminated, after 15 min incubation at 37°C, with 0.5% sodium dodecyl sulfate, 50 μg/ml Proteinase K and 10 mM EDTA and incubation for 30 min at 37°C.

2.3. Decatenation of kDNA

Reaction mixtures (20 μl final volume) contained 0.100 μg of kDNA and 1.5 Units of topoisomerase II in 30 mM Tris-HCl pH 7.8, 50 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol and 30 μg/ml bovine serum albumin. Following 30 min incubation at 37°C, the reactions were terminated as described above.

2.4. Electrophoretic analysis of the topoisomerase II activity in the presence of berenil

After addition of 0.1 volumes of loading dye (50% glycerol, 0.03% Bromophenol blue), the eukaryotic topoisomerase II products were separated on 1% agarose gel in Tris-Borate-EDTA buffer at 100 V for about one hour. Separation of relaxed circular DNA from the co-migrating nicked DNA was accomplished on a gel containing 30 μg/ml chloroquine, run at 40 V overnight. Gels were stained with ethidium bromide (0.5 μg/ml) for 45 min, destained in water and photographed. For quantitative purposes, the photographic negatives were scanned and the amount of DNA in each band determined using a computerized Molecular Dynamics laser densitometer.

*Corresponding author. Fax: (34) (3) 204 59 04.

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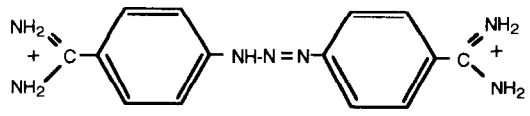

Fig. 1. Chemical structure of berenil.

3. Results and discussion

Fig. 2A displays the effects of berenil on the relaxation of pBR3222 DNA by calf-thymus topoisomerase II. In the absence of the drug (lane +) the topoisomerase converted supercoiled DNA into relaxed circular DNA, which, in the experimental conditions, comigrated with the nicked DNA. The addition of different concentrations of berenil (between 2 and 200 μM) produced a progressive inhibition of the topoisomerase II-induced topological changes in DNA, though a complete inhibition of the enzyme activity was not attained even at concentrations around 200 μM. In contrast, other minor-groove binding drugs are able to produce complete enzyme inhibition even at smaller concentrations [7]. These results suggest that berenil reduces but does not completely inhibit the reaction of topoisomerase II. An agarose gel containing chloroquine was used (Fig. 2B) to separate the co-migrating relaxed circular (IR) and nicked (II) forms, thus allowing correct quantification of the relaxation catalyzed by the topoisomerase. This gel confirmed that the amount of nicked DNA remained mostly unchanged in the presence of berenil and topoisomerase II. Quantification of the experimental data reveals that a 51% inhibition is obtained at 20 μM berenil, a concentration used in footprinting experiments to disclose the preferred binding sites [3,4], while about 90% inhibition was observed at 200 μM berenil.

The decatenation of kDNA was used to study the effects of berenil on topoisomerase II. Densitometric profiles obtained from a typical agarose gel pattern are shown in Fig. 3. Addition of berenil resulted in a drug-dependent decrease in the release of monomers, which is evident at approx. 20 μM, or at larger concentrations. The experiments to analyze the effects of berenil on the decatenation activity of topoisomerase II (Fig. 3) were performed in conditions chosen for the detection of inhibition, but low concentrations of the drug, below 20 μM, seemed to stimulate the release of uncatenated circles, while increasing berenil concentrations inhibited the production of monomers (right panel in Fig. 3). In fact, a complete inhibition of the decatenation procedure was not obtained, even at concentrations around 100 μM. Once again, the inhibitory effect was smaller than that seen for distamycin or other minor-groove binding ligands [7].

The influence of various reaction conditions on berenil action, including the order of addition in the reaction mixture, was also examined. For example the drug was equilibrated with supercoiled pBR322 DNA, for 20 min at 37°C, prior to the addition of the enzyme, but no increment in the inhibition was observed. Results in Fig. 2 show that drug concentrations up to 50 μM, produced distinct intermediate bands, indicating that the topoisomerase initiates, but does not complete, the relaxation of several supercoiled molecules. Results presented in Figs. 2B and 3 suggest that DNA topoisomerase II is unable to interact with DNA sites where the minor-groove is occupied by berenil. Furthermore, berenil is a relatively weak inhibitor of type II eukaryotic topoisomerase if compared with other minor-groove ligands [7]. The difference might be related with the lower affinity of berenil for A+T-rich DNAs compared to distamycin or netropsin [10].

Eukaryotic topoisomerase II poisons produce a rapid inhibition of DNA replication [11]. The replication of kDNA in the trypanosomatids, including those involved in tropical diseases, requires topological manipulations of this peculiar DNA [12]. It has been described that before the replication takes place, all the minicircles within a network are covalently closed. Berenil might be capable of inhibiting (Fig. 3) the release of minicircles that is needed to replicate the mitochondrial DNA [8,12].

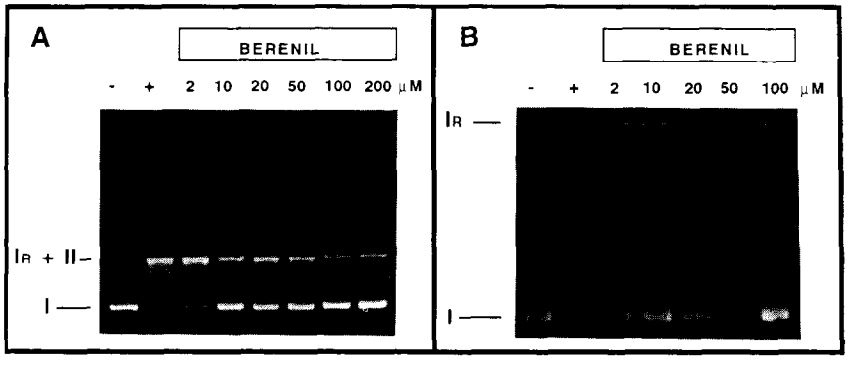

Fig. 2. Effects of berenil on the relaxation of supercoiled pBR322 DNA by topoisomerase II. Electrophoretic patterns without (lane −) and with (lane +) topoisomerase II, and in the presence of the indicated concentrations of berenil. (A): Gel stained with ethidium bromide; (B) Gel run in the presence of 30 μg/ml chloroquine. Forms I, IR and II represent highly supercoiled, relaxed circular and nicked DNA respectively.

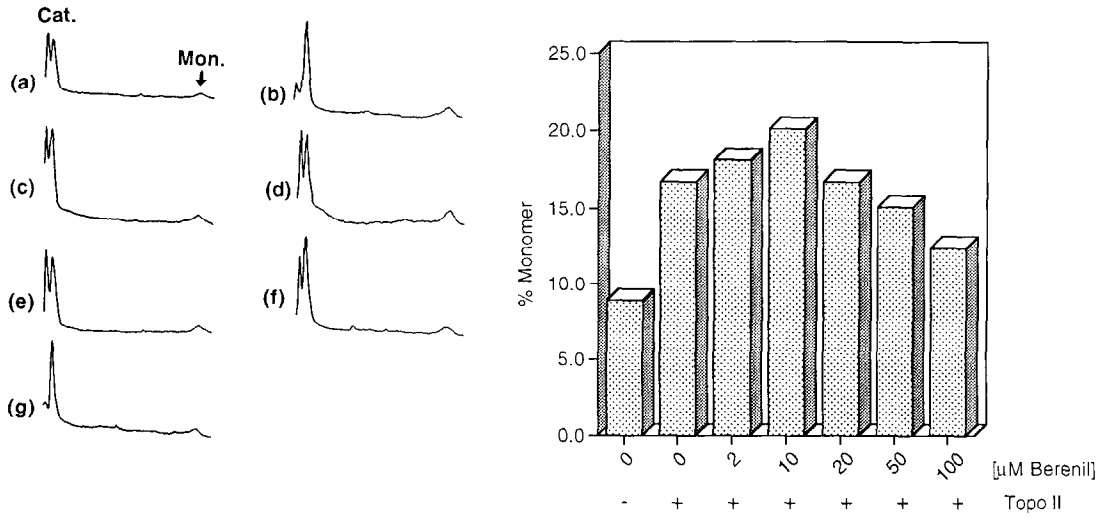

Fig. 3. Effects of berenil on the catalytic activity of topoisomerase II in a decatenation assay using catenated kinetoplast DNA. Cat. and Mon. correspond to catenated and monomer (decatenated) kDNA. The figure shows densitometric profiles (a) to (g) obtained from electrophoretic patterns without (a) and with topoisomerase II (b–g), and in the presence of berenil at the concentrations indicated in the right panel, which displays a quantification of the percentage of monomer (decatenated) kDNA in the samples.

To elucidate the exact mechanism by which berenil acts as a poison of topisomerase II will require further consideration using complementary experimental approaches to those presented here. The relatively small effect of berenil (Figs. 2 and 3), when compared with other ligands devoid of antitrypanosomal effect in vivo [7], indirectly indicates that the type II topoisomerase activity present in mitochondria [9] should display some kinetic peculiarities which would make it more susceptible to berenil than to other drugs.

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